Synthesis of Novel Polymeric Materials for Antimicrobial Applications

by

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DEDICATION

This work is dedicated to my parents and sisters for their endless love, support and encouragement throughout my studies and everyday life. The important life skills you have taught me have shaped the person I am today and without you I would have never been successful. Words cannot begin to express my gratitude.

Finally, this work is dedicated to my dearest husband Mohamed Adnan Salejee, an amazing man, without whom there would be no degree. His support, encouragement and patience are admirable and I truly appreciate it.

DECLARATION 1 – PLAGIARISM

I, Ms Nadia Suleman, declare that

1. The research reported in this thesis, except where otherwise specified, is my original work.
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Signed:…… Date:……..

I, Prof Thirumala Govender as supervisor of the MPharm study hereby consent to the submission of this MPharm thesis.

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I, Dr Chunderika Mocktar as co-supervisor of the MPharm study hereby consent to the submission of this MPharm thesis.

Signed:…… Date:……..

DECLARATION 2 – PUBLICATIONS

Details of contributions to publications that form part and/or include research presented in this thesis:

1. Suleman, N., Kalhapure, R.S., Mocktar, C., Rambharose, S., Singh, M. and Govender, T., 2015. Silver salts of carboxylic acid terminated generation 1 poly (propyl ether imine) (PETIM) dendron and dendrimers as antimicrobial agents against *S. aureus* and MRSA. Royal Society of Chemistry Advances, 5, 34967-34978.

   Ms N. Suleman contributed to the design of the project, and the preparation and characterisation of all G1 PETIM dendron/dendrimers and PETIM-silver salts in terms of synthesis, FT-IR, NMR, HRMS, *in vitro* cytotoxicity and *in vitro* antimicrobial studies, along with interpretation of the data and writing of the paper. Dr R. Kalhapure assisted with the design of the project, as well as the interpretation of characterisation data of all synthesised materials in terms of IR, NMR and HRMS. Dr C. Mocktar assisted with the *in vitro* antimicrobial study. Mr S. Rambharose and Dr M. Singh assisted with the *in vitro* cytotoxicity study. The remaining author served as supervisor.

2. Suleman, N., Kalhapure, R., Mocktar C., Rambharose, S., Govender, T., 2015. A poly (ethylene glycol) six-arm star-shaped polymer as an efficient stabiliser for the synthesis of antibacterial and non-cytotoxic silver nanoparticles. RSC Advances, SUBMITTED MANUSCRIPT. Reference number: RA-ART-11-2015-023113.

   Ms N. Suleman contributed to the design of the project, and the preparation and characterisation of the G1 PETIM-m-PEG star shaped polymer and star polymer stabilised nanoparticles, in terms of synthesis, FT-IR, NMR, DLS, TEM, XRD, *in vitro* cytotoxicity and *in vitro* antimicrobial studies, along with interpretation of the data and writing of the paper. Dr R. Kalhapure assisted with the design of the project, as well as the interpretation of characterisation data of the synthesised materials in terms of IR, NMR and XRD. Dr C. Mocktar assisted with the *in vitro* antimicrobial study. Mr S. Rambharose assisted with the *in vitro* cytotoxicity study. The remaining author served as supervisor.

3. Kalhapure, R.S., Suleman, N., Mocktar, C., Seedat, N., Govender, T., 2014.

Nanoengineered Drug Delivery Systems for Enhancing Antibiotic Therapy. Journal of Pharmaceutical Sciences, 3, 872-905.

Ms. N Suleman undertook the literature search for all papers with regard to nano antibiotic delivery systems. Ms N Suleman also contributed to writing the introduction section.

RESEARCH OUTPUT FROM THE THESIS

1. Publications

- Suleman, N., Kalhapure, R., Mocktar, C., Rambharose, S., Singh, M., Govender, T., 2015. Silver salts of carboxylic acid terminated generation 1 poly (propyl ether imine) (PETIM) dendron and dendrimers as antimicrobial agents against *S.aureus* and MRSA. Royal Society of Chemistry Advances, 5, 34967-34978. (IF = 3.708)

*The published paper can be found in appendix A.

- Kalhapure, R.S., Suleman, N., Mocktar, C., Seedat, N., Govender, T., 2014. Nanoengineered Drug Delivery Systems for Enhancing Antibiotic Therapy. Journal of Pharmaceutical Sciences, 3, 872-905. (IF = 2.59)

*The published paper can be found in chapter 5.

2. Submitted manuscripts

- Suleman, N., Kalhapure, R., Mocktar C., Rambharose, S., Govender, T., 2015. A poly (ethylene glycol) six-arm star-shaped polymer as an efficient stabiliser for the synthesis of antibacterial and non-cytotoxic silver nanoparticles. RSC Advances, SUBMITTED MANUSCRIPT. Reference number: RA-ART-11-2015-023113. (IF = 3.708)

*The manuscript proof of submission can be found in appendix B.

3. Conference Presentations

The following conference presentations were produced from data generated during this study:

International:

- Suleman, N., Kalhapure, R.S., Mocktar, C., Rambharose, S., Singh, M. and Govender, T. Silver salts of carboxylic acid terminated generation 1 poly (propyl ether imine) (PETIM) dendron and dendrimers as novel antimicrobial agents against S. aureus and MRSA. 4th International Symposium on Biomedical Applications of Dendrimers, Lugano, Switzerland, 18 – 20 June 2014.

- Suleman, N., Kalhapure, R.S., Mocktar, C., Rambharose, S., Singh, M. and Govender, T. Silver salts of carboxylic acid terminated generation 1 poly (propyl ether imine) (PETIM) dendron and dendrimers as novel antimicrobial agents against S. aureus and MRSA. International Union of Microbiological Societies, Montreal, Canada, 27 July – 1 August 2014.

*the poster can be found in appendix C.

- Suleman, N., Kalhapure, R.S., Mocktar, C., Rambharose, S., and Govender, T. Novel poly (ethylene glycol) star shaped polymer coated silver nanoparticles: synthesis, *in vitro* cytoxicity and antibacterial activity. Nanotech France 2016 International Conference and Exhibition, Paris, France, 1 – 3 June 2016 (abstract accepted).

*The abstract and proof of acceptance can be found in appendix D.

Local:

- Suleman, N., Kalhapure, R.S., Mocktar, C., Rambharose, S., Singh, M. and Govender, T. Silver salts of carboxylic acid terminated generation 1 poly (propyl ether imine) (PETIM) dendron and dendrimers as novel antimicrobial agents against S. aureus and MRSA. 35th Annual Conference of the Academy of Pharmaceutical Sciences, Port Elizabeth, South Africa, 14 – 16 September 2014.

*The poster can be found in Appendix C.

ABSTRACT

Infectious diseases are one of the leading causes of death globally for adults and children and remains a major public health issue for developed and developing countries. Although antibiotics transformed the treatment of infections, saving millions of lives, eighty years after their discovery, their usefulness is seriously threatened by antimicrobial resistance. This rising development of antibiotic resistance to presently used antibiotics and the decline in introduction of new antibiotic drugs is unmistakably a risk to human health worldwide. It is therefore essential that alternative novel antimicrobial therapeutic strategies are explored to address the imminent crisis associated with conventional antibiotics. The quest for novel and effective approaches to enhance antimicrobial drug therapy is therefore identified globally as a key focus area of research priority. Antimicrobial materials such as novel dendrimer silver salts and a novel star polymer for application in silver nanoparticles, might be a favourable approach to overcome the existing challenges related to antibiotic therapy due to their unique properties. PETIM silver salts have not been studied and there are no PEG star polymers available for stabilisation of silver nanoparticles. The aims of the study were therefore to: (1) explore the potential of novel antimicrobial dendrimer silver salts for enhanced antimicrobial activity and (2) explore the potential of a novel star shaped polymer for use as a stabilising agent in the preparation of silver nanoparticles.

The purpose of aim one was to exploit the multiple peripheral functionalities of G1 PETIM dendron and dendrimers for the formation of silver salts containing multiple silver ions in a single molecule for enhanced antimicrobial activity at the lowest possible concentration. In order to accomplish the first aim, G1 PETIM dendron, dendrimers and their silver salts were synthesised and characterised by FT-IR, ^{1}H NMR and ^{13}C NMR. PETIM silver salts were evaluated against Hep G2, SKBR-3 and HT-29 cell lines for their cytotoxicity using the MTT assay. The G1 PETIM dendron/dendrimers, silver nitrate and silver salts of the G1 dendron (compound **13**), G1 dendrimer with an aromatic core (compound **14**) and an oxygen core (compound **15**) were evaluated for activity against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) by the broth dilution method. PETIM silver salts were found to be non-cytotoxic even up to 100 µg/ml. Minimum inhibitory concentration values of compounds **13**, **14** and **15** against *S. aureus* were 52.1, 41.7, and 20.8 µg/ml while against MRSA they were 125.0, 26.0 and 62.5

μg/ml respectively. The calculated fractional inhibitory concentration index further indicated that compound **14** specifically displayed additive effects against *S. aureus* and synergism against MRSA. The enhanced antimicrobial activities of the PETIM dendron/dendrimer-silver salts against both sensitive and resistant bacterial strains widen the pool of available pharmaceutical materials for optimising treatment of bacterial infections.

The purpose of aim two was to synthesise a G1 PETIM dendrimer derived 6-arm polyethylene glycol (PEG) star polymer for application as a stabiliser for silver nanoparticles. In order to accomplish the second aim the synthesised star polymer was characterised using FT-IR, ^{1}H, ^{13}C and XRD analysis. Silver nanoparticles were prepared via chemical reduction using the star polymer as a stabiliser and their formation was verified using UV-vis spectroscopy, dynamic light scattering, transmission electron microscopy and XRD analysis. The synthesised star polymer and star polymer stabilised silver nanoparticles were evaluated for their cytotoxicity against MCF-7, HeLa and Hep G2 cell lines using the MTT assay. The silver nanoparticles, silver nitrate, star polymer and a physical mixture of the latter two were evaluated for antibacterial activity against *S. aureus*, MRSA, *E. coli* and *P aeruginosa*. The synthesised silver nanoparticles were non-agglomerated, spherical and monodisperse with an average particle size of 36.44 ± 2.51 nm, and found to be non-cytotoxic even up to 100 μg/ml. The minimum inhibitory concentration values against *S. aureus* and MRSA (Gram-positive bacteria) were 18.5 and 74 μg/ml respectively, and against *E.coli* and *P. aeruginosa* (Gram-negative bacteria), the values were 9.25 and 74 μg/ml respectively. These low MIC values confirmed that the silver nanoparticles retained their antibacterial potential even upon stabilisation by the star polymer. These results suggested that the synthesised star polymer is an attractive biocompatible material for the stabilisation of silver nanoparticles.

The results obtained in this study confirm the potential formation of novel materials, such as the PETIM silver salts as well as the star polymer stabilised silver nanoparticles. They both display good antimicrobial activity against sensitive and resistant bacterial strains. These are new materials with the potential for commercialisation. This study is the building block for future research and can also be explored for application in nanomedicine and the biomedical sciences.

Keywords: Dendrimer · Silver nitrate · Antimicrobial · Poly (propyl ether imine) · star-shaped · poly (ethylene glycol) · stabiliser · silver nanoparticles · antibacterial · *P. aeruginosa* · *E. coli* · *S. aureus* · MRSA

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LIST OF ABBREVIATIONS

DMAP	4-(Dimethylamino) pyridine	PBP	Penicillin binding protein
AcCl	Acetyl chloride	PDI	Polydispersity index
AFM	Atomic force microscopy	PEG	Polyethylene glycol
$AgNO_3$	Silver nitrate	PETIM	Poly(propyl ether imine)
AMR	Antimicrobial resistance	PLA	Poly(lactic acid)
CTAB	Cetyltrimethylammonium bromide	PLGA	Poly(lactide-co-glycolic acid)
CTAC	Cetyltrimethylammonium chloride	PPI	Poly propylene imine
DMSO	Dimethyl sufoxide	ROS	Reactive oxygen species
DLS	Dynamic light scattering	SD	Standard deviation
EPR	Electron paramagnetic resonance	*S. aureus*	*Staphylococcus aureus*
E. coli	*Escherichia coli*	$AgNO_3$	Silver nitrate
FDA	Food and drug administration	$NaBH_4$	Sodium borohydrate
FIC	Fractional inhibitory concentration	NaCl	Sodium chloride
FT-IR	Fourier transmission infrared spectroscopy	$Na_3C_6H_5O_7$	Sodium citrate
$LiAlH_4$	Lithium aluminium hydride	SD	Standard deviation
MHA	Mueller-Hinton agar	SDS	Sodium dodecyl sulfate
MHB	Mueller-Hinton broth	NaOH	Sodium hydroxide
MIC	Minimum inhibitory concentration	SPPR	Surface plasmon polariton resonances
m-PEG	Poly (ethylene glycol) methyl ether	TEM	Transmission electron microscopy
MRSA	Methicillin resistant *staphylococcus aureus*	UV	Ultraviolet
MRSE	Methicillin resistant *staphylococcus epidermis*	VRE	Vancomycin resistant *enterococcus*
MSSA	Methicillin sensitive *staphylococcus aureus*	VRSA	Vancomycin resistant *staphylococcus aureus*
NMR	Nuclear magnetic resonance	H_2O	Water
OD	Optical density	WHO	World health organisation
P. aeruginosa	*Pseudomonas aeruginosa*	XRD	X-ray diffraction
PAMAM	Poly(amidoamine)	ZP	Zeta potential

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CHAPTER 1. INTRODUCTION

1.1 Introduction

This chapter outlines the background of this study, including the present status of infectious diseases and the difficulties associated with current antibiotic therapy. It explores the use of novel polymeric materials to overcome the challenges related to antimicrobial resistance, and indicates the aims and objectives. The significance and novelty of the study are also highlighted.

1.2 Background to the study

Infectious diseases, a major percentage of which are of bacterial origin, are one of the foremost causes of death worldwide for children and adults, and remain an important public health problem for both developed and developing countries (Lozano *et al.*, 2013). In 2013, approximately half of the 6.3 million children who died before the age of 5 did so as a result of infectious causes, and nearly two-fifths passed away during the neonatal period (Liu *et al.*, 2015). According to the WHO, on April 30, 2014, inconsequential infections and injuries, which were easily treatable for years, were again a major threat to public health (WHO). The introduction of antibiotics was one of the most significant interventions to decrease diseases, has saved millions of human and animal lives, and contributed significantly to the progress of modern medicinal procedures (Ray *et al.*, 2012; Xiong *et al.*, 2014).

However, eighty years subsequent to their discovery, their usefulness is compromised by antimicrobial resistance (AMR) (Cars *et al.*, 2011) that threatens to invalidate the use of even the most effective antibiotics, which will result in patients suffering and/or dying owing to infection control failure, and lead to escalated health care costs (Huh and Kwon, 2011). Worldwide, resistant bacterial strains, for example methicillin-resistant *Staphylococcus aureus* (MRSA) (Cohen, 2000), vancomycin-resistant *Enterococcus* (VRE) (Wood *et al.*, 1996) and vancomycin-resistant *Staphylococcus aureus* (VRSA), (Périchon and Courvalin, 2009), have become noteworthy threats in community settings and hospitals to treat infections. Additionally, if present rising trends in AMR continue, numerous vital procedures, for example organ transplantation, cancer chemotherapy, hip and other joint replacements, may no longer be performed due to fear that the associated compromised immune system could place the patients at a severe threat of attaining

infections that are difficult to treat and eventually fatal (Heymann and Rodier, 2001). Furthermore, they are affected by insufficient drug concentrations at the target infection sites, increased frequency of administration, severe side effects, as well as poor patient compliance, all of which which compromises drug therapy and contributes significantly to AMR (Huh and Kwon, 2011; Sharma *et al.*, 2012). The global AMR crisis is further intensified by the decline in the development of novel antibiotics by pharmaceutical companies (Cars *et al.*, 2008). The deterioration in drug development is a result of high costs and lengthy delays related to developing a novel chemical entity, high attrition rates at final testing, and growing AMR to newly established drugs, which makes discovering a new drug extremely costly and restricts the return on investment (Huh and Kwon, 2011; Okeke *et al.*, 2005).

It is therefore imperative that alternative novel antimicrobial therapeutic strategies are explored to address the looming crisis with conventional antibiotics. Alternative options currently being investigated are novel nanosized drug delivery systems, which could be a promising approach to overcome the current challenges associated with antibiotic therapy owing to their unique physicochemical properties. These include their small size, large ratio of surface area to mass, and unique interactions with microorganisms and cells of the host, in addition to their capability to be structurally and functionally modified (Kalhapure *et al.*, 2014c; Zhang *et al.*, 2007; Zhang *et al.*, 2010). Examples of such systems are silver nanoparticles (Kalhapure *et al.*, 2014a; Mala *et al.*, 2012; Morones *et al.*, 2005), solid lipid nanoparticles (Kalhapure *et al.*, 2014b; Wang *et al.*, 2012; Xie *et al.*, 2011), liposomes (Drulis-Kawa *et al.*, 2006; Pumerantz *et al.*, 2011; Sande *et al.*, 2012) and even the synthesis of new antimicrobial materials, such as dendrimers (Cheng *et al.*, 2007; Felczak *et al.*, 2013; Svenson, 2009) and antimicrobial peptides (Faccone *et al.*, 2014).

Polymeric materials, which comprise natural, seminatural and synthetic polymers, propose endless opportunities to control the properties of drug delivery systems besides meeting numerous criteria such as biocompatibility, biodegradability and reproducibility, due to their diversity in topology, chemistry and dimension. Several types of polymeric materials, for instance linear polymers (Qiu and Bae, 2006), block copolymers (Kumar *et al.*, 2001; Qiu and Bae, 2006) and hyperbranched polymers (Gao and Yan, 2004; Jikei and Kakimoto, 2001), are being studied for drug delivery systems. In this study, dendrimers (Gillies and Frechet, 2005; Huh and Kwon, 2011; Inoue, 2000;

Qiu and Bae, 2006) and star polymers (Gao, 2012; Inoue, 2000; Qiu and Bae, 2006) were specifically addressed.

Silver, which is a powerful antimicrobial agent, mainly in its positively charged ionic form, demonstrates good toxicity to an extensive range of micro-organisms, and simultaneously has a principally low human toxicity (Dallas *et al.*, 2011; Gibbins and Warner, 2005; Liau *et al.*, 1997). Additionally, it is able to interrupt significant functions in a microorganism that causes AMR. Nevertheless, it is imperative to be mindful of the fact that silver is only non-toxic to human cells in small concentrations (Pal *et al.*, 2007), and thus restricts the use of metallic silver and silver ions as antibacterial agents up to concentrations that are non-toxic to eukaryotic cells. Dendrimers, instead are repetitively branched molecules or nano-sized, radially symmetric molecules, having well-defined, uniform and monodisperse structures, and comprise of branches that surround a core (Bosman *et al.*, 1999; Hari *et al.*, 2012). They are a great source for finding novel and distinct properties due to their accessibility of numerous functional surface groups and low polydispersity (Hari *et al.*, 2012; Inoue, 2000).

Owing to their unique properties, and because they can be modified to therapeutic needs, they have become model carriers for small molecule drugs and biomolecules (Svenson, 2009). They have also gained added attention as possible antimicrobial agents due to the availability of numerous end groups and compressed structure (Chen and Cooper, 2000; Polcyn *et al.*, 2013). Commonly, dendrimers display promising biocompatibility (Svenson, 2009), which is vital for their application, and can be used as antimicrobial agents themselves (Charles *et al.*, 2012; Chen *et al.*, 2000; Felczak *et al.*, 2012; Lopez *et al.*, 2009). Poly propylene imine (PPI), polylysine, triazine (Jain *et al.*, 2010) and polyamidoamine (PAMAM) are the most widely used dendrimers in drug delivery, with PAMAM being the first and most frequently studied (Svenson, 2009). However, its advantages are constricted by limitations such as cytotoxicity, which is caused by its amine-terminated nature (Sosnik *et al.*, 2010) and there are consequently no commercially available dendrimer based preparations for systemic use (Jain *et al.*, 2010). Conversely, poly (propyl ether imine) (PETIM) dendrimers, a relatively new class of dendrimers, have been described to have good biocompatibility compared to commercial PAMAM dendrimers.

While they have numerous advantages, such as non-cytotoxicity and easy functional group modification at the periphery, their potential for antimicrobial therapy is yet to be exploited. While the potential to develop complexes of silver and dendrimers to enhance antimicrobial activity has been demonstrated, but research is limited (Balogh *et al.*, 2001; Ottaviani *et al.*, 2002). Balogh *et al.* and Ottaviani *et al.* are the very few reported papers who prepared poly(amidoamine) (PAMAM) dendrimer based silver complexes. To the best of our knowledge, no other classes of dendrimers have been used to prepare silver salts, highlighting the need for further studies on dendrimer silver salts.

Numerous types of metal nanomaterials have been studied to date (Esumi *et al.*, 2000; Gong *et al.*, 2007; Huang *et al.*, 2008), yet silver nanoparticles are at the forefront of research, being the most effective due to their exceptional antimicrobial efficacy against not just bacteria, but viruses and other eukaryotic micro-organisms as well (Gong *et al.*, 2007; Rai *et al.*, 2009). Silver nanoparticles appear to be a more suitable choice when compared to the silver cation salts and complexes. Moreover, when compared to commercially available antibiotics that can cause harm to valuable enzymes, colloidal silver allows these enzymes to remain unharmed (Dallas *et al.*, 2011).

Bacterial resistance has also yet to be identified when using silver nanoparticles as antibacterial agents. This is apparently a consequence of the difference mechanisms of the antibacterial actions of the various forms of silver (Gogoi *et al.*, 2006; Kvitek *et al.*, 2008; Yamanaka *et al.*, 2005). An important application problem associated with silver nanoparticles is the sufficient stability of their dispersions and the prevention of aggregation (Shrivastava *et al.*, 2007; Teeguarden *et al.*, 2007). To combat this problem, various polymers are often used to stabilise these metal colloids, as polymers act as a type of matrix, and have been commonly used for trapping nanoparticles (Jeon *et al.*, 2008; Lu *et al.*, 2006; Spadaro *et al.*, 2010). Numerous methods have also been established to immobilise silver nanoparticles in the polymeric matrix. Among those techniques, chemical reduction is the most regularly utilised approach, which consists of adding a reducing agent, such as sodium borohydride (NaH_4), sodium citrate ($Na_3C_6H_5O_7$), ascorbate or lithium aluminium hydride ($LiAlH_4$), to reduce silver nitrate ($AgNO_3$) in an aqueous solution (Huang *et al.*, 2012; Lu *et al.*, 2006; Wang *et al.*, 2010).

Star polymers are the simplest type of branched materials in which no less than three linear polymer chains, with basically identical lengths, are linked to only one branching point (core) (Inoue, 2000; Kuzuu, 1980; Wu *et al.*, 2015). They can contain chemically identical or different arms (miktoarm star polymer) that are linked to the core, and have gained interest due to their distinctive topological structure and attractive physical and chemical properties, which are unlike their linear counterparts (Jia *et al.*, 2014; Wu *et al.*, 2015). Furthermore, they contain a greater degree of end group functionalities that are essential in specific applications (Wang *et al.*, 2005). Hence, if the components of the star polymers are biodegradable and/or biocompatible, they can have various possible biomedical applications (Jia *et al.*, 2014; Wu *et al.*, 2015). These star polymers could be regarded as a new family of stabilising agent for preparing colloidal silver nanoparticles, as very few star polymers have been utilised as stabilising agents thus far (Huang *et al.*, 2012; Sun *et al.*, 2010; Zhang *et al.*, 2008). Therefore, the search for new polymers as stabilising agents is of great importance to facilitate their application as antimicrobials. There is thus a gap with regards to not only dendrimer silver salts, but also stabilising agents for silver nanoparticles.

1.3 Aims and objectives

The aim of the study was to: (1) explore the potential of novel antimicrobial dendrimer silver salts for enhanced antimicrobial activity and (2) explore the potential of a novel star shaped polymer for use as a stabilising agent in the preparation of silver nanoparticles.

In order to accomplish the first aim, the objectives of the study were to:

1. Design and synthesise novel PETIM silver salts of generation 1 poly (G1) (propyl ether imine) (PETIM) dendron and dendrimers.
2. Characterise the synthesised PETIM dendron/dendrimers and PETIM silver salts in terms of infrared spectroscopy (IR) and/or nuclear magnetic resonance spectroscopy (NMR).
3. Evaluate the cytotoxic effects of the PETIM silver salts in terms of MTT assay.
4. Assess the potential of the prepared samples as antimicrobial agents by *in vitro* antimicrobial testing.

To accomplish the second component of the aim, the objectives were to:

5. Design and synthesise a biocompatible and biodegradable G1 PETIM-m-PEG star shaped polymer.
6. Characterise the synthesised polymer in terms of IR, ^{1}H NMR, ^{13}C NMR, and X-ray diffraction (XRD) analysis.
7. Evaluate the cytotoxic effects of the star shaped polymer in terms of MTT assay.
8. Prepare star polymer stabilised silver nanoparticles and determine their particle size, polydispersity index (PDI), morphology and stability.
9. Evaluate the cytotoxic effects of the star polymer stabilised silver nanoparticles in terms of MTT assay.
10. Assess the potential of the prepared samples as antimicrobial agents by *in vitro* antimicrobial testing.

1.4 Novelty of this study

Dendrimers, dendritic polymers and star polymers, due to their uniqueness, are a promising new scaffold for drug delivery. Numerous branched polymers have been developed worldwide, and the synthesis of these polymers are ongoing. However, the dendritic materials reported in this study are novel for various reasons:

- While other classes of dendrimers, such as PAMAM, have been extensively used and studied, data on PETIM dendrimers is lacking. Although the latter have several advantages, such as non-cytotoxicity and easy functional group modification at the periphery, their potential for antimicrobial therapy has not been exploited. This study is therefore the first combination of PETIM dendrimers and silver to identify novel antimicrobial materials effective against both sensitive and resistant bacterial strains.
- While several studies have been reported on the synthesis and characterisation of star polymers, as well as the application of certain hyperbranched and star polymers as stabilising agents, to our knowledge, there are no studies on the use of a novel PETIM-m-PEG type of star polymer as a stabilising agent in silver nanoparticles production. The PEG six-arm star-shaped polymer synthesised in this study has not been reported previously.

1.5 Significance of this study

The formation of new polymeric materials for application in antimicrobial systems offers a novel and promising means of overcoming the resistance that is associated with most the frequently used antibiotics. The potential benefits of formulating the novel polymeric materials proposed in this study may include the following:

- With the rise in AMR and the lack of commercially available superior polymeric materials to overcome the ever threatening complications which arise from resistance, identifying new polymeric materials for applicability in developing novel nano-delivery systems could be of great value to combating this global problem.
- The development of these novel materials could mean cost-effective and superior delivery systems may be developed, and could not only lead to a reduction in health care costs worldwide, but also improvements in infectious diseases treatment and management.
- These new materials with antimicrobial properties will widen the pool of available materials for formulation scientists to explore for additional applications.
- New knowledge on the applicability and antimicrobial properties of PETIM dendron/dendrimers, as well as the applicability of star polymers as stabilising agents for nanoparticle formation, could be obtained.

1.6 Overview of this thesis

The study will be presented in the following five chapters:

Chapter 2. Literature Review: reviews infectious diseases, the resistance associated with these diseases and strategies to overcome their current limitations. This chapter focuses on the synthesis of novel polymeric material and their use as antimicrobial agents. Additionally, various types of polymeric materials are described, with particular focus on dendrimers and star polymers and their role in medicine.

Chapter 3. (publication) is a first author article accepted in an ISI international journal. This chapter is presented in the mandatory format of the journal and is the final revised accepted version. It reports on novel work published in an international journal. It describes the synthesis of biocompatible PETIM silver salts as novel antimicrobial agents.

Chapter 4. (manuscript) is a first author manuscript submitted to an ISI international journal. This chapter is presented in the mandatory format of the journal and is the final version submitted

for review. It describes the synthesis of a novel biocompatible star polymer and its use as a stabilising agent in the preparation of silver nanoparticles.

Chapter 5. (publication) is a co-authored review article reporting on nanoengineered drug delivery systems for enhancing antibiotic therapy.

Chapter 6. Conclusion: addressed the study aim with respect to the seven objectives, outlines the limitations, the significance of the findings, and provides recommendations for future studies to widen the use of silver salts as antimicrobial agents, and to optimise the use of star polymer stabilised silver nanoparticles as an ideal delivery system.

1.7 References

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CHAPTER 2. INFECTIOUS DISEASES AND NOVEL POLYMERIC MATERIALS FOR DRUG DELIVERY

2.1 Introduction

This chapter encompasses a review of the literature on infectious diseases, the resistance associated with these diseases and the strategies to overcome their current limitations. It also focuses on the synthesis of novel polymeric material and their use as antimicrobial agents. Additionally, various types of polymeric materials are described, with particular focus on dendrimers and star polymers and their role in medicine. Finally, the use of silver as a model antimicrobial agent for this study is explained.

2.2 Current status of infectious diseases and bacterial resistance

Infectious diseases are disorders that result from the presence of a pathogenic agent, being either a virus, bacterium, fungus or parasite. These diseases are also known as communicable diseases due of their ability to be transmitted from one person to another (malaria, tuberculosis) and even occasionally from one species to another (flu, influenza) (Salouti and Ahangari, 2014). Infectious diseases, a significant portion of which are of bacterial origin, are one of the leading causes of untimely death globally for adults and children, and remain a major public health issue for developed and developing countries (Lozano *et al.*, 2013). Africa, and South Africa in particular, have a high burden of infectious diseases and due to this, gastrointestinal, respiratory, sexually transmitted, and hospital acquired infections are leading causes of death in the developing world (Kalhapure *et al.*, 2014; Winters and Gelband, 2011). While antibiotics revolutionised the treatment of infections, thereby extending the lives of millions of people, eighty years after their discovery, their effectiveness is seriously threatened by antimicrobial resistance (AMR) (Figure 1) (Cars *et al.*, 2011). This nullifies the use of even the most potent antibiotics, which leads to patient suffering and/or dying due to poor infection control failure, and results in escalated health care costs (Huh and Kwon, 2011).

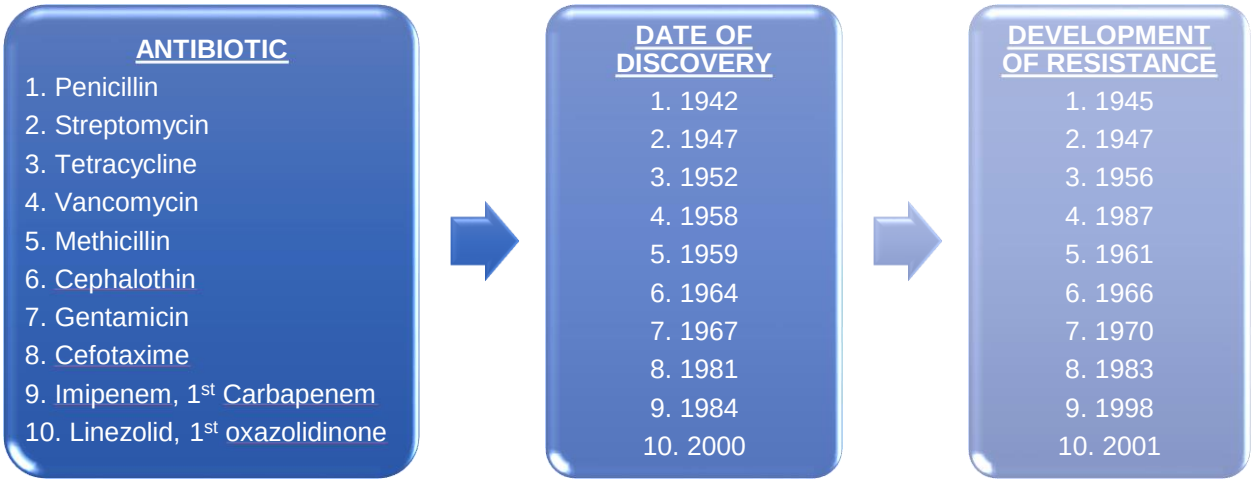

Figure 1. Date of discovery and resistance of antibiotics. Adapted from Huh and Kwon, 2011 and Brooks and Brooks, 2014.

Globally, resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Cohen, 2000), vancomycin-resistant *Enterococcus* (VRE) (Wood *et al.*, 1996) and vancomycin-resistant *Staphylococcus aureus* (VRSA), (Périchon and Courvalin, 2009) have become significant threats in community settings and hospitals for treating infections. Additionally, emerging and re-emerging infectious diseases (Huh and Kwon, 2011), together with concerns such as the rising global trade, international travel and the likelihood of bioterrorist attacks in numerous countries, have compounded the seriousness of infectious diseases (Kalhapure *et al.*, 2014). If the current escalating trends in AMR continue, several important procedures, such as cancer chemotherapy, organ transplantation and hip and other joint replacements, could no longer be performed for fear that the related compromised immune system might put the patients at severe risk of acquiring a difficult to treat and ultimately fatal infection (Heymann and Rodier, 2001). The global AMR crisis is amplified by the decreasing development of new antibiotics by pharmaceutical companies (Cars *et al.*, 2008), with 20 novel classes of antibiotics being developed in between 1930-1962 (Coates *et al.*, 2002; Powers, 2004), yet only two novel classes have been developed since then (Cars *et al.*, 2008). This decline in drug development is due to the high costs and lengthy delays associated with developing a new chemical entity, high attrition rates at final testing, and increasing AMR, which makes finding a new drug very expensive and limits the return on investment (Huh and

Kwon, 2011; Okeke *et al.*, 2005). It is therefore essential that alternative novel antimicrobial therapeutic strategies are explored to address the imminent crisis with conventional antibiotics.

2.3 Current antibiotic therapy and limitations

Antibiotic use began when penicillin was developed more than 70 years ago, their use being increasingly effective when newer, superior antibiotics were introduced to treat a host of infectious diseases, thereby contributing to lessening the related morbidity and mortality (Huh and Kwon, 2011; Kalhapure *et al.*, 2014). Antibiotics are considered essential in nearly all medical areas, such as general surgery, including organ transplant procedures, treatment of premature babies, and even chemotherapy in cancer patients cannot be accomplished without effectively treating and preventing bacterial infections (Cars *et al.*, 2011).

Unfortunately, there are several restrictions related to current antibiotic drug therapies. Various antibiotic dosage forms are compromised by inadequate drug concentrations at target infection sites, increased frequency of administration, severe side effects, and poor patient compliance, all of which inadvertently compromise drug therapy (Huh and Kwon, 2011; Sharma *et al.*, 2012). These limitations, in addition to the common use and abuse of antibiotics, have contributed to their greatest drawback, i.e. resistance to bacterial microorganisms. Furthermore, the antibiotic resistance crisis has been intensified by pan drug-resistant and extensively drug-resistant organisms to antibiotics, which has reached distressing levels worldwide (Cars *et al.*, 2011; Seil and Webster, 2012). Although new methods to overcome antibiotic resistance are constantly being researched and developed, antibiotic discovery is not on par with the rates of drug resistance, and a gradual and steady decrease in the introduction of novel drugs has been reported by the US Food and Drug Administration (FDA) (Brooks and Brooks, 2014; Taubes, 2008). This is due to the exorbitant costs and prolonged times for ultimate regulatory approval of new compounds, along with low returns on investment, which further compounds the current crisis (Kalhapure *et al.*, 2014; Sondi and Salopek-Sondi, 2004). It is therefore evident that the pace at which drug development and registration is going is not timeously receptive to the hasty development of resistance by microbial pathogens.

This rising development of antibiotic resistance to presently used antibiotics, and the decline in introduction of new antibiotic drugs, is unmistakably a risk to human health worldwide (Kalhapure *et al.*, 2014). The quest for novel and effective approaches to enhance drug therapy with existing antibiotics is therefore identified globally as a key focus area of research priority (Kalhapure *et al.*, 2014), and combatting this multifaceted issue of antibiotic resistance must go past the development of novel pharmaceuticals and include a multidisciplinary culture of change (Brooks and Brooks, 2014).

2.4 The use of nanotechnology to overcome limitations with current antibiotic drugs

The vital components to combat the continued expanding threat of AMR include various approaches such as: establishing an effective surveillance system for early detection of AMR; methods to optimise the use of antibiotics; resilient infection control and prevention methods to avoid the spread of AMR; improvements in healthcare providers and public education, and alertness concerning antibiotics to avoid their incorrect use; as well as research to guide the aforementioned points and to develop novel antibiotics that offer effective treatment to patients (Paphitou, 2013). While there is general agreement that a multifaceted strategy is essential to address the problem, little is known about which approach will be effective and economical to accomplish this goal (Paphitou, 2013).

Conversely, the use of nanotechnology for drug delivery is widely anticipated to alter the landscape of the pharmaceutical and biotechnology industries in the upcoming years (Farokhzad and Langer, 2009). The noteworthy advantages of using nanotechnology for treating numerous illnesses by improving the solubility, efficacy, bioavailability, and specificity of drugs are extensively acknowledged in the literature (Kalhapure *et al.*, 2014). Nanotechnology refers to the design, production, and application of nanosized materials, and is considered to be a new paradigm to enhance the outcomes in infectious diseases treatment (Huh and Kwon, 2011). New nanosized drug delivery systems might be a favourable approach to overcome the existing challenges related to antibiotic therapy due to their unique physicochemical properties (Figure 2).

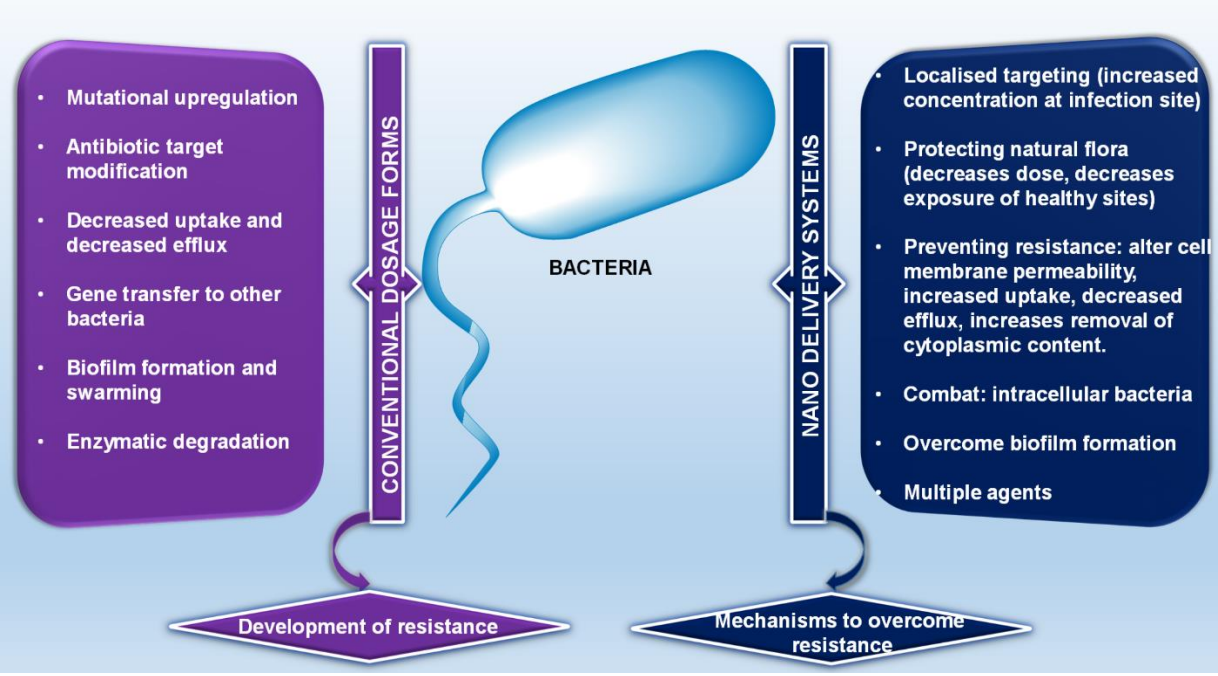

Figure 2. Development of resistance with conventional dosage forms and the mechanisms used to overcome resistance using nano delivery systems.

These include their small size, large ratio of surface area to mass, and unique interactions with microorganisms and cells of the host, in addition to their capability to be structurally and functionally modified (Kalhapure *et al.*, 2014; Zhang *et al.*, 2007; Zhang *et al.*, 2010). These systems offer various advantages, such as targeted delivery to the infection site, fairly even distribution in the identified tissue, better cellular bacterial internalisation and solubility, sustained drug release, and improved patient compliance, thereby increasing the efficiency and efficacy of therapy, and concurrently reducing side effects (Mansour *et al.*, 2009; Salouti and Ahangari, 2014; Sosnik *et al.*, 2010). Figure 2 represents the characteristics of an optimal nanocarrier.

Figure 3. The characteristics of an ideal nanocarrier. Adapted from Abed and Couvreur, 2014.

More notably, inherent mechanisms to overcome resistance include: photocatalytically manufacturing reactive oxygen species (ROS), which harm cellular and viral constituents, compromising of the bacterial cell wall/membrane, disrupting energy transduction, and inhibiting enzyme action and DNA synthesis (Figure 4) (Huang *et al.*, 2008; Huh and Kwon, 2011; Kim *et al.*, 2007; Li *et al.*, 2008; Maness *et al.*, 1999; Pal *et al.*, 2007; Rabea *et al.*, 2003; Weir *et al.*, 2008).

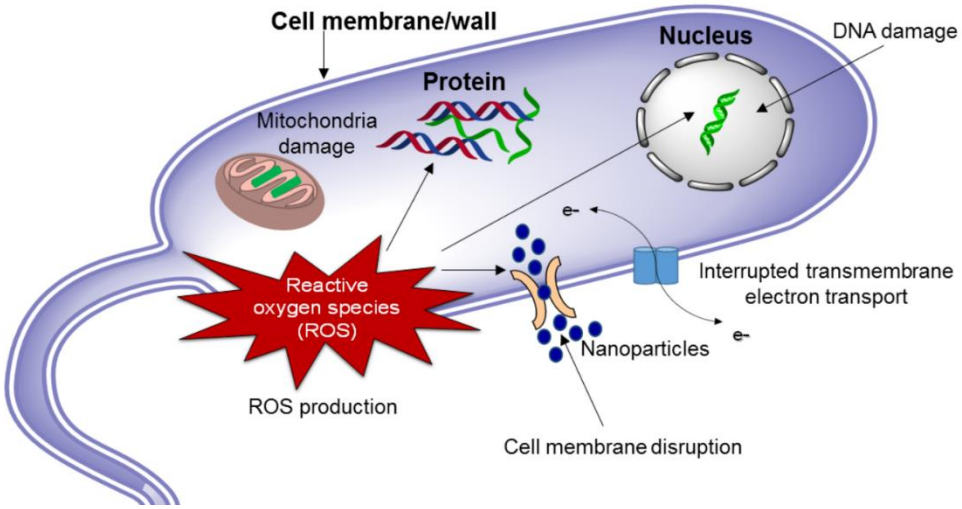

Figure 4. Various antimicrobial mechanisms of nanomaterials. Adapted from Huh and Kwon, 2011, and Brooks and Brooks, 2014.

The utilisation of nanosystems as delivery vehicles for antimicrobial agents proposes a novel and promising model in designing effective therapeutics against numerous pathogenic microbes (Salouti and Ahangari, 2014), as these systems have been found to overcome prevailing specific drug-resistance mechanisms by microorganisms (Pelgrift and Friedman, 2013). Moreover, combining a few antibiotics into these nanosystems, which are capable of overcoming resistance mechanisms and having antimicrobial activity, can encourage synergistic activities and resistance overcoming effects (Zhang *et al.*, 2010). These benefits are documented as the main contributors to incapacitating bacterial resistance related to poor delivery of antibiotics (Blecher *et al.*, 2011). Nanodrug delivery systems thus propose an innovative and superior method to overcoming numerous limitations linked to the currently available antibiotic drug therapies, including the severe worldwide threat of antibiotic resistance (Table 1). However, when compared to cancer and cardiovascular disease conditions, the use of nanodrug delivery systems for specifically encapsulating and distributing antibiotic drugs is still in early stages (Huh and Kwon, 2011).

Table 1. The advantages and disadvantages of antimicrobial nanoparticles over free antimicrobial agents. Adapted from Huh and Kwon, 2011.

Antimicrobial nanoparticles	Free antimicrobial agents
Advantages	**Disadvantages**
Targeted drug delivery by specific accumulation	No specific accumulation
Lower chance of antimicrobial resistance	Higher chance of antimicrobial resistance
Less side effects of chemical antimicrobials	More side effects of chemical antimicrobials
Extended therapeutic lifetime owing to slow elimination	Shorter half-life owing to quick elimination
Controlled drug release	The usual pharmacokinetic properties of free drugs
Enhanced solubility	At times poor solubility
Wide-ranging therapeutic index	Narrow therapeutic index
Cost effective	Costly
Low immunosuppression	Immunosuppression
Disadvantages	**Advantages**
The accumulation of intravenously injected nanomaterials in tissues and organs	Complete absence of nanomaterials in the entire body
A high systemic contact to locally administered drugs	A low systemic contact to locally administered drugs
Nanotoxicity in the brain, kidneys, lungs, liver, metabolism, germ cells, etc.	The nonexistence of nanotoxicity
The deficiency of characterisation methods that are not affected by nanoparticles' properties	Well-established characterisation methods

2.5 Types of polymeric materials for nano-drug delivery

The use of formulation materials such as lipids and polymers are essential for the formulation of nanosystems. When compared to other classes of materials, polymeric materials, which include natural, seminatural, and synthetic polymers, offer limitless opportunities to control the properties of drug delivery systems properties, other than to meet numerous criteria, such as biocompatibility, biodegradability and reproducibility, due to their diversity in topology, chemistry and dimension. Different types of polymeric materials are continually being studied for drug delivery systems and include block copolymers (Kumar *et al.*, 2001; Qiu and Bae, 2006), dendrimers (Gillies and Frechet, 2005; Huh and Kwon, 2011; Inoue, 2000; Qiu and Bae, 2006), hyperbranched polymers (Chen *et al.*, 2008; Inoue, 2000; Qiu and Bae, 2006), as well as star polymers (Gao, 2012; Inoue, 2000; Qiu and Bae, 2006).

2.5.1 Block copolymers

Block copolymers can be defined as polymers with two or more blocks or segments assembling in the main chain (Figure 5), being categorised according to their architecture as AB-type diblock,

ABA- or BAB-type triblock, and multiblock, where A denotes the soluble block in a particular solvent and B designates the insoluble block (Kumar *et al.*, 2001; Qiu and Bae, 2006). They have the ability to manipulate their amphiphilic behaviour, as well as their mechanical and physical properties by modifying the ratio of the constituting block, or by adding new blocks of preferred properties (Kumar *et al.*, 2001).

Figure 5. Block copolymer. Adapted from Qiu and Bae, 2006.

Block copolymers in particular have gained considerable attention, not just due to the scale of their microdomains (tens of nanometres) and their numerous chemical and physical properties, but also because of their appropriate size and shape tunability of their microdomains attained by simply varying their molecular weights and compositions (Park *et al.*, 2003). Widespread work has been carried out over the last few decades, to synthesise block copolymers for drug delivery in nanoparticles, hydrogels, implants, micelles etc. (Kumar *et al.*, 2001). A method to attain these copolymers that allows surface modification is attaching polyethylene glycol (PEG) chains to a biodegradable polymer, such as poly(lactic acid) (PLA) or poly(lactide-co-glycolic acid) (PLGA). Although several possible uses of block copolymers for different nanotechnologies have been suggested, based primarily on their capability to form interesting patterns, the foremost challenge of using these polymers is with controlling their microstructure (Park *et al.*, 2003).

2.5.2 Dendrimers

Dendrimers are repeatedly branched molecules or nano-sized, radially symmetric molecules that have a well-defined, uniform and monodisperse structure that consists of branches surrounding a core (Figure 6) (Bosman *et al.*, 1999; Hari *et al.*, 2012).

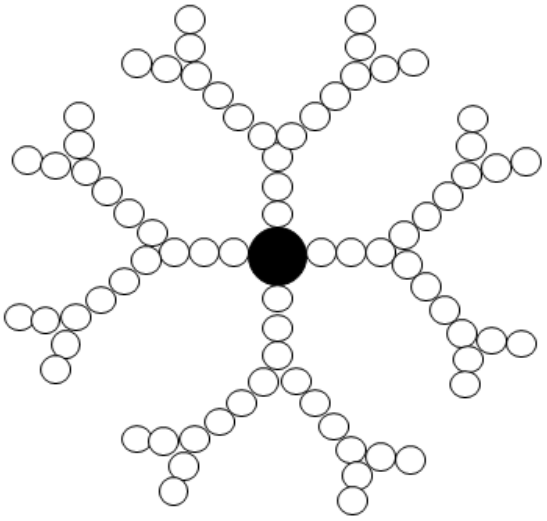

Figure 6. Dendrimer. Adapted from Qiu and Bae, 2006.

The availability of several functional surface groups, and their low polydispersity, make them a rich source for finding novel and unique properties (Hari *et al.*, 2012; Inoue, 2000). Due to these very distinctive properties, and the fact that they can be adapted to therapeutic needs, they are regarded as good carriers for small molecule drugs and biomolecules (Svenson, 2009). Dendrimers have gained further interest as likely antimicrobial agents due to the availability of numerous end groups and their compressed structure (Chen and Cooper, 2000; Polcyn *et al.*, 2013). Therefore, if any one of the functional groups is capable of interacting with a target, other groups within close proximity of one another could make synergistic interactions for antimicrobial activity possible (Chen and Cooper, 2000). Specific interactions (e.g. quaternary ammonium based dendrimers) aim to eliminate bacterial/viral infections by inhibiting the growth of microbes, thereby killing them and nonspecific interactions (e.g. oligosaccharide based dendrimers), and preventing the initial attachment between bacteria/viruses and host cells (Chen and Cooper, 2000).

It has also been highlighted that dendrimers show promising biocompatibility in general (Svenson, 2009), which is essential for their application, and can therefore be used as antimicrobial agents (Charles *et al.*, 2012; Chen *et al.*, 2000; Felczak *et al.*, 2012; Lopez *et al.*, 2009). Consequently, highly potent dendrimer based antibacterial agents have been synthesised (Charles *et al.*, 2012; Chen *et al.*, 2000). Currently, the dendrimers most extensively used for drug delivery include poly propylene imine (PPI), polylysine, triazine (Jain *et al.*, 2010) and polyamidoamine (PAMAM), the

latter being the first and most commonly studied (Svenson, 2009). Unfortunately, its uses are constrained by limitations such as cytotoxicity, which results from its amine-terminated nature (Sosnik *et al.*, 2010), and as a result, there are no commercially available dendrimer based formulations for systemic administration (Jain *et al.*, 2010). Conversely, hand carboxylic- or hydroxyl-terminated PAMAM dendrimers, which seem to be more biocompatible and less toxic than unmodified ones, can be simply conjugated with antimicrobial agents through abundant functional groups (Gillies and Frechet, 2005; Sosnik *et al.*, 2010). Numerous other antimicrobial drugs have been successfully combined into dendrimer nanoparticles for better solubility and, thus, therapeutic efficacy (Table 2).

Table 2. Polymer-based nanocarriers for antimicrobial drug delivery. Adapted from Huh and Kwon, 2011.

Type of Nanocarrier: Dendrimers	Polymer	Encapsulated antibiotics	Target microorganism	Mechanism for enhanced therapeutic effects	Ref.
	PAMAM	Silver salts	*Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli*	A high payload and lengthy circulation half-life	(Suri *et al.*, 2007)
	PLCP	Artemether	*Plasmodium falciparum*	Increased drug stability, improved solubility and lengthy drug circulation half-life.	(Bhadra *et al.*, 2005)
	PAMAM	Sulfamethoxazole	*Escherichia coli*	Sustained drug release, improved antibacterial activity by enhanced penetration of antibiotics through the bacterial membrane, aided by surface amine groups at a high density.	(Ma *et al.*, 2007)
	PAMAM	Nadifloxacin and Prulifloxacin	*Escherichia coli*	Enhanced water solubility with strong antimicrobial activity by greater penetration of antibiotics through the bacterial membrane.	(Cheng *et al.*, 2007)

A relatively new class of dendrimers, known as the poly (propyl ether imine) (PETIM) dendrimers, has been reported to have good biocompatibility when compared to commercial PAMAM dendrimers, and has been effectively applied for encapsulating ketoprofen for sustained drug delivery. (Jain *et al.*, 2010) Although it has several advantages, such as non-cytotoxicity and easy functional group modification at the periphery, its potential for antimicrobial therapy has not been exploited.

2.5.3 Hyperbranched and Star polymers

Hyperbranched polymers are highly branched macromolecules with a three-dimensional dendritic structure (Figure 7), and have gained growing attention due to their unique properties, relative ease of preparation and greater availability compared to dendrimers (Gao and Yan, 2004; Jikei and Kakimoto, 2001). Their structure is not as controlled as that of dendrimers, and their functional groups are not situated at an ordered position. Therefore, the degree of branching for dendrimers and hyperbranched polymer is quite different, although their overall compositions are similar. Thus, hyperbranched polymers may have intermediate properties between that of linear and dendritic polymers (Inoue, 2000). These biocompatible polymers display low toxicity and can be considered as candidates for drug delivery (Paleos *et al.*, 2010). As polymers act as a kind of matrix, they have been extensively utilised for trapping nanoparticles (Jeon *et al.*, 2008; Lu *et al.*, 2006; Spadaro *et al.*, 2010), with various hyperbranched polymers having been reported to have been used as stabilising agents for metal nanoparticles (Aymonier *et al.*, 2002; Zhang *et al.*, 2008).

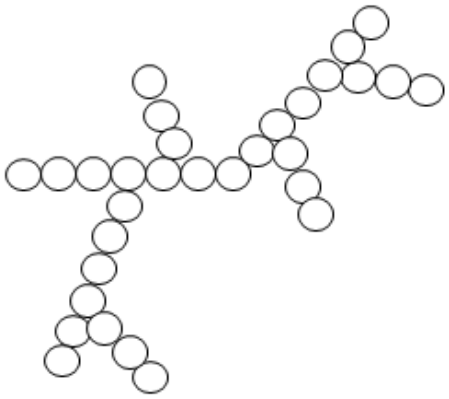

Figure 7. Hyperbranched polymer. Adapted from Qiu and Bae, 2006.

Star polymers are known as the simplest type of branched materials, wherein at least three linear polymer chains with essentially identical lengths are attached to only one branching point (core) (Figure 8) (Inoue, 2000; Kuzuu, 1980; Wu *et al.*, 2015). These polymers can contain chemically

identical or different arms (miktoarm star polymer) linked to the core.

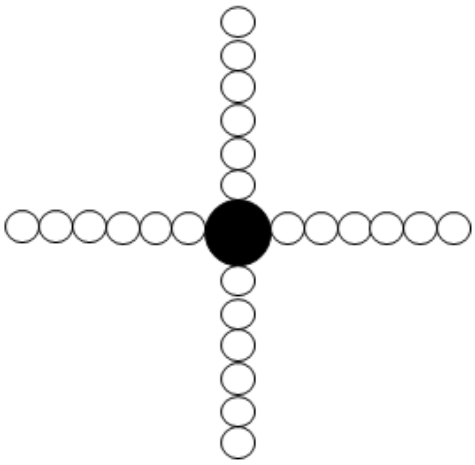

Figure 8. Star polymer. Adapted from Qiu and Bae, 2006.

Various methods have been applied to synthesise star polymers (Inoue, 2000), and have attracted considerable attention due to their unique topological structure, and the fact that their attractive physical and chemical properties are different from their linear counterparts (Jia *et al.*, 2014; Wu *et al.*, 2015). Usually, star polymers have a smaller hydrodynamic dimension, reduced solution and lower melt viscosities than their linear counterparts with equivalent molecular weights (Deng and Chen, 2004; Sun *et al.*, 2010). Moreover, they also have a higher degree of end group functionalities that are fairly important in specialised applications (Wang *et al.*, 2005b). Therefore, if the components of the star polymers are biodegradable or biocompatible, these copolymers can have potential biomedical applications, such as drug/gene delivery, tissue engineering, diagnosis, medical devices, and antibacterial/antifouling biomaterials (Jia *et al.*, 2014; Wu *et al.*, 2015).

Star polymers also display promising performance in sustained, controlled and targeted drug delivery, and have been extensively applied to improve drug delivery systems. Dendritic polymers are associated with the usual peripheral drug conjugation and loose internal drug loading space, while star polymers provide other internal drug-conjugated sites and looser drug loading space (She *et al.*, 2013; Wu *et al.*, 2015; Zhang *et al.*, 2014; Zhou *et al.*, 2014). Owing to exceptional drug loading capacity and controllable properties of star polymers, many researchers are currently using the latter for *in vivo* drug delivery studies. For example, a class of unimolecular nanocarriers based on star polymers has been explored to control drug release (Jones *et al.*, 2003; Lee *et al.*,

2011; Li *et al.*, 2012; Wu *et al.*, 2015). Star polymers could be a novel family of stabilising agent to prepare colloidal silver nanoparticles. Although an amphiphilic modified hyperbranched polyethyleneimine polymer has been used to stabilise silver nanoparticles and displayed certain attractive advantages, for instance, the quasi-spherical branched assembly with numerous inner cavities and nearly nonexistence of chain entanglements (Aymonier *et al.*, 2002), very few star polymers, particularly those that are water-soluble, have been utilised as stabilising agents so far (Huang *et al.*, 2012; Sun *et al.*, 2010; Zhang *et al.*, 2008). Finally, although PEG/dendrimer star polymers have been synthesised and applied previously (Hedden and Bauer, 2003; Yang and Lopina, 2003), according to our knowledge, they are yet to be utilised as stabilising agents to prepare metal nanoparticles.

2.6 G1 PETIM silver salts as antimicrobial agents

A comprehensive literature search indicated a broad array of metal complexes being investigated and various types of silver complexes being studied, with few studies on dendrimer-silver salts being noted. A knowledge gap remains despite researchers having recognised the potential of developing complexes of silver and dendrimers to enhance antimicrobial activity, with Balogh *et al.* having prepared PAMAM dendrimer based silver complexes (Balogh *et al.*, 2001), which showed enhanced antimicrobial effect, creating a new and potent antimicrobial agent for biomedical applications. The preparation of silver nanoparticles and silver nanocomposites are much more widely reported on compared to silver salts, and to the best of the researcher's knowledge, there are only two published papers thus far on dendrimer-silver salts (Balogh *et al.*, 2001; Ottaviani *et al.*, 2002). An overview of silver salts are presented in the following section.

2.6.1 Preparation of silver salts

A wide range of medicinal applications for metal complexes has been investigated with varying methods of preparation. To the researchers' knowledge, no review article was found that discusses the preparation of silver salts. Two methods discussing metal complexes and two discussing silver salts have therefore been explained hereunder.

For the first method, according to Creaven *et al.*, $Cu(hnc)_2(H_2O)_2]\ 2H_2O$ and [Ag(hnc)] complexes of hydroxynitrocoumarins were prepared in aqueous solution by deprotonating the hydroxyl group of 4-hydroxy-3-nitro-2H-chromen-2-one (hncH) with sodium hydroxide and then adding

copper(II) chloride dihydrate and silver(I) nitrate, respectively. [Ag(hmnc)] was synthesised in a similar manner, the mixed-ligand Ag(I) complex [Ag-(phen)2hnc] was prepared by treating silver(I) nitrate with phen and a subsequent reaction with a solution containing hncH and sodium hydroxide (Creaven *et al.*, 2005). Kleyi *et al.* prepared a solution of silver nitrate in ethanol, and 2-hydroxymethyl-N-alkylimidazole was then added. The reaction was stirred at room temperature for 24 hours and was then filtered. Ethyl acetate was added and the solvent evaporated slowly at atmospheric pressure to obtain silver complexes containing ligands (Kleyi *et al.*, 2012).

For the second method, silver dendrimer complexes were synthesised by Ottaviani *et al.* They explained that individual dendrimers formed complexes with an equal and well-defined number of metal atoms per dendrimer molecule, which were expressed as average numbers. 5 ml of silver nitrate stock solution was added dropwise with stirring to a weighed amount of PAMAM dendrimer stock solution in methanol. The volumetric flask was filled up to 10.00 mL with methanol, resulting in a solution of sample containing nitrogen ligands silver ions for the investigated solutions. The metal ion/dendrimer ratio was predetermined by the ratio of metal ion moles per dendrimer moles due to the uniformity of dendrimers and the isotropic nature of the diffusion (Ottaviani *et al.*, 2002). Balogh *et al.* prepared silver containing PAMAM complexes by adding aqueous solutions of the dendrimers to the calculated amount of silver acetate powder. Although silver acetate is poorly soluble in water, it dissolved rapidly in PAMAM solutions. This was due to the combined action of the silver-carboxylate formation and/or to the complex formation with the internal nitrogens. The procedure resulted in slightly yellow dendrimer-complex/salt solutions (Balogh *et al.*, 2001).

2.6.2 Characterisation of silver salts

As mentioned above, as no review articles was found discussing silver salts, a few characterisation methods of metal complexes and silver salts are briefly discussed. Infrared spectra is most often used to characterise silver salts. Salts of carboxylic acids do not display any of the carbonyl bands rather bands owing to the asymmetric and symmetric stretching vibrations of the equivalent carbon-oxygen bonds. They are observed at 1610-1550 cm^{-1} and 1420-1300 cm^{-1} respectively, which provides evidence for the carboxylate anion (Vogel and Furniss, 1989). Noteworthy changes in absorption frequencies reported by Creaven *et al.*, between the free and coordinated ligands,

were $\nu_{asym}(NO_2)$ stretch of the nitro group, the $\nu(C=O)$ and $\nu(C–O)$ stretches of the lactone (in hncH), and the phenoxy alcohol $\nu(O–H)$ stretch (in hmncH) (Creaven *et al.*, 2005). Kleyi *et al.* also reported differences in spectra between their complexes and free ligands. The IR spectra of silver(I) complexes displayed bands in the 3500–3100 cm^{-1} region, showing the occurrence of –OH functional groups. The occurrence of these bands also showed that the –OH groups were not contributing in the coordination to the metal centre. Imidazoles vibrational bands at 1450–1300 cm^{-1} seem somewhat shifted to lower frequencies in the complexes compared to the free ligands, and the bands also seem to display a noticeable broadening. The shifting and broadening of these bands showed the coordination of the ligands through the C=N nitrogen atoms of imidazoles (Kleyi *et al.*, 2012).

Electron paramagnetic resonance (EPR) technique has been effectively applied previously (Balogh and Tomalia, 1998) to increase an understanding of the detailed assembly of copper(II) complexes of amine and methylester terminated PAMAM dendrimers. As these ions have been studied widely, they can be used as reporter ions. Computer-aided analysis of the EPR spectra offers information on the development of copper complexes in numerous internal or external locations of the dendrimers, together with the structure of the complexes and nanocomposites as a function of the size and surface of the dendrimers, temperature, silver content, etc. (Ottaviani *et al.*, 2002).

2.7 Overview of silver nanoparticles

Noble metal nanoparticles have attracted attention due to their unique properties and potential applications in numerous areas, such as sensors, medicine, catalysts and electronics (Huang *et al.*, 2012; Jain *et al.*, 2008; Wuithschick *et al.*, 2015). Various kinds of metal nanomaterials have been studied to date, however, silver nanoparticles are shown as being most effective and promising in biomedical and pharmaceuticals (Rai *et al.*, 2009). This can be ascribed to their good antimicrobial efficacy against not only bacteria, but also viruses and other eukaryotic micro-organisms (Gong *et al.*, 2007; Rai *et al.*, 2009). While elemental silver and silver salts have been recognised as antimicrobial agents in preventive and curative health care since ancient times (Dallas *et al.*, 2011), their use could cause unwanted adsorption of ions in the sweat glands and epidermal cells (Dallas *et al.*, 2011; Russell and Hugo, 1994; Silver and Phung, 2005). Hence, silver nanoparticles appear to be a better candidate compared to the silver cation salts and complexes (Dallas *et al.*, 2011).

Furthermore, the importance of silver nanoparticles, as promising antibacterials, lies in the fact that, unlike commercial antibiotics, they do not damage useful enzymes in the host (Dallas *et al.*, 2011). Silver nanoparticles have unique physical and chemical properties, and are considered an alternate for developing novel antibacterial agents. Furthermore, they have varied medical applications, such as coatings for medical devices, wound dressings and textile fabrics (Rai *et al.*, 2009). Bacterial resistance has also not yet been detected with the use of silver nanoparticles. This is apparently a result of the difference in the mechanism of the antibacterial actions of the diverse forms of silver (Gogoi *et al.*, 2006; Kvitek *et al.*, 2008; Yamanaka *et al.*, 2005). It is extremely unlikely that resistance to antimicrobial silver might ever develop, as this would mean that an organism would have to undertake simultaneous mutations in every critical function within just a single generation to evade the compounds multiple actions (Gibbins and Warner, 2005).

2.7.1 Silver nanoparticles as antimicrobial agents

The use of silver to treat bacterial infections became unpopular due to the introduction of penicillin in the 1940s (Chopra, 2007; Huh and Kwon, 2011). However, the recent occurrence of antibiotics-resistant bacteria, and the inadequate efficacy of antibiotics, resuscitated the clinical use of silver, for example in wound dressings (Huh and Kwon, 2011; Taubes, 2008). Various researchers have studied the bactericidal efficacy of silver nanoparticles, and their effective potential against a wide range of organisms has been proven (Table 3) (Rai *et al.*, 2012).

Table 3. Activity of silver nanoparticles against a wide spectrum of bacteria. Adapted from Rai *et al*., 2012.

Form of silver	Target organisms	References
Silver ions	*Escherichia coli* and *Staphylococcus aureus*	(Feng *et al.*, 2000)
Silver nitrate	Periodontal pathogens	(Spacciapoli *et al.*, 2001)
Silver zeolite	*Escherichia coli*	(Matsumura *et al.*, 2003)
Silver nanoparticles	*Escherichia coli*	(Pal *et al.*, 2007; Sondi and Salopek-Sondi, 2004)
Silver ions	RNA viruses	(Butkus *et al.*, 2004)
Silver nanoparticles	*Escherichia coli*, *Vibrio cholera, Salmonella typhus*, and *Pseudomonas aeruginosa*	(Morones *et al.*, 2005)
Silver nanoparticles	*Escherichia coli* in liquid and solid medium	(Baker *et al.*, 2005)
Silver ions	*Escherichia coli*	(Yamanaka *et al.*, 2005)
Silver nanoparticles	*Escherichia coli* and *Staphylococcus aureus*	(Shahverdi *et al.*, 2007)
Super paramagnetic silver nanoparticles, bifunctional Fe_3O_4, Ag nanoparticles	*Escherichia coli*, *Bacillus subtilis* and *Staphylococcus epidermis*	(Gong *et al.*, 2007)
Nanofiber impregnated silver nanoparticles	*Escherichia coli* and *Staphylococcus aureus*	(Jia *et al.*, 2007)
Silver nanoparticles on cotton fabrics	*Staphylococcus aureus*	(Durán *et al.*, 2007)
Silver nanoparticles impregnanted on wound dressings	*Escherichia coli* and *Staphylococcus aureus*	(Maneerung *et al.*, 2008)
Silver nanoparticles	*Escherichia coli*, *Salmonella typhi*, *Staphylococcus epidermis* and *Staphylococcus aureus*	(Ingle *et al.*, 2008)
Silver nanoparticles	*Phoma golmerata*, *Phoma herbarum*, *Fusarium semitectum*, *Trichoderma sp.* and *Candida albicans*	(Gajbhiye *et al.*, 2009)
Silver nanoparticles	*Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*	(Birla *et al.*, 2009)
Silver nanoparticles	*Escherichia coli* and *Staphylococcus aureus*	(Gade *et al.*, 2010)
Silver nanoparticles	*Escherichia coli* and *Pseudomonas aeruginosa*	(Geethalakshmi and Sarada, 2010)
Silver nanoparticles	*Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*	(Bonde *et al.*, 2012)
Silver nanoparticles	*Pseudomonas aeruginosa*, *Staphylococcus aureus*, pathogenic fungi *Aspergillus flavus* and *Aspergillis niger*	(Govindaraju *et al.*, 2010)
Silver nanoparticles	*Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Bacillus subtilis*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*	(Namasivayam and Ganesh, 2011)
Silver nanoparticle coated medical devices	*Staphylococcus aureus* and *Streptococcus mutans*	(Ki-Young, 2011)
Bacterial cellulose-silver nanoparticle composite	*Escherichia coli* and *Staphylococcus aureus*	(Barud *et al.*, 2011)

In addition, silver nanoparticles display bactericidal potential against drug resistant bacteria (Rai *et al.*, 2012). A review of the antimicrobial potential of silver nanoparticles against drug resistant bacteria is stated in Table 4.

Table 4. The antimicrobial activity of silver nanoparticles against drug resistant bacteria. Adapted from Rai *et al*., 2012.

Drug resistant bacteria	References
MRSA	(Panáček *et al.*, 2006)
MRSA and non-MRSA	(Ayala-Núñez *et al.*, 2009)
Streptococcus mutans	(Espinosa-Cristóbal *et al.*, 2009)
MRSA, Methicillin sensitive *Staphylococcus epidermis* (MRSE) and *Streptococcus pyogenes*	(Nanda and Saravanan, 2009)
MRSA and MRSE	(Saravanan and Nanda, 2010)
Erythromycin-resistant *Streptococcus pyogenes*, Ampicillin-resistant *Escherichia coli* and multidrug-resistant *Pseudomonas aeruginosa*	(Lara *et al.*, 2010)
Staphylococcus aureus, Methicillin sensitive *S. aureus* (MSSA) and MRSA	(Ansari *et al.*, 2011)

The antimicrobial action of silver nanoparticles has an inverse relationship with shape (Pal *et al.*, 2007) and size (Raimondi *et al.*, 2005; Sondi and Salopek-Sondi, 2004). The combination of silver nanoparticles with antibiotics, for instance vancomycin, erythromycin, penicillin G and amoxicillin, lead to improved and synergistic antimicrobial effects against both Gram-positive and Gram-negative bacteria, such as *Escherichia coli* (*E.coli*) and *Staphylococcus aureus* (*S. aureus*) (Fayaz *et al.*, 2010; Rai *et al.*, 2009; Shahverdi *et al.*, 2007). While assorted uses of silver nanoparticles exist, lengthy contact to soluble silver comprising compounds could yield an irreparable pigmentation in the skin (argyria) and the eyes (argyrosis), along with added toxic effects, such as organ damage (liver and kidneys), irritation in the eyes and skin and alterations in blood cell counts (Drake and Hazelwood, 2005; Huh and Kwon, 2011). In contrast, metallic silver seems to pose a reduced risk to health, and silver nanoparticles have been proposed to possibly be non-toxic in certain studies (Johnston *et al.*, 2010; Oberdörster *et al.*, 2005). However, some studies stated concentration-dependent side effects of silver nanoparticles on mitochondrial activity (Braydich-Stolle *et al.*, 2005; Hussain *et al.*, 2005). The introduction of silver nanoparticles as likely antimicrobial nanomaterials necessitates clear and full clarifications of their possible toxicity (Huh and Kwon, 2011).

2.7.2 Mechanism of action of silver nanoparticles

Although the exact mechanism of action of silver on microbes is relatively unknown, the likely mechanism of action of metallic silver, silver ions and silver nanoparticles have been proposed in keeping with the structural and morphological modifications that originate in the bacterial cells

(Rai *et al.*, 2009). Silver nanoparticles specifically display effective antimicrobial properties compared to other salts due to their particularly large surface area, which affords better interaction with microorganisms. The nanoparticles attach themselves to the cell membrane and pierce into the bacteria. Silver nanoparticles interact with sulfur-containing proteins that are housed in the bacterial membrane, as well as with phosphorus containing compounds, such as DNA. Upon entry into the bacterial cell, silver nanoparticles form a low molecular weight area in the middle of the bacteria to which the bacteria agglomerate, thereby guarding the DNA from the silver ions. The nanoparticles favourably attack cell division, the respiratory chain inevitably causing cell death. The nanoparticles also discharge silver ions in the bacterial cells, which lead to improved bactericidal activity (Feng *et al.*, 2000; Morones *et al.*, 2005; Rai *et al.*, 2009; Sondi and Salopek-Sondi, 2004; Song *et al.*, 2006). The various actions of silver nanoparticles against bacteria is portrayed in Figure 9.

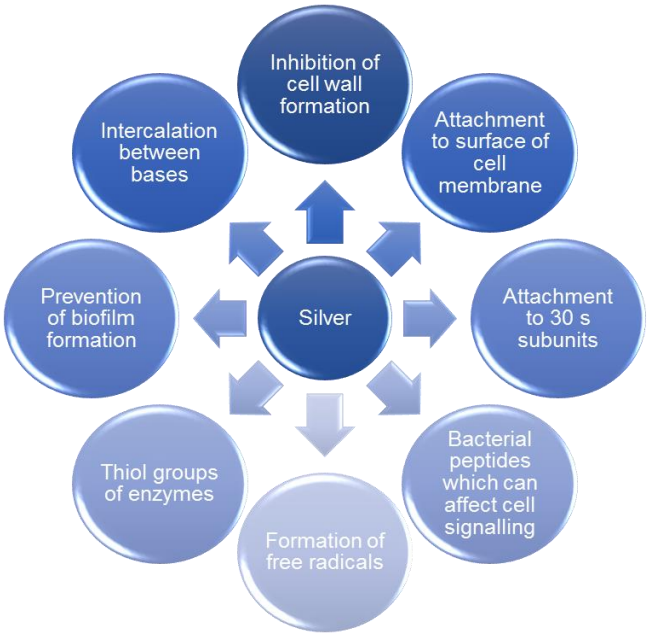

Figure 9. The multiple bactericidal actions of silver nanoparticles. Adapted from Rai *et al.*, 2012.

2.7.3 Preparation of silver nanoparticles

Various techniques for synthesising silver nanoparticles have been described, and include chemical reduction (Lee and Meisel, 1982; Song *et al.*, 2009; Wang *et al.*, 2005a), thermal decomposition (Navaladian *et al.*, 2007; Yang *et al.*, 2007), laser ablation (Chen and Yeh, 2002;

Mafuné *et al.*, 2000a; Simakin *et al.*, 2004), as well as sonochemical synthesis (Salkar *et al.*, 1999). Of these, chemical reduction and laser ablation are the most frequently used synthetic routes. Chemical reduction comprises the reduction of metal salt, such as silver nitrate, in a suitable medium by means of numerous reducing agents e.g. citrate, borohydride, to yield colloidal suspensions integrated by nanoparticles (Evanoff and Chumanov, 2005). Lee and Meisel explained the citrate reduction technique in which, silver nitrate was dissolved in distilled water and brought to a boil. A solution of 1% sodium citrate was then added and continued to boil for 1 h. The subsequent colloid was greenish yellow in colour and its absorption maximum was at 420 nm (Lee and Meisel, 1982). An alternate technique, usually referred to as the Creightons method, utilises sodium borohydride as the reducing agent in place of citrate (Creighton *et al.*, 1979). Generally, $NaBH_4$ is diluted with water in a volumetric flask, with nitrogen being bubbled through it and then placed in an ice bath to avoid degradation. Thereafter, a solution of silver nitrate is diluted with water in a volumetric flask, and lastly, the cold solution of $NaBH_4$ was added to the silver nitrate solution with vigorous stirring. This caused a colour change to light yellow, with stirring ensuing until the reaction reached room temperature. These synthetic procedures regularly produces particles of narrow size distribution (Ravindran *et al.*, 2013).

Thermal decomposition of metal complexes is another possible way of producing metal nanoparticles (Lee and Kang, 2004). If the product is metal and its decomposition temperature is low, thermal decomposition can be used for the synthesis of silver nanoparticles (Navaladian *et al.*, 2007). Silver nanoparticles can also be synthesised via irradiation. Metal atoms having a few metal clusters, which are ablated from a metal rod via laser ablation and are aggregated into metal clusters with adequately larger sizes (Mafuné *et al.*, 2000a). Laser irradiation can also formulate silver nanoparticles with a well-defined size and shape distribution (Simakin *et al.*, 2004), with no additional chemical reducing agent being necessary (Ravindran *et al.*, 2013). Additionally, the sonochemical reduction method produces high pressures and temperatures to reduce silver nitrate to metallic silver. This procedure is advantageous as it is relatively simple, efficient, and yields nanoparticles that are very small in size (Salkar *et al.*, 1999).

Finally, a pursuit for environmentally sustainable synthetic procedures has led to a few ‘green’ approaches (Raveendran *et al.*, 2003). Such a method needs to be evaluated based on a green chemistry viewpoint that comprises: choosing an appropriate solvent medium, choosing an

environmentally safe capping and reducing agent that contains extracts from bio organisms or plants, or a blend of biomolecules found in these extracts, for instance polysaccharides, amino acids, enzymes/proteins, and vitamins, and choosing nontoxic materials to stabilise the particles (Kalimuthu *et al.*, 2008; Ravindran *et al.*, 2013).

2.7.4 Characterisation of silver nanoparticles

There are a few methods for nanoparticle size characterisation, such as dynamic light scattering (DLS) (Cumberland and Lead, 2009; Martínez-Castañón *et al.*, 2008), UV-Vis spectroscopy (Navaladian *et al.*, 2007; Song *et al.*, 2009; Wang *et al.*, 2005a) using the move of the band gap of absorption in the UV-visible spectrum (Tomaszewska *et al.*, 2013), transmission electron microscopy (TEM) (Chen and Yeh, 2002; Navaladian *et al.*, 2007; Wang *et al.*, 2005a), and atomic force microscopy (AFM). It is also possible to calculate particle sizes using X-ray diffraction (XRD) patterns (Navaladian *et al.*, 2007; Wang *et al.*, 2005a). DLS measures fluctuations in the intensity of scattered light, this being initiated by particle movement, and covers a size range from a few nanometres to approximately 3 microns. However, it is important to note that both approaches do not 'measure' particle sizes, they detect light scattering effects that are used to calculate particle sizes (Mehnert and Mäder, 2001; Müller *et al.*, 2000).

Conversely, UV-Vis spectroscopy measures the intensity of light that passes through a sample. Nanoparticles have unique optical properties that are sensitive to shape, size, concentration changes and agglomeration. The properties of metal nanoparticles are a result of the collective oscillations of conduction electrons that are excited by electromagnetic radiation, and are known as surface plasmon polariton resonances (SPPR) (Evanoff and Chumanov, 2005). These changes affect the refractive index next to the nanoparticles surface, thereby making it possible to characterise nanomaterials by UV-Vis spectroscopy (Tomaszewska *et al.*, 2013).

DLS and UV-Vis spectroscopy are easy to operate and fast methods for particle characterisation, particularly for colloidal samples (Huang *et al.*, 2007; Leung *et al.*, 2006). DLS and UV-Vis methods have numerous advantages, including simplicity, sensitivity and selectivity to nanoparticles, short measurement time, and no need for calibration. While DLS is extensively used for particle characterisation, there are certain difficulties, specifically when measuring samples

that have large-size distribution or multimodal distributions (Khlebtsov and Khlebtsov, 2011; Zanetti-Ramos *et al.*, 2009). The mean diameter of monodisperse colloids can be determined by DLS, however, with polydisperse colloids, there is a possibility that tiny objects can be concealed by bigger ones, which may result in them not being seen.

When compared to DLS, TEM offers direct information about not only the morphology but also the size of particles at the same time. However, sample preparation for analysis is critical, which can be time consuming, necessitates high precision and the use of suitable reagents (Grobelny *et al.*, 2011; He *et al.*, 2000; Pethkar *et al.*, 2001). Microscopic methods can also be challenging in terms of polydisperse samples, as there is a possibility of sample fractionation or particle aggregation during drying.

AFM is gaining more appeal of late, and can be used to determine the geometric sizes of nanoparticles deposited on the surface (Tomaszewska *et al.*, 2013). It uses the force acting amongst a surface and a probing tip, resulting in a spatial resolution of up to 0.01 nm for imaging. A distinct advantage of this method is ease of sample preparation (Mehnert and Mäder, 2001; Müller *et al.*, 2000).

XRD is used to not only confirm the identity of nanoparticles by matching their diffractogram peaks to that of elemental silver, but it can also be used to confirm the size of nanoparticles using the Rietveld analysis (Martínez-Castañón *et al.*, 2008).

It is important to note that differences in the size of nanoparticles was observed with the different methods used, this being a function of the specificity of each rather than to any measurement errors. TEM and AFM measure geometric sizes of the nanoparticles deposited on the surface, thus results in these methods providing similar results. DLS measures hydrodynamic size, and light scatter from bigger silver nanoparticles is so intense that the scattered light from the smaller nanoparticles is screened. Consequently, the size of the measured nanoparticles can vary from those determined by AFM/TEM. Generally, each technique indicates that only monodisperse particles are present in the colloids (Tomaszewska *et al.*, 2013).

2.7.5 Stabilising agents in silver nanoparticle production

Despite the fact that silver nanoparticles have displayed good performance as antibacterials, a significant challenge currently associated with their application is identifying strategies to provide the satisfactory stability of their dispersions to prevent their nanoparticles from aggregating. Generating spacious aggregates shows a noteworthy drop in the activity of the nanoparticles, which results in inferior performance and a loss of antibacterial activity (Kvitek *et al.*, 2008; Shi *et al.*, 2015). Therefore, silver nanoparticles are frequently fabricated by reducing silver nitrate, which are then stabilised by capping agents to reduce the risk of aggregation that arises from the high surface area of nanoparticles (Shi *et al.*, 2015). To prevent particle agglomeration, numerous surfactants and polymers have been investigated to stabilise these metal colloids (Li *et al.*, 2015).

The improved stability of aqueous dispersions of the silver nanoparticles can be attained through two types of protecting mechanisms. The first mechanism of the dispersion system stabilisation is centred on an electrostatic repulsion. The addition of an ionic surfactant can enhance the surface charge of the disperse phase and provide electrostatic protection of the nanoparticles, which can result in their adhering to one another. Cetyltrimethylammonium chloride or bromide (CTAC, CTAB) (Yu and Yam, 2005), as the cationic surfactant group, and sodium dodecyl sulfate (SDS) (Mafuné *et al.*, 2000b), as the anionic surfactant group, have been utilised in numerous studies and are considered stabilising agents of considerable importance (Yu and Yam, 2005; Zheng *et al.*, 2003). The mechanism of surfactant adsorption, with regard to silver nanoparticle associations with ionic surfactants, has not yet been adequately explained. Nevertheless, a likely mechanism for arranging of the SDS molecules on the nanoparticle surface can be the hydrophilic groups of the surfactant molecules are adsorbed on the silver nanoparticle surface and the hydrophobic tails, are arranged outward to provide the first layer. Therefore, a counter-layer is arranged in the opposite way, causing interpenetration of the surfactant hydrophobic tails among the two layers, with the hydrophilic groups directed outward (Chen and Yeh, 2002; Kvitek *et al.*, 2008).

The second one, which is based on the steric repulsion, shows a stabilising effect with the aid of polymers and non-ionic surfactants that are instantly adsorbed at the phase interphase (Hunter, 2001). The equilibrium between the attractive and the repulsive forces is mainly reliant on the thickness of the adsorbed layer (Chou and Lai, 2004; Luo *et al.*, 2005), and, in the case of polymers,

is reliant on the chain length as well as on its adsorption mode. When compared to polymers, the non-ionic surfactants are adsorbed in a more compressed manner at the surface of the nanoparticles that gives an exceptional stabilising effect (Kvitek *et al.*, 2008; Liz-Marzán and Lado-Tourino, 1996). Polymers studied so far for stabilising silver nanoparticles include polyethylene glycols (PEG) (Popa *et al.*, 2007), poly(vinylalcohols) (PVA) (Chou and Ren, 2000), poly(vinylpyrrolidones) (PVP) (Silvert *et al.*, 1996), polyacrylamides (Chen *et al.*, 2006), polyurethanes (PU), (Chou *et al.*, 2006), as well as highly branched molecules such as dendrimers (Esumi *et al.*, 2000), hyperbranched polymers (Zhang *et al.*, 2008) and star polymers (Huang *et al.*, 2012).

2.8 Silver as a model antimicrobial agent

Silver is a potent antimicrobial agent, particularly in its' positively charged ionic form [e.g. silver nitrate (Figure 10a) and silver acetate (Figure 10b)], as it displays a strong toxicity to a wide range of micro-organisms, and concurrently has a particularly low human toxicity (Dallas *et al.*, 2011; Gibbins and Warner, 2005; Liau *et al.*, 1997).

(a) (b)

Figure 10. Types of ionic silver: (a) Silver nitrate, (b) silver acetate

Antimicrobial silver is widely used to combat organisms associated with burns and wounds (Gibbins and Warner, 2005). In addition, silver-based medical preparations are available and frequently used in biomedical material coatings, such as silver impregnated catheters and dressings for wound healing (Dallas *et al.*, 2011). Silver is also capable of disturbing key functions in a microorganism that causes AMR. It has a high affinity for negatively charged side groups on biological molecules, such as carboxyl, phosphate, sulfhydryl and others dispersed throughout microbial cells. It thereby transforms the macromolecule's molecular structure via this binding reaction, rendering it useless to the cell (Gibbins and Warner, 2005). Concomitantly, silver can attack numerous sites within the cell, incapacitating critical physiological functions, such as cell

wall synthesis, protein folding and function, membrane transport, nucleic acid (such as RNA and DNA) synthesis, as well as translation and electron transport, which are vital for cell energy production. Dispossessed of such key functions, bacterial growth can either be inhibited or, more frequently, the microorganism is killed (Gibbins and Warner, 2005). It is highly improbable that resistance to antimicrobial silver could ever develop, as this would mean that an organism would have to undertake concurrent mutations in every critical function within just a single generation to evade the compounds multiple actions. (Gibbins and Warner, 2005) This is a crucial factor to consider when developing new antimicrobial materials to overcome resistance. However, it should be noted that silver is nontoxic to human cells only in minute concentrations (Pal *et al.*, 2007). This clearly limits the use of metallic silver and silver ion as an antibacterial agent only up to concentrations that are non-toxic to eukaryotic cells.

2.9 Conclusions

This chapter highlighted the current status of infectious diseases, its drug therapy and their associated limitations. Strategies aimed at overcoming the current limitations associated with antibiotic drugs include complexes of silver and dendrimers, as well as star polymer stabilised silver nanoparticles. This literature review showed that although studies on silver nanoparticles and silver nanocomposites have been widely reported, very little work has been done on dendrimer silver salts and their potential uses as antimicrobial agents. Another noteworthy point was that a significant application difficulty related to silver nanoparticles is the satisfactory stability of their dispersions. The search for new polymers as stabilising agents is therefore of great importance to facilitate the application of silver nanoparticles as antimicrobials.

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CHAPTER 3. PUBLISHED PAPER

3.1 Introduction

The following paper was published in an international peer reviewed ISI journal and reports the original research from data generated during this study:

Suleman, N., Kalhapure, R., Mocktar, C., Rambharose, S., Singh, M., Govender, T., 2015. Silver salts of carboxylic acid terminated generation 1 poly (propyl ether imine) (PETIM) dendron and dendrimers as antimicrobial agents against *S.aureus* and MRSA. Royal Society of Chemistry Advances, 5, 34967-34978. (IF = 3.708)

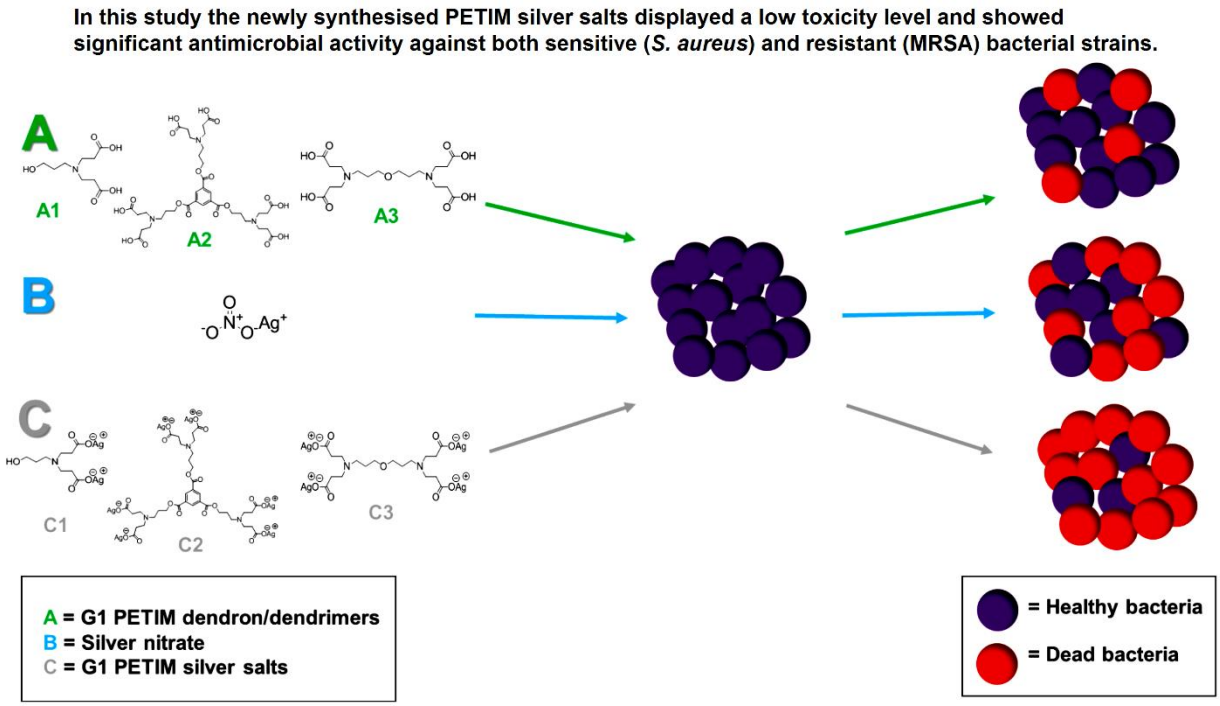
Ms. N Suleman contributed to the design of the project, and the preparation and characterisation of all G1 PETIM dendron/dendrimers and PETIM-silver salts in terms of synthesis, IR, NMR, HRMS, *in vitro* cytotoxicity and *in vitro* antimicrobial studies, along with interpretation of the data and writing of the paper. Dr R. Kalhapure assisted with the design of the project, as well as the interpretation of characterisation data of all synthesised materials in terms of IR, NMR and HRMS. Dr C. Mocktar assisted with the *in vitro* antimicrobial study. Mr S. Rambharose and Dr M. Singh assisted with the *in vitro* cytotoxicity study. The remaining author served as supervisor.

This chapter is presented in the required format of the journal and is the final revised and accepted version. The published article (DOI:10.1039/c5ra03179f) can be found in appendix A.

3.2 Published paper

Silver salts of carboxylic acid terminated generation 1 poly (propyl ether imine) (PETIM) dendron and dendrimers as antimicrobial agents against *S.aureus* and MRSA

Nadia Suleman[a], Rahul Kalhapure[a], Chunderika Mocktar[a], Sanjeev Rambharose[a], Moganavelli Singh[b] and Thirumala Govender[a]



Abstract

Novel therapeutic strategies are essential to address the current global antimicrobial resistance crisis. Branched molecules with multiple peripheral functionalities, known as dendrimers, have gained interest as antimicrobials and have varying levels of toxicity. Silver displays activity against several micro-organisms only in its positively charged form. In this study, silver salts of generation 1(G1) poly (propyl ether imine) (PETIM) dendron and dendrimers were synthesised and evaluated for their antimicrobial potential against sensitive and resistant bacteria. The purpose was to exploit the multiple peripheral functionalities of G1 PETIM dendron and dendrimers for the formation of silver salts containing multiple silver ions in a single molecule for enhanced antimicrobial activity

at the lowest possible concentration. G1 PETIM dendron, dendrimers and their silver salts were synthesised and characterised by FT-IR, ^{1}H NMR and ^{13}C NMR. PETIM silver salts were evaluated against Hep G2, SKBR-3 and HT-29 cell lines for their cytotoxicity using the MTT assay. The G1 PETIM dendron/dendrimers, silver nitrate and silver salts of the G1 dendron (compound **13**), G1 dendrimer with an aromatic core (compound **14**) and an oxygen core (compound **15**) were evaluated for activity against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) by the broth dilution method. PETIM silver salts were found to be non-cytotoxic even up to 100 µg/ml. Minimum inhibitory concentration values of compounds **13**, **14** and **15** against *S. aureus* were 52.1, 41.7, and 20.8 µg/ml while against MRSA they were 125.0, 26.0 and 62.5 µg/ml respectively. The calculated fractional inhibitory concentration index further indicated that compound **14** specifically displayed additive effects against *S. aureus* and synergism against MRSA. The enhanced antimicrobial activities of the PETIM dendron/dendrimer-silver salts against both sensitive and resistant bacterial strains widen the pool of available pharmaceutical materials for optimizing treatment of bacterial infections.

Keywords Dendrimer · Silver nitrate · Antimicrobial · Poly (propyl ether imine) · *S. aureus* · MRSA

Introduction

Infectious diseases, a significant portion of which are of bacterial origin, are one of the leading causes of death globally for adults and children and remains a major public health issue for developed and developing countries. [1] While antibiotics revolutionized the treatment of infections, thereby saving millions of lives, eighty years after their discovery, their effectiveness is seriously threatened by antimicrobial resistance (AMR). [2] This nullifies the use of even the most potent antibiotics, which leads to patient suffering and/or dying due to infection control failure, and results in escalated health care costs. [3]

Globally, resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [4], vancomycin-resistant *Enterococcus* (VRE) [5] and vancomycin-resistant *Staphylococcus*

aureus (VRSA), [6] have become significant threats in community settings and hospitals for treatment of infections. Furthermore, if current escalating trends in AMR continue, several important procedures, such as cancer chemotherapy, organ transplantation and hip and other joint replacements, could no longer be performed for fear that the related compromised immune system might put the patients at severe risk of acquiring a difficult to treat and ultimately fatal infection. [7] The global AMR crisis is amplified by the decreasing development of new antibiotics by pharmaceutical companies[8], with 20 novel classes of antibiotics being developed in between 1930-1962 [9, 10], and only two of them have been marketed.[11-14] This decline in drug development is due to the high costs and lengthy delays associated with developing a new chemical entity, high attrition rates at final testing, and increasing AMR, which makes finding a new drug very expensive and limits the return on investment. [3, 15]

It is therefore essential that alternative novel antimicrobial therapeutic strategies are explored to address the imminent crisis with conventional antibiotics. Alternative options currently being investigated are novel drug delivery systems for existing antibiotics, such as silver nanoparticles [16-18], solid lipid nanoparticles [19-21], liposomes [22-24] and the synthesis of new antimicrobial materials, such as dendrimers [25-27] and antimicrobial peptides. [28]

Silver is a potent antimicrobial agent, particularly in its positively charged ionic form, as it displays a strong toxicity to a wide range of micro-organisms and concurrently has a particularly low human toxicity. [29-31] Antimicrobial silver is widely used to combat organisms associated with burns and wounds. [30] In addition, silver-based medical preparations are available and frequently used in coatings of biomedical materials, such as silver impregnated catheters and dressings for wound healing. [29] Silver is also capable of disturbing key functions in a microorganism that causes AMR. It has a high affinity for negatively charged side groups on biological molecules, such as carboxyl, phosphate, sulfhydryl and others dispersed throughout microbial cells. It thereby transforms the macromolecule's molecular structure via this binding reaction, rendering it useless to the cell. [30] Concomitantly, silver can attack numerous sites within the cell, incapacitating critical physiological functions, such as cell wall synthesis, protein folding and function, membrane transport, nucleic acid (such as RNA and DNA) synthesis, and translation and electron transport, which are vital for cell energy production. Dispossessed of such key functions, bacterial growth can either be inhibited or, more frequently, the microorganism is killed. [30] It is highly improbable that resistance to antimicrobial silver could ever develop, as this would mean that an organism

would have to undertake concurrent mutations in every critical function within just a single generation to evade the compounds multiple actions. [30] This is a crucial factor to consider when developing new antimicrobial materials to overcome resistance. However, it should be noted that silver is nontoxic to human cells only in minute concentrations. [32] This clearly limits the use of metallic silver and silver ion as an antibacterial agent only up to concentrations that are non-toxic to eukaryotic cells.

Dendrimers are repeatedly branched molecules or nano-sized, radially symmetric molecules that have a well-defined, uniform and monodisperse structure that consists of branches surrounding a core. [33, 34] The availability of several functional surface groups and their low polydispersity make them a rich source for finding novel and unique properties. [33, 35] Due to these very distinctive properties, and the fact that they can be adapted to therapeutic needs, they are regarded as model carriers for small molecule drugs and biomolecules. [26] Dendrimers have gained further interest as likely antimicrobial agents due to the availability of numerous end groups and their compressed structure. [36, 37] Therefore, if any one of the functional groups is capable of interacting with a target, other groups within close proximity of one another could make synergistic interactions for antimicrobial activity possible. [36] Specific interactions (e.g. quaternary ammonium based dendrimers) aim to eliminate bacterial/viral infections by inhibiting the growth of microbes, thereby killing them and nonspecific interactions (e.g. oligosaccharide based dendrimers), and preventing the initial attachment between bacteria/viruses and host cells. [36]

It has also been highlighted that dendrimers show promising biocompatibility in general [26], which is essential for their application, and can themselves be used as antimicrobial agents. [38-41] Consequently, highly potent dendrimer based antibacterial agents have been synthesised. [38, 40] Currently, the most extensively used dendrimers in drug delivery include poly propylene imine (PPI), polylysine, triazine [42] and polyamidoamine (PAMAM), the latter being the first and most commonly studied. [26] Unfortunately, its uses are constrained by limitations such as cytotoxicity resulting from its amine-terminated nature [43] and as a result, there are no commercially available dendrimer based formulations for systemic administration. [42] Researchers have recognized the potential of developing complexes of silver and dendrimers to enhance antimicrobial activity, with Balogh *et al.* having prepared PAMAM dendrimer based silver complexes [44], which showed

enhanced antimicrobial effect, creating a new and potent antimicrobial agent for biomedical applications.

A fairly new class of dendrimers, known as the poly (propyl ether imine) (PETIM) dendrimers, has been reported to have good biocompatibility when compared to commercial PAMAM dendrimers, and has been effectively applied for encapsulation of ketoprofen for sustained drug delivery. [42] Although it has several advantages, such as non-cytotoxicity and easy functional group modification at the periphery, its potential for antimicrobial therapy has not been exploited. This study is therefore the first combination of PETIM dendrimers and silver to identify novel antimicrobial materials effective against both sensitive and resistant bacterial strains, and will widen the pool of available pharmaceutical materials to optimize the treatment of bacterial infections.

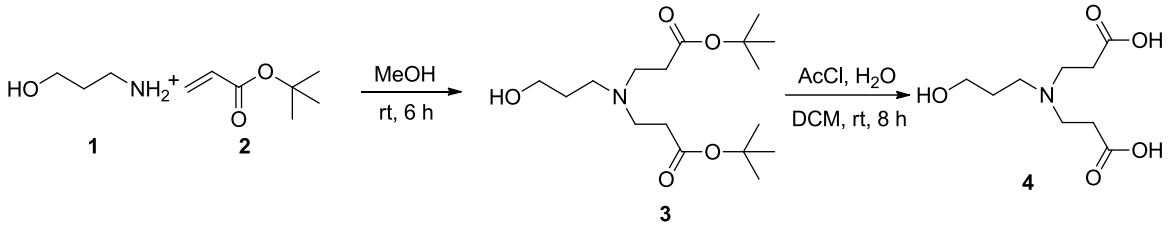
In this study, a generation 1 (G1) PETIM dendron and two PETIM dendrimers containing a carboxylic acid function at the periphery were synthesised and reacted with silver nitrate to form dendrimer-silver salts. The PETIM dendrimers were used as templates to contain the silver ions. The rationale for using PETIM dendrimer and dendron as a template to contain silver ions were: i) more than one silver ion can be accommodated on a single PETIM dendrimer or dendron, as it contains multiple carboxylic acid functions at the periphery; ii) PETIM silver complexes is nontoxic to mammalian cells due to the biocompatibility of PETIM dendrimers; and iii) PETIM on its own could display antimicrobial activity, thus the potential antimicrobial activity of PETIM silver complexes may display additive or synergistic effects. Published studies on silver complexes of organic compounds as antimicrobial agents mostly include two organic molecules complexed with one silver ion through a chemical bond formation. [45, 46] In the present investigation, our goal was therefore to exploit the multiple peripheral functionalities of biocompatible PETIM dendron and dendrimers to form silver salts containing multiple silver ions in a single molecule for enhanced antibacterial activity at the lowest possible concentration. The intention was to study the effect of the number of silver ions per molecule of these dendrimer silver salts on antimicrobial efficacy against both sensitive and resistant strains. For this reason, a G1 PETIM dendron (two carboxylic acid functions at the periphery), G1 PETIM dendrimer with oxygen core (four carboxylic acid functions at the periphery) and G1 PETIM dendrimer with aromatic core (six carboxylic acid functions at the periphery) were selected. The results of the investigations are reported in this paper.

Results and Discussion

Synthesis

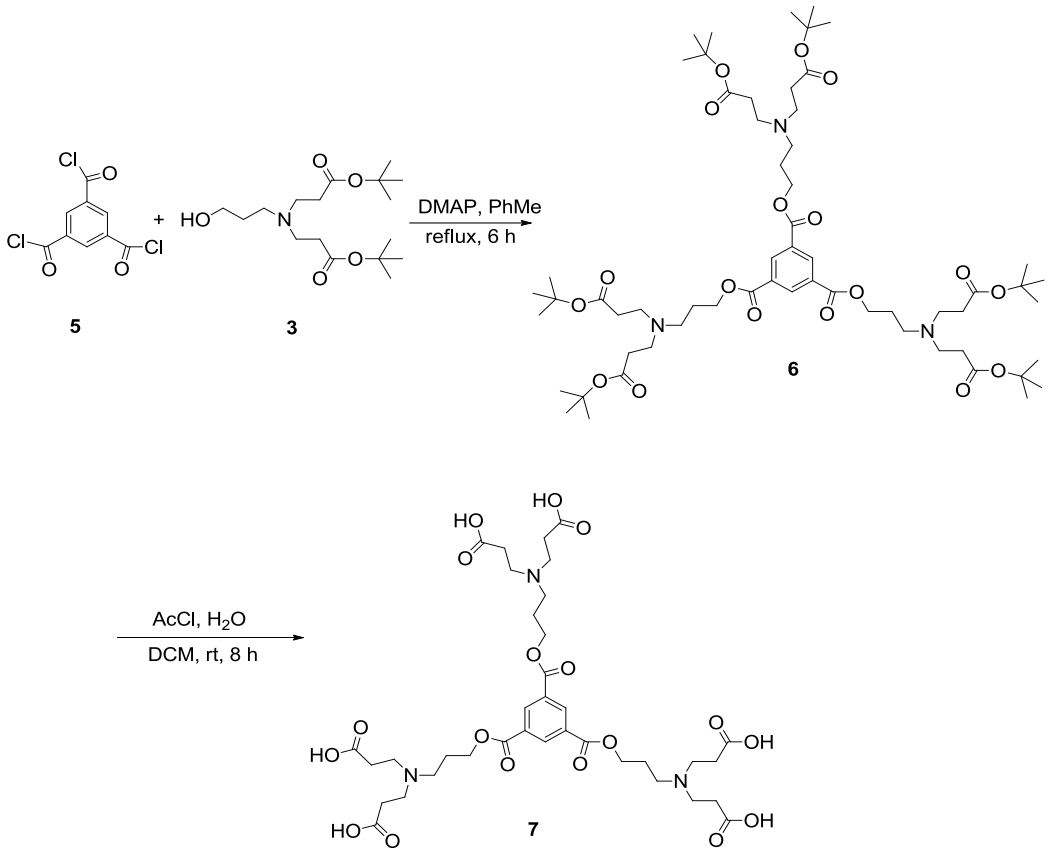
In this study three different compounds were employed, viz., a G1 PETIM dendron **4**, a PETIM dendrimer with an aromatic core **7**, and another PETIM dendrimer with an oxygen core **12**. Synthetic steps for these compounds are depicted in **Scheme 1-3** and explained hereunder.

The dendron was prepared using 3-amino-1 propanol **1** and excess *tert*-butyl acrylate **2** to afford an ester in good yield. Thereafter the resulting ester was deprotected (AcCl, H_2O) to obtain the free carboxylic acid containing G1 PETIM dendron **4** (**Scheme 1**).



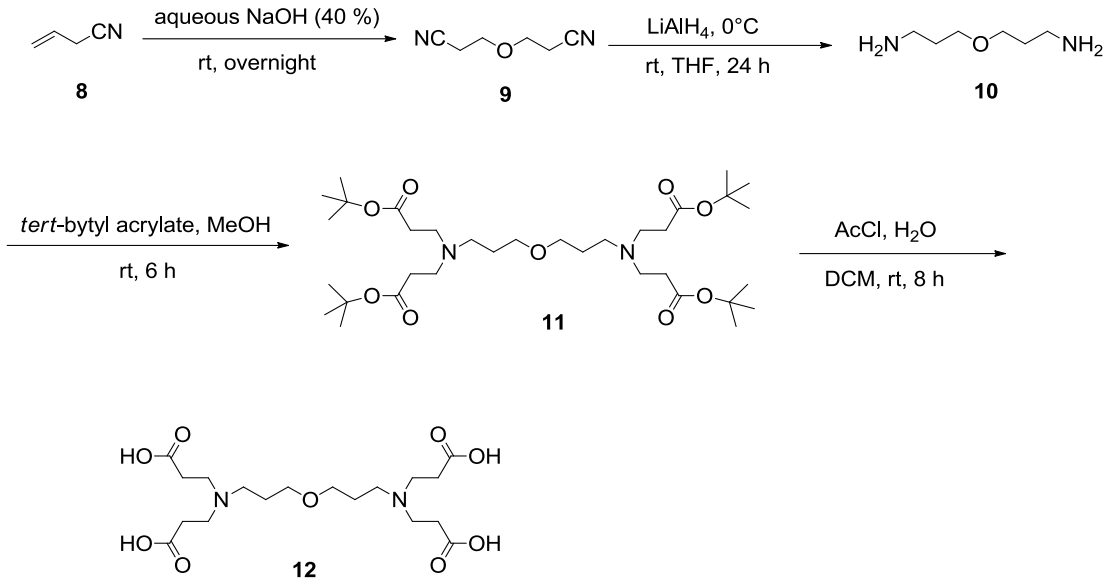
Scheme 1 Synthesis of G1 PETIM dendron.

The two dendrimers synthesised were prepared with slight modifications in a previously reported method [47]. Upon synthesis of the dendron **3**, its attachment to a selected core was carried out. Compound **3** was coupled with 1,3,5-benzenetricarbonyl trichloride **5** in the presence of DMAP to attain **6.** Thereafter the resulting ester was deprotected via an acetyl chloride and water system to attain the free carboxylic acid containing G1 PETIM dendrimer with an aromatic core **7** (**Scheme 2**).



Scheme 2 Synthesis of G1 PETIM dendrimer containing an aromatic core.

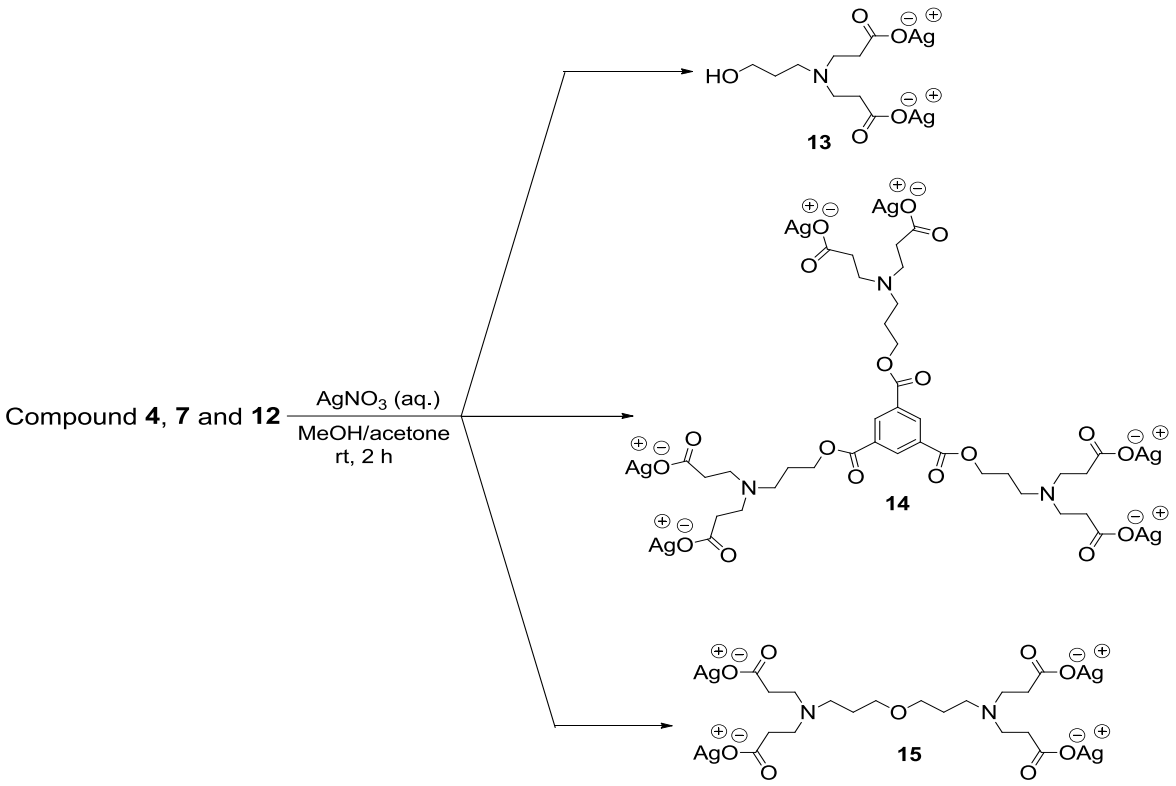
Bis-nitrile **9** was attained from acrylonitrile **8** and aqueous NaOH (40%). Bis-nitrile was subjected to successive reactions; i.e. reduction of the nitrile using $LiAlH_4$ to a diamine **10**; Michael addition of *tert*-butyl acrylate to afford the tetrakis **11**; and deprotection of the ester (AcCl, H_2O) to attain the free carboxylic acid containing G1 PETIM dendrimer with an oxygen core **12** (**Scheme 3**).



Scheme 3 Synthesis of G1 PETIM dendrimer containing an oxygen core.

Preparation of PETIM-silver salts (**Scheme 4**)

PETIM silver salts (**13**, **14** and **15**) were all prepared in a similar method where silver was reacted with **4**, **7** and **12** to afford these PETIM-silver salts.



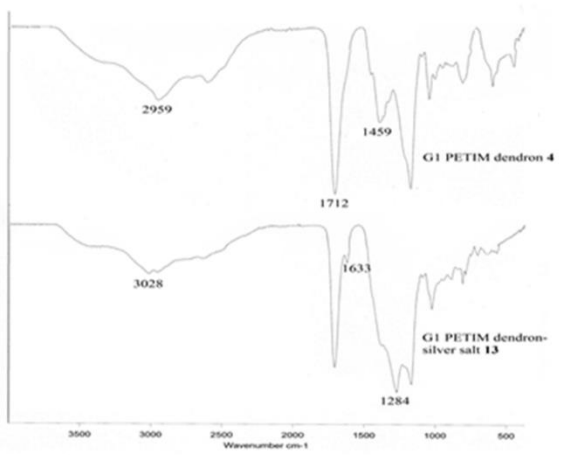
Scheme 4. Synthesis of silver salts of G1 PETIM dendron and dendrimers.

Characterisation

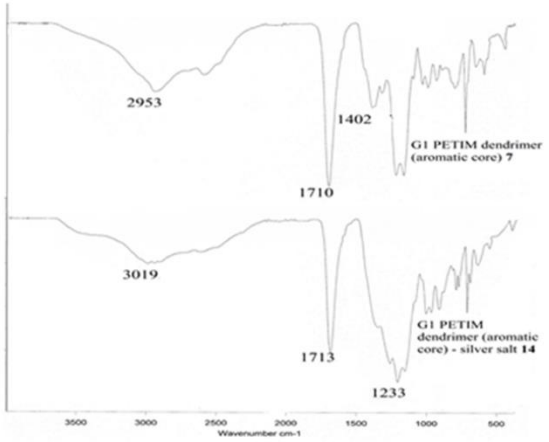
The synthesised dendron and dendrimers were characterised by FT-IR, ^{1}H NMR, ^{13}C NMR and HRMS and were compared with the literature values. [47] Synthesis of the silver salts were accomplished via reaction of silver nitrate with the corresponding dendron/dendrimer acid. Formation of silver salts was supported by observing the shifts in the positions of characteristic IR frequencies of carboxylic groups in the dendron and dendrimers.

The main feature which allows one to differentiate a carboxylic acid from all other carbonyl compounds is a broad absorption band due to the strongly hydrogen bonded O-H stretching vibrations which extends from 3300 cm-2500 cm $^{-1}$. The transformation of the ester function to a carboxylic acid was confirmed by the presence of this characteristic peak in FT-IR spectrum. In addition, all carboxylic acid terminated dendron/dendrimers exhibited a peak in the range of 1707-1714 cm^{-1} indicating the presence of a C=O stretching band of the –COOH group. The aliphatic C-H stretching band appeared as a jagged peak near 3000 cm^{-1}. Coupled vibrations involving C-O stretching were observed in the range of 1459-1399 cm^{-1}. Salts of carboxylic acids do not display

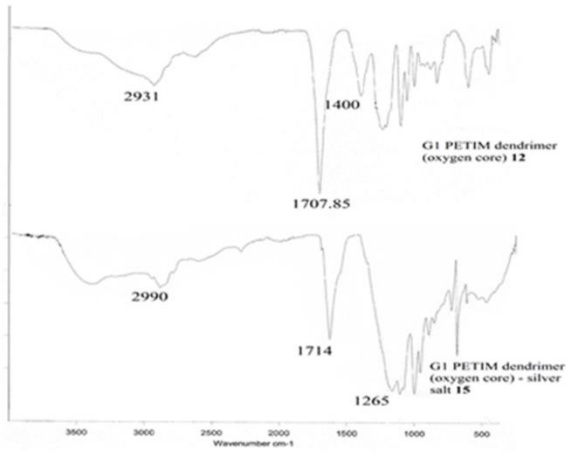
any of the carbonyl bands rather bands owing to the asymmetric and symmetric stretching vibrations of the equivalent carbon-oxygen bonds. They are observed at 1610-1550 cm^{-1} and 1420-1300 cm^{-1} respectively which provides evidence for the carboxylate anion [48]. In our study the peaks in the range of 1459-1332 cm^{-1} from carboxylic acid terminated dendron and dendrimers disappeared and appearance of symmetric stretching vibrations in the range of 1288-1233 cm^{-1} was observed (Fig. 1, 2 and 3) after transforming them into their respective silver salts. Thus, the presence of the bands because of symmetric stretching vibrations of the equivalent carbon-oxygen bonds strongly confirms the formation of silver salts of G1 PETIM dendron and dendrimers. Further attempts to characterise silver salts using elemental analysis were not successful because of their hygroscopic nature. [47]


2959
1459
1712
G1 PETIM dendron 4
3028
1633
1284
G1 PETIM dendron-silver salt 13
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(a)


2953
1402
1710
G1 PETIM dendrimer (aromatic core) 7
3019
1713
1233
G1 PETIM dendrimer (aromatic core) - silver salt 14
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(b)


2931
1400
1707.85
G1 PETIM dendrimer (oxygen core) 12
2990
1714
1265
G1 PETIM dendrimer (oxygen core) - silver salt 15
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(c)

Fig. 1 (a) FT-IR spectra comparing G1 PETIM dendron **4** and G1 PETIM dendron-silver salt **13; (b)** G1 PETIM dendrimer (aromatic core) **7** and G1 PETIM dendrimer (aromatic core) -silver salt **14** and **(c)** G1 PETIM dendrimer (oxygen core) **12** and G1 PETIM dendrimer (oxygen core) -silver salt **15**

In vitro cytotoxicity study

An *in vitro* cell culture system was used to determine the biological efficacy of the PETIM silver salts. The MTT assay, which is based on the biochemical reduction of MTT by viable cells, was used to determine the cytotoxicities of the PETIM silver salts against Hep G2, HT-29 and SK-BR-3 cell lines. [49] Determining cell viability using cytotoxicity assays are basic steps in toxicology that explain the cellular response to a compound by providing information on cell death and their metabolic activities. [50] Cell viability of between 80% and 95% were observed for all the PETIM silver salts across all the cell lines (**Fig. 2**). The comparative results between the individual PETIM dendron/dendrimers, their respective concentrations of silver nitrate, and their subsequent combinations (PETIM silver salts) were tested for cytotoxicity and are represented in **Fig. 3**.

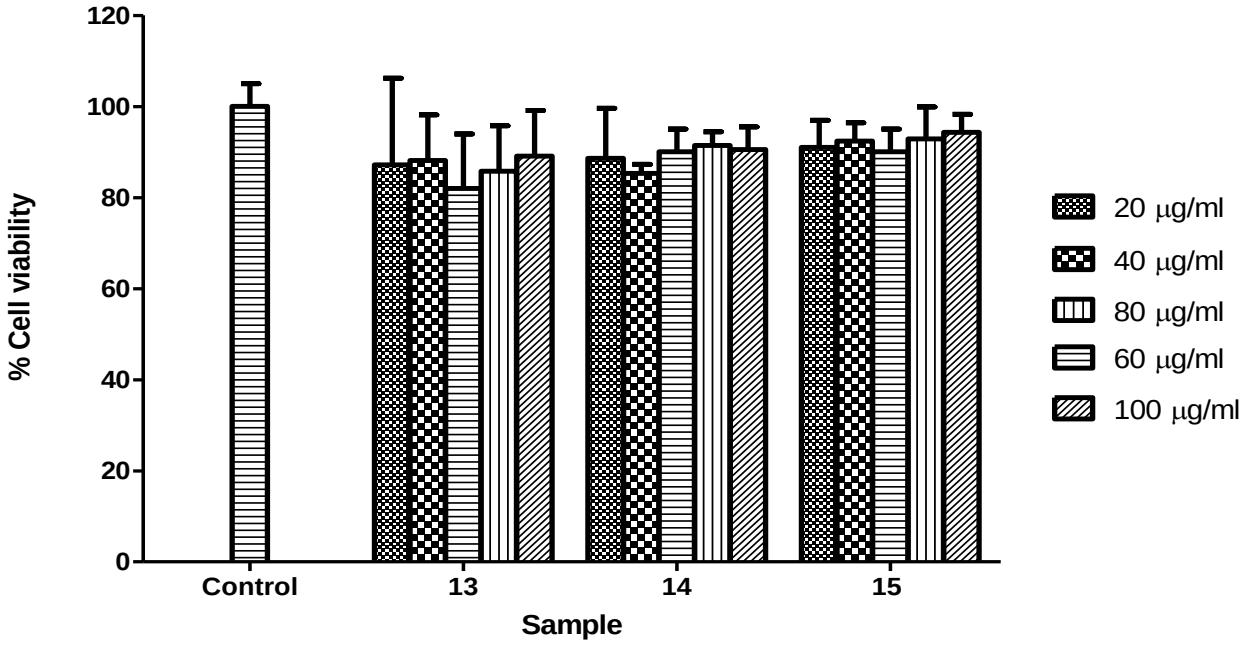


Fig. 2a Cytotoxicity assay against Hep G2 cells, displaying percentage cell viability after exposure

to various concentrations of PETIM silver salts [G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**] to Hep G2 cells. Results are presented as mean ± SD. (n = 6).

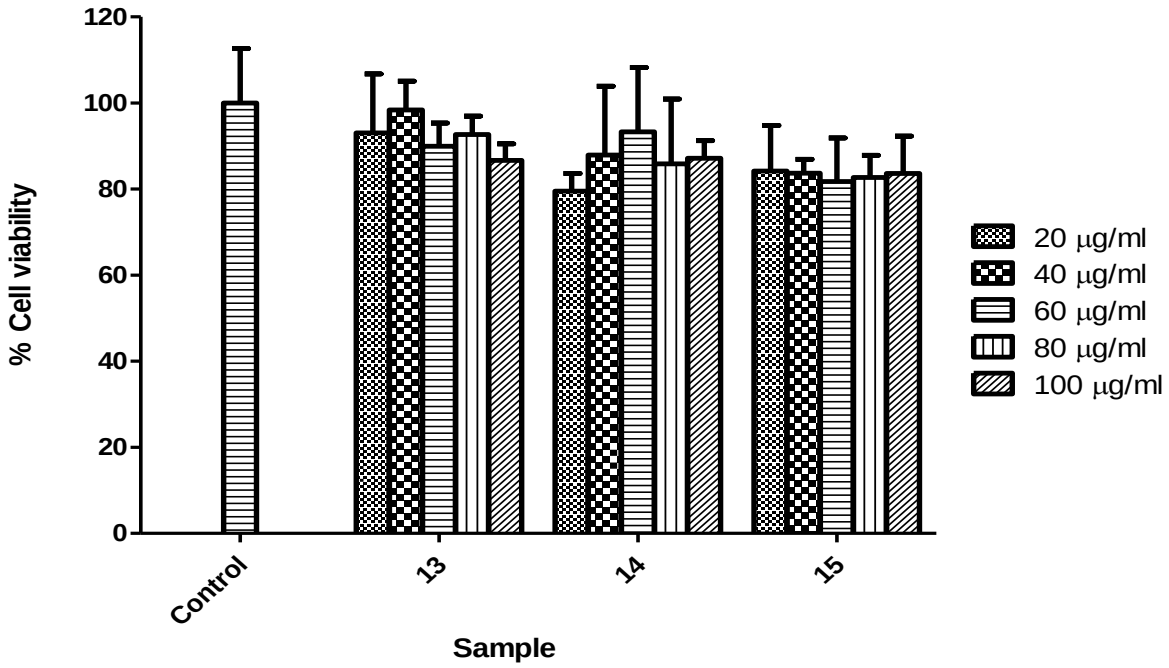


Fig. 2b Cytotoxicity assay against HT-29 cells, displaying percentage cell viability after exposure to various concentrations of PETIM silver salts [G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**] to Hep G2 cells. Results are presented as mean ± SD. (n = 6).

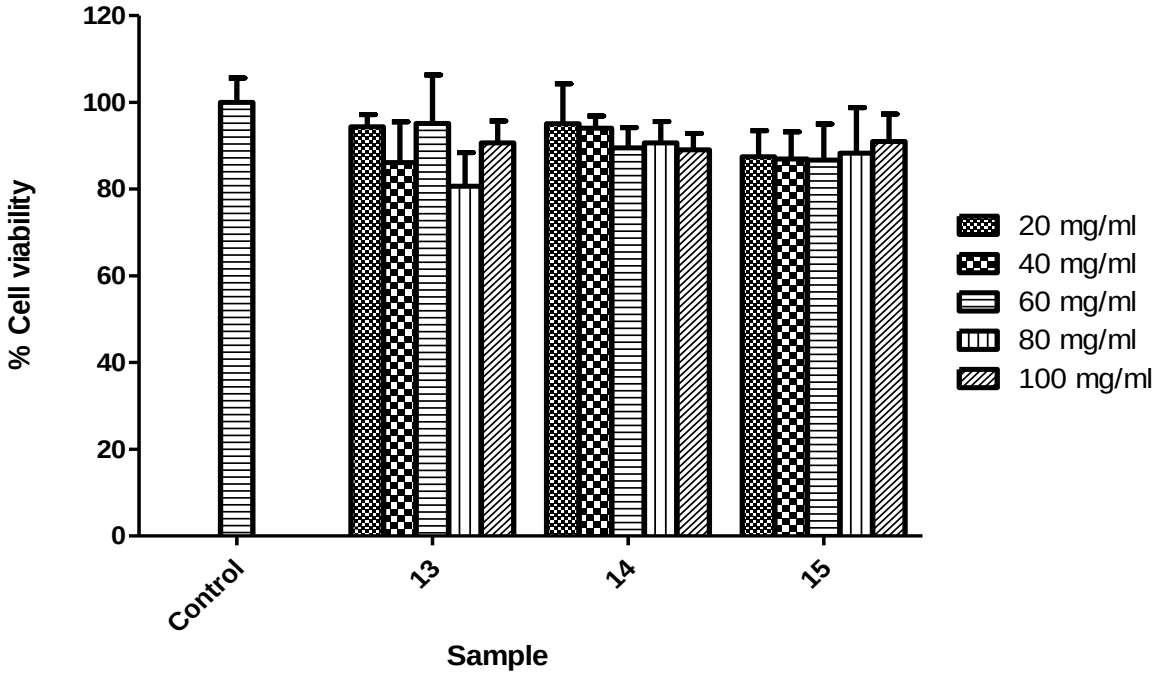


Fig. 2c Cytotoxicity assay against SK-BR-3 cells, displaying percentage cell viability after exposure to various concentrations of PETIM silver salts [G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**] to Hep G2 cells. Results are presented as mean ± SD. (n = 6).

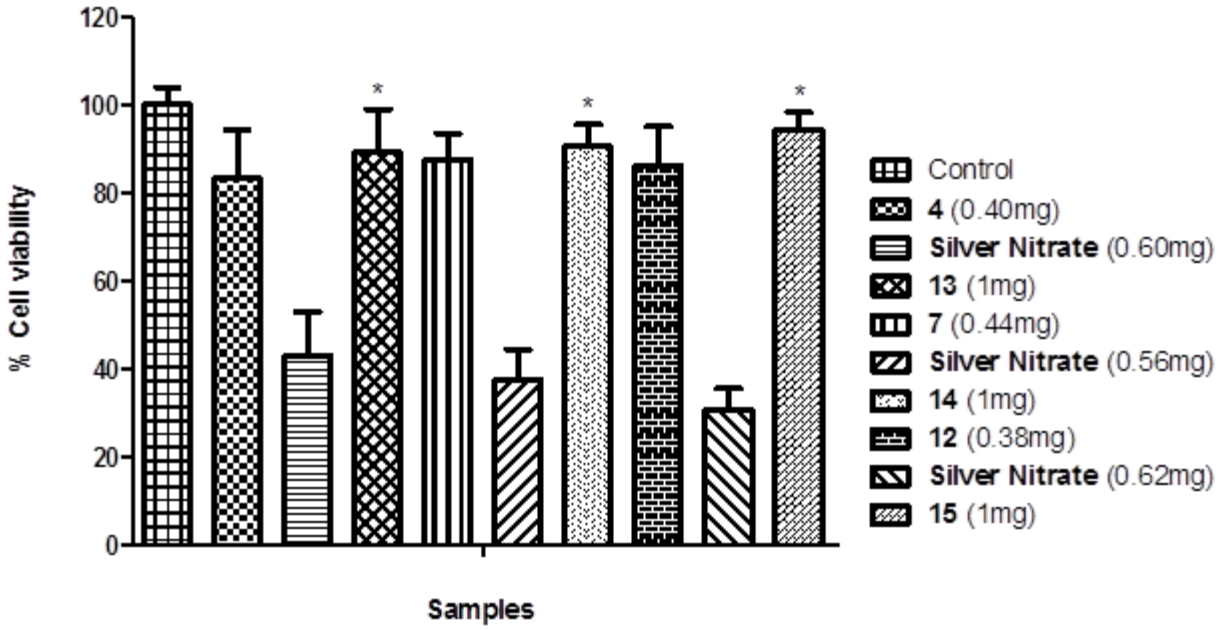


Fig. 3a Cytotoxicity assay on Hep G2 cell lines, comparing percentage cell viability after exposure to PETIM silver salts against their individual parent dendron/dendrimer as well as silver nitrate concentrations. Results are presented as mean ± S.D. (n = 6). *denotes significant difference compared to the respective silver nitrate ($P < 0.05$) [G1 PETIM dendron **4**; G1 PETIM dendrimer with aromatic core **7**; G1 PETIM dendrimer with oxygen core **12** ; G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**].

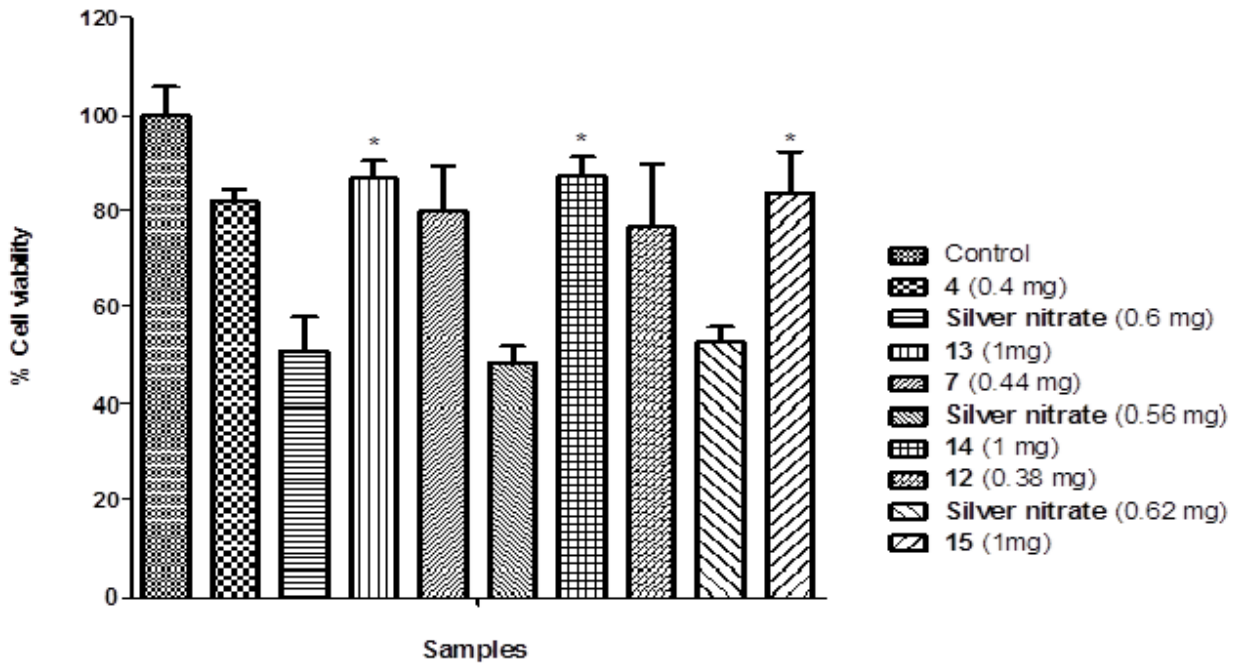


Fig. 3b Cytotoxicity assay on HT-29 cell lines, comparing percentage cell viability after exposure to PETIM silver salts against their individual parent dendron/dendrimer as well as silver nitrate concentrations. Results are presented as mean ± S.D. (n = 6). *denotes significant difference compared to the respective silver nitrate ($P < 0.05$) [G1 PETIM dendron **4**; G1 PETIM dendrimer with aromatic core **7**; G1 PETIM dendrimer with oxygen core **12** ; G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**].

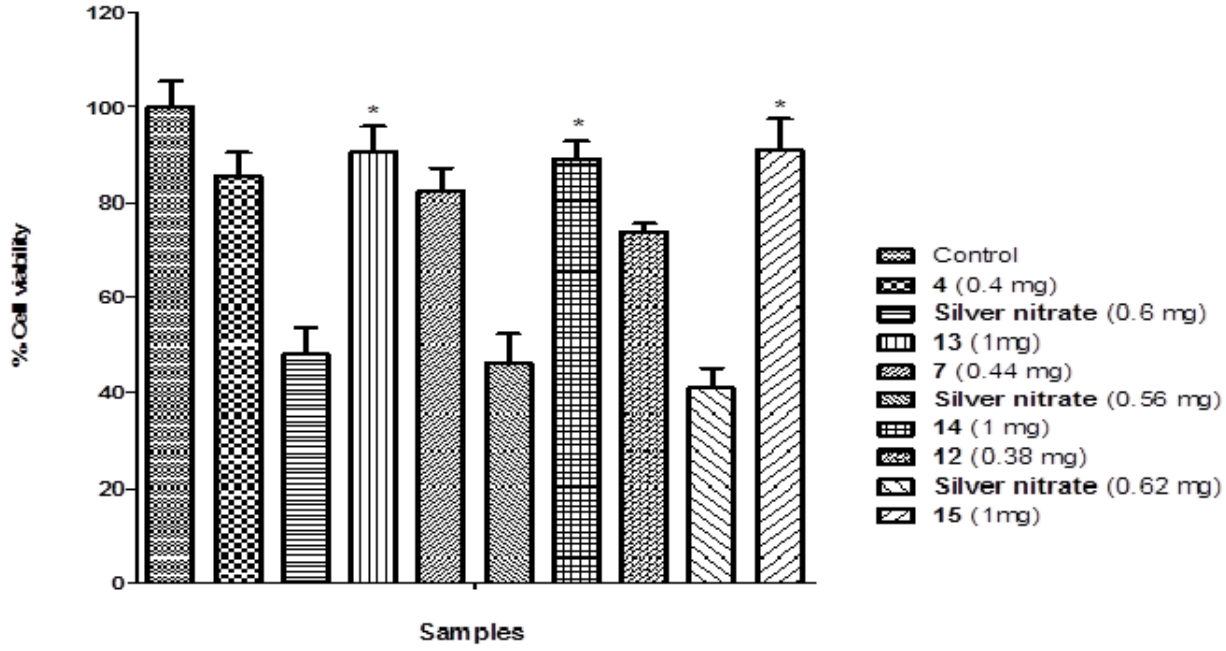


Fig. 3c Cytotoxicity assay on SK-BR-3 cell lines, comparing percentage cell viability after exposure to PETIM silver salts against their individual parent dendron/dendrimer as well as silver nitrate concentrations. Results are presented as mean ± S.D. (n = 6). *denotes significant difference compared to the respective silver nitrate ($P < 0.05$) [G1 PETIM dendron **4**; G1 PETIM dendrimer with aromatic core **7**; G1 PETIM dendrimer with oxygen core **12** ; G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**].

The range of cell viability obtained in this study indicates that the PETIM silver salts displayed a low toxicity level on all cell lines studied. [51] The results also showed that the effects of the compounds on the cell line were not dose dependent, as no dose dependent trends were observed for any of the PETIM silver salts at the various treatment concentrations against any of the cell lines (**Fig. 2**). These PETIM silver salts displayed a greater percentage cell viability when compared to their respective concentrations of silver nitrate (**Fig. 3**). Reduced cytotoxicity of the PETIM silver salts may be due to their close-to-neutral net surface charge, which had little effect on membrane integrity. [52] These results are in line with previous findings, where acetamide-terminated G5 PAMAM dendrimers revealed to have little effect on membrane integrity, whereas

positively charged G5 PAMAM dendrimers reduced the integrity of the cell membrane and prompted the release of cytoplasmic membrane proteins, lactate dehydrogenase and luciferase. [52, 53] The PETIM silver salts therefore have a statistically greater cell viability than the silver nitrate ($P < 0.05$) (**Fig. 5**) and slightly higher cell viability when compared to the PETIM dendron/dendrimers. Therefore, they can be considered non-toxic with potential for use in the biomedical and pharmaceutical fields.

In vitro antimicrobial evaluation

The antimicrobial activities of silver nitrate, the PETIM dendron/dendrimers and the PETIM silver salts were investigated against *S. aureus* and MRSA. A summary of the results for the MIC values for *in vitro* antimicrobial activity is presented in **Table 1**. MIC values for the different concentrations of silver nitrate against *S. aureus* were 112.5, 87.5 and 77.5 μg/ml respectively, and against MRSA they were 93.7, 210 and 77.5 μg/ml respectively (**Table 1**). Ionized silver brings about structural changes in bacterial cell walls and nuclear membranes as it is highly reactive when it binds to tissue proteins. Thus it results in cell distortion and even cell death. Silver can also bind to bacterial DNA and RNA, and can therefore inhibit bacterial replication. These antimicrobial properties of silver are dependent on the quantity and the rate at which silver is released. [54, 55] The MIC values for the PETIM dendron/dendrimers, i.e. **4**, **7** and **12** against *S. aureus* and MRSA, were all 500 μg/ml (**Table 1**). The MIC values obtained for the PETIM dendron/dendrimers indicate that the PETIM dendron/dendrimers alone do have some antimicrobial activity, although low, against the selected bacteria. Higher antimicrobial activity has been reported for both unmodified dendrimers and dendrimers, with additional surface modifications such as PAMAM dendrimer ammonium salts [38], and PPI dendrimers modified with maltotriose 25% and 100%. [39] However, the unmodified dendrimers displayed higher levels of cytotoxicity when compared to the surface modified dendrimers due to the cationic nature of these dendrimers. [56] As the PETIM dendrimers in our study displayed good cell viability due to their anionic nature, this nullifies the need for surface modification procedures to minimize the toxicity. Although these MIC values are higher when compared to surface modified and unmodified dendrimers, such as PAMAM and PPI against gram positive bacteria, this does confirm for the first time the antimicrobial activity of G1 PETIM dendron and dendrimers (**4**, **7** and **12**).

The MIC values for the PETIM silver salts, i.e. **13**, **14** and **15** investigated in this study, were 52.1, 41.7, and 20.8 μg/ml against *S. aureus* respectively, while against MRSA they were 125.0, 26 and 62.5 μg/ml respectively (**Table 1**). An increase in antimicrobial activity was observed for all salts when compared to silver nitrate and PETIM dendron/dendrimers alone. This may be a result of a high local concentration of silver ions available at the periphery of the PETIM silver salts. Antimicrobial activity was reported to be less when internal complexes were applied, showing that accessibility of the silver is a vital factor, and that a high local concentration of silver needs to be accessible to have a significant effect on microorganisms. [44] The MIC values of the salts of PETIM dendron/dendrimers were markedly reduced for G1 PETIM-dendron silver salt **13** and G1 PETIM dendrimer (oxygen core)-silver salt **15** against *S. aureus*, and G1 PETIM dendrimer (aromatic core)-silver salt **14** against both organisms. Compound **13** and **15** exhibited 42% and 33% greater activity against *S. aureus* respectively when compared to MRSA. However, compound **14** displayed 62 % greater activity against MRSA than *S. aureus*. The PETIM silver salts showed different degrees of antibacterial activity in relation to the bacterial species used in this study. Compound **14** displayed greater antibacterial activity against MRSA than *S. aureus*. Certain dendrimers displayed potent and broad antimicrobial activity against *S. aureus* [37], as well as a selectivity toward this particular bacterial species. [39] Polcyn *et al* also recently synthesised a range of modified dendrimers and interestingly, they too identified one particular dendrimer as having strong activity against MRSA [37], similar to the antimicrobial activity of compound **14** used in this study. Wang *et al*, performed antimicrobial testing on norfloxacin-loaded solid lipid nanoparticles for a 144 h time period, and the results indicated antimicrobial activity for an extended time period. [19] Similarly, the antimicrobial activity of **13**, **14** and **15** were tested over a 72 h period, with the results being consistent throughout this time span, indicating that they have the potential for sustained antimicrobial activity.

MIC values alone did not contribute toward a clear indication of the combined effects of the PETIM dendron/dendrimers and silver nitrate. Hence, the effects of the combination of G1 PETIM dendron/dendrimers and silver nitrate were also investigated, and these effects were evaluated using ΣFIC. A summary of the results for the ΣFIC values for *in vitro* antimicrobial activity experiments is presented in **Table 2**. All of the combinations displayed different degrees of effectiveness against the bacteria tested, and no antagonistic relations were observed.

Compound **13** presented a ΣFIC value of 1.58 against MRSA (**Table 2**), which represents indifference (**Table 3**). Compound **13** and **14** presented a ΣFIC value of 0.57 and 0.56 against *S. aureus* (**Table 2**), and **15** presented a ΣFIC value of 0.93 against MRSA (**Table 2**), which are all indicative of additive effects (**Table 3**). Compound **14** presented a ΣFIC value of 0.18 against MRSA (**Table 2**) and **15** presented a ΣFIC value of 0.31 against *S. aureus* (**Table 2**), which signify synergistic effects (**Table 3**). Of the three PETIM silver salts tested, **13** was observed to be the least active salt, whereas **14** was most active. This pattern of antibacterial activity of G1 PETIM-silver salts against both *S. aureus* and MRSA can be correlated to the structures of the compounds. The order of antibacterial potency of dendron/dendrimer-silver salts was G1 PETIM dendrimer (aromatic core)-silver salt **14** (six Ag^+ ions in the structure) > G1 PETIM dendrimer (oxygen core)-silver salt **15** (four Ag^+ ions in the structure) > G1 PETIM dendron-silver salt **13** (two Ag^+ ions in the structure). The G1 PETIM-silver salt with the highest number of carboxylic acid functions, and ultimately the highest number of Ag^+ ions, had the greatest antibacterial activity. As the G1 PETIM-silver salts contain positively charged Ag^+ ions and the bacterial cell wall has an overall negative charge, which has more affinity towards positively charged compounds, it may be possible that **14** had the best activity because of the highest number of Ag^+ ions present. The synergistic effect of **14** could therefore be a result of the combination of different mechanisms of actions of both silver and the dendron/dendrimers. Silver is known for its growth inhibitory capacity against microorganisms [57], and by using dendrimers as a template to incorporate silver, dendrimers themselves can become potent antimicrobials. [58] This activity can then be further enhanced if the functional groups of the dendrimers are within close proximity to one another. [36]

The interesting differences in activity of the three compounds against *S. aureus* and MRSA as well as specifically the significant synergistic activity against MRSA as compared to *S. aureus* in **14** may be due to differences in the structure and composition of their cell walls. For example one of the most widely reported mechanisms of resistance in *S. aureus* is the development of a modified penicillin binding protein (PBP) known as PBP 2a found in MRSA. [59, 60] Biosynthesis of peptidoglycan, which comprises the outermost layer of Gram-positive bacteria, is achieved by the membrane-bound enzymes PBP.[59] With MRSA the modified PBP known as PBP 2a, is intrinsically resistant to inhibition by β-lactams and stays active even in the presence of antibiotics that typically inhibit most endogenous PBP enzymes, thereby replacing their functions in cell wall synthesis and permitting growth in the presence of β-lactam inhibitors such as Methicillin. [59]

The significant increase in activity of **14** against MRSA may be attributed to its higher valency compared to **15**. The higher valency of **14** might have resulted in better binding affinity to PBP 2a of MRSA than PBP of *S. aureus*. This plausible mechanism of action could be supported by the recent findings where multivalent vancomycin-conjugated G5 PAMAM dendrimers exhibited enhancement in avidity in the cell wall models of *S. aureus* and VRSA as compared to free vancomycin. In this particular study authors have observed that the vancomycin-conjugated PAMAM dendrimers had binding avidity of 2-3 and 5 orders of magnitude with $_{(D)}$-Ala-$_{(D)}$-Ala, a cell wall precursor of *S. aureus* and $_{(D)}$-Ala-$_{(D)}$-Lac, a cell wall precursor of VRSA respectively. [61] The absence of PBP 2a in *S. aureus* could have been the reason behind low activity of **14** against *S. aureus* as compared to **15**. In the case of *S. aureus* **15** may have greater binding affinity to PBP resulting in its higher antibacterial activity against *S. aureus* than **14**.

Whilst the paper by Choi *et al* attempts to provide a mechanistic understanding of the vancomycin-conjugated G5 PAMAM dendrimers against *S. aureus* and VRSA, there are no such mechanistic studies available in the literature using novel materials and delivery systems against S. aureus and MRSA. There is also the possibility of multiple simultaneous mechanisms of actions of **14** and **15** against both *S. aureus* and MRSA, therefore, the mechanism of action postulated to explain the differences in antibacterial activity of **14** and **15** against MRSA and *S. aureus* respectively is a hypothesis based on previous literature and needs to be confirmed by future in depth experimental mechanistic studies.

Table 1. MIC results for *in vitro* antimicrobial activity of PETIM dendron/dendrimers, PETIM silver salts and their corresponding individual silver nitrate concentrations against *S. aureus* and MRSA.

Sample	MIC (µg/ml)	
	Organism	
	S. aureus	**MRSA**
G1 PETIM dendron **4**	500*	500*
Silver Nitrate	112.5*	93.7*
G1 PETIM dendron-silver salt **13**	52.1±18.04	125*
G1 PETIM dendrimer with an aromatic core **7**	500*	500*
Silver Nitrate	87.5*	210*
G1 PETIM dendrimer (aromatic core)-silver salt **14**	41.7±18.04	26±9.04
PETIM dendrimer with oxygen core **12**	500*	500*
Silver Nitrate	77.5*	77.5*
G1 PETIM dendrimer (oxygen core)-silver salt **15**	20.8±11.07	62.5*

*denotes SD = 0

Table 2. ΣFIC results for *in vitro* antimicrobial activity of the PETIM silver salts.

Sample	ΣFIC		Results	
	S. aureus	**MRSA**	***S. aureus***	**MRSA**
G1 PETIM dendron-silver salt **13**	0.57	1.58	Additive	Indifference
G1 PETIM dendrimer (aromatic core)-silver salt **14**	0.56	0.18	Additive	Synergy
G1 PETIM dendrimer (oxygen core)-silver salt **15**	0.31	0.93	Synergy	Additive

Experimental

Materials and methods

Acrylonitrile, *tert*-butyl acrylate and 3-amino-1-propanol were purchased from Alfa Aesar (Germany). 4 – (dimethylamino) pyridine (DMAP), lithium aluminum hydride ($LiAlH_4$), acetyl chloride (AcCl), 1,3,5-benzenetricarbonyl trichloride, silver nitrate and silica gel were purchased from Sigma-Aldrich (USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Merck Chemicals (Germany). All other chemicals and solvents used were of analytical grade, used without further purification and purchased from Merck Chemicals (Germany). Purified water used during the study was produced in the laboratory with a Milli-Q purification system (Millipore corp., USA). Nutrient Broth, Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA) were obtained from Biolab (South Africa). The bacterial cultures used were *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *Staphylococcus aureus* (MRSA) (*Staphylococcus aureus* Rosenbach ATCC BAA 1683). Optical density (OD) was measured using a Mindray MR-96A microplate spectrophotometer (China). FT-IR spectra of all the compounds were recorded on a Bruker Alpha-p spectrometer with diamond ATR (Germany) as per standard protocols. ^{1}H NMR and ^{13}C NMR measurements were performed on a Bruker 400/600 Ultrashield™ (United Kingdom) NMR spectrometer. HRMS was performed on a Waters Micromass LCT Premier TOF-MS (United Kingdom).

Synthesis of dendron 4 (Scheme 1)

The G1 PETIM dendron with a carboxylic acid function at the periphery was synthesised by hydrolysis of the dendron as reported in the literature [47]. In summary, a mixture of 3-amino-1-propanol **1** (5 g; 67 mmol) in methanol (20 ml) was added drop wise to a solution of *tert*-butyl acrylate **2** (51.2 g; 399 mmol) in methanol (100 ml), and was stirred for 6 h at room temperature. Surplus *tert*-butyl acrylate and solvent were removed in vacuo, with the crude product obtained being diluted with dichloromethane and washed with brine (3 x 25 ml). The organic layer was dried over anhydrous sodium sulphate and concentrated to yield **3** as a clear colourless liquid (21 g; 96%). Acetyl chloride (0.95 g; 12 mmol) and water (0.22 ml; 12 mmol) were added to a solution of **3** (0.5 g; 1.5 mmol) in dichloromethane (30 ml), and the solution was stirred at room temperature for 8 h. Solvents were removed under vacuum to afford **4** as a viscous material (0.3 g; 91%). FT-IR (neat) ν: 2959, 1712, 1399, 1179, 929 cm^{-1}. ^{1}H NMR (400MHz, CD_3SOCD_3) δ: 1.82 (q, 2H),

2.50 (t, 4H), 2.82 (t, 2H), 3.47 (t, 4 H), 3.76 (t, 2H). ^{13}C NMR (100MHz, CD_3SOCD_3) δ: 28.3, 37.0, 52.2, 58.9, 59.0, 174.4. HRMS (ES-TOF): $[M]^+$ calcd for $C_9H_{17}NO_5$ 220.1185; found 220.1181.

Synthesis of G1 PETIM dendrimer with an aromatic core 7 (Scheme 2)

A mixture of **3** (3 g; 9 mmol) and DMAP (3.3 g; 27 mmol) in PhMe (60 ml) was refluxed for 3 h and cooled to room temperature. 1,3,5-benzenetricarbonyl trichloride **5** (0.6 g; 2.3 mmol) was then added to the mixture and the reaction was refluxed for 6 h. PhMe was removed in vacuo and the crude product was purified via column chromatography (silica, mesh size 60-100) hexane/EtOAc, 4:6) to obtain **6** as a colourless oil (1.5 g; 60%). Acetyl chloride (3.63 g; 46 mmol) and water (0.73 ml; 41 mmol) were added to a solution of **6** (1.06 g; 0.92 mmol) in dichloromethane (40 ml), and the resulting solution was stirred vigorously at room temperature for 8 h. Solvents were then removed in vacuo and the subsequent residue was triturated with dichloromethane and hexane to obtain **7** (0.7 g; 93%) as a white foamy solid. FT-IR (neat) ν: 2601, 1710, 1232, 1402, 944, cm^{-1}. ^{1}H NMR (400MHz, CD_3SOCD_3) δ: 2.23 (b, 6 H), 2.86 (t, 12H), 3.29 (t, 6H), 3.36 (t, 12H), 4.44 (t, 6H), 8.70 (s, 3H). ^{13}C NMR (100MHz, CD_3SOCD_3) δ: 22.8, 28.3, 50.6, 52.1, 62.9, 131.28, 134.3, 167.5, 174.1.

Synthesis of G1 PETIM dendrimer with an oxygen core 12 (Scheme 3)

Acrylonitrile **8** (11.66 g; 0.22 mmol) was added drop wise to aqueous sodium hydroxide (40%) (2 ml), while maintaining the temperature below 30 °C. The reaction mixture was stirred overnight at room temperature and then neutralized with hydrochloric acid (32%) (w/w). The product was extracted with chloroform (3x50 ml) and washed with 5% sodium hydroxide (100 ml) followed by brine (50 ml). The organic layer was dried over anhydrous sodium sulphate and concentrated under vacuum to yield **9** (7.44 g; 55%). To a solution of $LiAlH_4$ (1.38 g; 48 mmol) in dry THF (40 ml) at 0 °C, **9** (3 g; 24 mmol) was added drop wise, a solution of **9** in THF (10 ml). The reaction was allowed to come to room temperature and was then stirred for 1 h, after which cold water (2.2 ml; 122 mmol) was added drop wise to the reaction mixture. The reaction mixture was stirred overnight at room temperature to afford **10**, a diamine (2.63 g; 80%**)** after filtration of the reaction mixture and evaporating the solvent. A solution of **10** (2.63 g; 20 mmol) in methanol (60 ml) was added drop wise to *tert*-butyl acrylate (14.02 g; 0.11 mmol) in methanol (50 ml), and the reaction was stirred for 6 h at room temperature. After column chromatographic purification (silica, mesh

size 60-100) (hexane/EtOAc, 7:3) and removal of the solvents, **11** was obtained as a colourless liquid (3.45 g; 27%). Finally, acetyl chloride (1.33 ml; 15 mmol) and water (0.28 ml; 16 mmol) were added to a solution of **11** (0.5 g; 0.77 mmol) in dichloromethane (10 ml), and the solution was stirred vigorously at room temperature for 8 h to afford **12** (0.3 g; 94%) after removing the solvent in vacuo and trituration of residue with hexane and dichloromethane several times. FT-IR (neat) ν: 2931, 1707, 1240, 1400, 930 cm^{-1}. ^{1}H NMR (400MHz, D_2O) δ: 2.0 (b, 4H), 2.88 (t, 8H), 2.93 (b, 4H), 3.31 (b, 4H), 3.47 (t, 8H). ^{13}C NMR (100MHz, D_2O) δ: 22.50, 2.28, 52.08, 58.9, 62.7, 176.5.

Silver salt of G1 PETIM dendron 13

To a solution of **4** (0.1 g; 0.46 mmol) in methanol (10 ml), an aqueous solution of silver nitrate (0.154 g; 0.9 mmol) in H_2O (5 ml) was slowly added and stirred vigorously for 2 h. The solvents were removed in vacuo to obtain **13** (0.19 g; 96%). FT-IR (neat) ν: 3028, 1722, 1633, 1284 cm^{-1}.

Silver salt of G1 PETIM dendrimer with an aromatic core 14

An aqueous solution of silver nitrate (0.25 g; 0.31 mmol) in H_2O (30 ml) was slowly added to a solution of compound **7** (0.2 g; 1.18 mmol) in methanol and stirred vigorously for 2 h. The solvents were removed in vacuo to obtain **14** (0.33 g; 92%). FT-IR (neat) ν: 3019, 1713, 1287, 1233, 1181 cm^{-1}.

Silver salt of G1 PETIM dendrimer with an oxygen core 15

Compound **12** (0.25 g; 0.59 mmol) was dissolved in acetone (50 ml), to which an aqueous solution of silver nitrate (0.405 g; 2.38 mmol) in H_2O (30 ml) was slowly added and stirred vigorously for 2 h. The solvents were removed in vacuo to afford **15** (0.5 g; 99%). FT-IR (neat) ν: 2932, 1714, 1265, 1210 cm^{-1}.

In vitro cytotoxicity study

Cell culture against hepatocellular carcinoma (Hep G2), colorectal adenocarcinoma (HT-29) and breast adenocarcinoma (SK-BR-3) cell lines were cultured with complete medium (minimum essential medium, supplemented with 10 % bovine calf serum, 100 units/ml of penicillin, and 100

mg/ml of streptomycin). Cells were maintained at 37 ˚C in a humidified atmosphere of 5% CO_2 in air.

Solutions: The compounds were dissolved in DMSO and distilled water as a stock solution [62], and diluted in the culture medium at concentrations of 20, 40, 60, 80 and 100 µg/ml as working-solutions. [63]

MTT assay: The cell lines were harvested from the exponential phase were seeded equivalently into a 96-well plate (2.2×10^3) and incubated for 24 h to allow for adherence. Thereafter, the culture medium was removed and replaced with fresh medium (100 µl per well), with the samples being added to the wells to achieve final concentrations. The control wells were prepared by adding the culture medium only. Wells containing the culture medium without cells were used as blanks. All experiments were performed with six replicates. Upon completion of the incubation for 48 h, the culture medium and compounds were removed and replaced with fresh medium (100 µl) and 100 µl of MTT solution (5 mg/ml in PBS) in each well. After 4 h incubation, the media and MTT solution was removed and 100 µl of DMSO was added to each well to solubilize the MTT formazan. The OD of each well was measured on a microplate spectrophotometer at a wavelength of 540 nm [64]. The percentage cell viability was calculated as follows:

% cell survival = [A540 nm treated cells] / [A540 nm untreated cells] X 100 (1)

(A540: absorbance at a wavelength of 540 nm)

Antimicrobial evaluation

Determination of minimum inhibitory concentrations (MICs): The MICs of the PETIM dendron/dendrimers, silver nitrate and dendrimer-silver salts were determined in triplicate using the broth dilution method. Stock solutions of **4** (0.4 mg/ml), compound **7** (0.44 mg/ml) and **12** (0.38 mg/ml), as well as silver nitrate in three different concentrations (0.60 mg/ml, 0.56 mg/ml, 0.62 mg/ml), were prepared in dimethyl sulfoxide (DMSO). The quantities were equivalent to the amount of individual components present in 1 mg/ml solutions of the respective dendrimer-silver salt. Stock solutions of the various dendrimer-silver salts (1 mg/ml) were prepared in distilled water **13** and DMSO **14** and **15**. The compounds were tested against *S. aureus* and MRSA, which were grown overnight in Nutrient Broth at 37° C and adjusted to 0.5 McFarlands standard with distilled water. Serial dilutions of the dendron/dendrimers, silver nitrate and dendrimer-silver salts

were prepared in MHB from the stock solutions. The test bacteria were added to each dilution and incubated overnight at 37 °C. Thereafter, each dilution was spotted on MHA plates and incubated overnight at 37° C. After incubation, the MHA plates were examined for growth and the MIC's was determined, with DMSO being used as a control.

Determination of fractional inhibitory concentration (FIC): The effects of the combination of G1 PETIM dendron/dendrimers and silver nitrate were investigated by determining the Σ FIC. The European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) [65] described the method for quantifying MIC results in terms of the FIC index, defined as the sum of FIC values of two drugs in combination. An example of the method used to calculate the ΣFIC is as follows:

For two antibacterials A and B alone and in combination (**4** and silver nitrate)

$$FIC_{(A)} = \frac{MIC_{(A \text{ in presence of B})}}{MIC_{(A \text{ alone})}} \quad (2)$$

$$= \frac{52.08}{500}$$

$$= 0.10416$$

$$FIC_{(B)} = \frac{MIC_{(B \text{ in presence of A})}}{MIC_{(B \text{ alone})}} \quad (3)$$

$$= \frac{52.08}{112.5}$$

$$= 0.46293$$

$$\Sigma FIC = FIC_{(A)} + FIC_{(B)} \quad (4)$$

$$= 0.10416 + 0.46293$$

$$= 0.56709$$

The FIC index is shown in **Table 3**. Indifference is when the effect of a combination of antimicrobials is equal to the effects of the most active compound. The additive effect refers to the effect of a combination of antimicrobials, where the effect of the combination is equal to that of the sum of the effects of the individual components. Synergistic action of a combination of two

antimicrobials is present if the effect of the combination exceeds the additive effects of an individual compound. [65]

Table 3. FIC index. [65]

Index	Synergy	Additive	Indifference	Antagonism
FIC	≤ 0.5	$> 0.5 – 1$	>1 to < 2	≥ 2

Statistical analysis

The results are expressed as mean ± standard deviation (SD) and were analysed using one-way analysis of variance (ANOVA), followed by the Mann-Whitney test using GraphPad Prism® (Graph Pad Software Inc. Version 5, San Diego, CA). A *p* value of less than 0.05 was considered to be statistically significant.

Conclusion

The results obtained in the present study confirm the enhanced antimicrobial activity of the PETIM-silver salts at low concentrations against both *S. aureus* and MRSA. These results also demonstrate that the PETIM-silver salt with the highest number of Ag^+ ions, had the greatest antibacterial activity. At the same time these salts display low cytotoxicity, which paves the way to synthesise silver salts of higher generation PETIM dendrimers, and to evaluate them as effective antimicrobials against a range of sensitive and resistant micro-organisms. A combination of such antimicrobial agents increases the spectrum of organisms that can be targeted and circumvent the emergence of resistance in microorganisms. The synthesized G1 PETIM-silver salts in this study show potential for applicability in pharmaceutical as well as biomedical fields.

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CHAPTER 4. SUBMITTED MANUSCRIPT

4.1 Introduction

The following paper was submitted to an international ISI journal and reports the original research from data generated during this study:

Suleman, N., Kalhapure, R., Mocktar C., Rambharose, S., Govender, T., 2015. A poly (ethylene glycol) six-arm star-shaped polymer as an efficient stabiliser for the synthesis of antibacterial and non-cytotoxic silver nanoparticles. RSC Advances, SUBMITTED MANUSCRIPT. Reference number: RA-ART-11-2015-023113. (IF = 3.708)

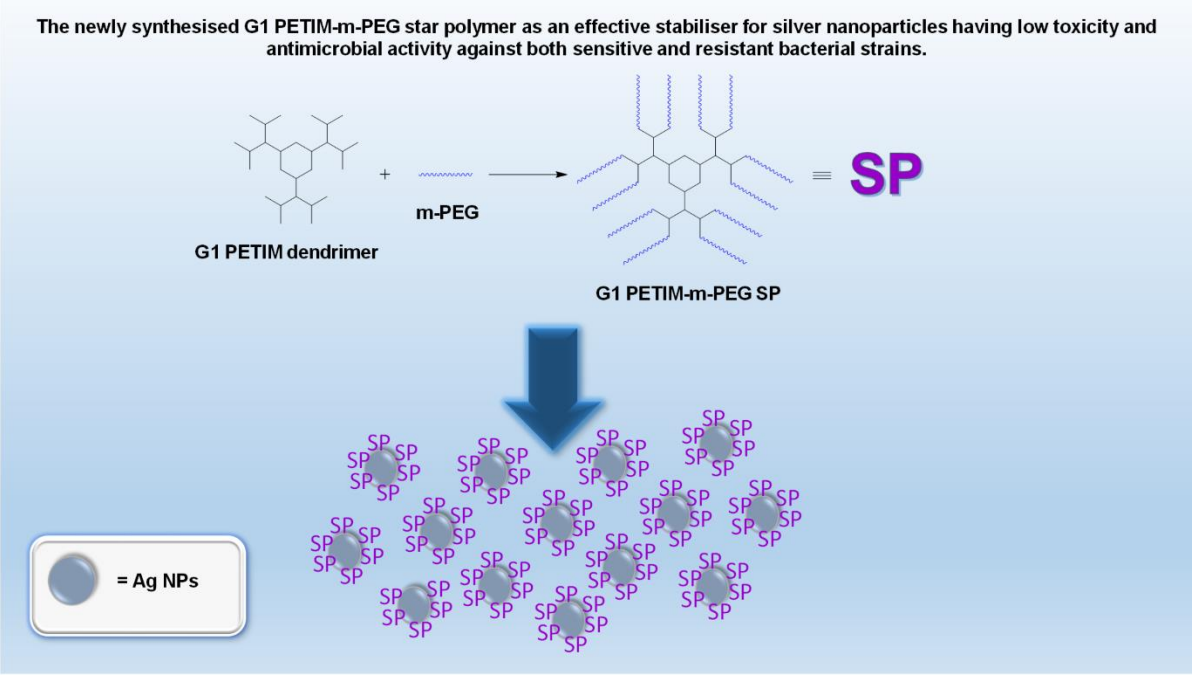
Ms. N Suleman contributed to the design of the project, and the preparation and characterisation of the G1 PETIM-m-PEG star shaped polymer and star polymer stabilised nanoparticles, in terms of synthesis, IR, NMR, DLS, TEM, XRD, *in vitro* cytotoxicity and *in vitro* antimicrobial studies, along with interpretation of the data and writing of the paper. Dr R. Kalhapure assisted with the design of the project, as well as the interpretation of characterisation data of the synthesised materials in terms of IR, NMR and XRD. Dr C. Mocktar assisted with the *in vitro* antimicrobial study. Mr S. Rambharose assisted with the *in vitro* cytotoxicity study. The remaining author served as supervisor.

This chapter is presented in the required format of the journal and is the final submitted version for review. The manuscript proof of submission can be found in Appendix B.

4.2 Submitted manuscript

A poly (ethylene glycol) six-arm star-shaped polymer as an efficient stabiliser for the synthesis of antibacterial and non-cytotoxic silver nanoparticles

Nadia Suleman, Rahul S. Kalhapure, Chunderika Mocktar, Sanjeev Rambharose and Thirumala Govender*



Abstract

Although silver nanoparticles (Ag NPs) are considered an attractive alternative for developing novel antibacterials, stability is a significant concern linked to their application as aggregation of NPs has been shown to considerably decrease their activity, leading to inferior performance. There is a lack of studies for the application of star polymers as stabilising agents for preparing colloidal Ag NPs. In this paper, we report on the synthesis of a generation 1 poly propyl ether imine (G1-PETIM) dendrimer derived 6-arm polyethylene glycol (PEG) star polymer (G1-PETIM-mPEG SP) and its application as a stabiliser for Ag NPs. The G1-PETIM-m-PEG SP was characterised using Fourier-transform infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C) and X-ray diffraction (XRD) analysis. Silver nanoparticles (G1-PETIM-m-PEG SP@Ag NPs) were prepared via chemical reduction using the G1-PETIM-m-PEG SP as a stabiliser, with their formation being verified using UV-vis

spectroscopy, dynamic light scattering, transmission electron microscopy and XRD analysis. The G1-PETIM-m-PEG SP and G1-PETIM-m-PEG SP@Ag NPs were evaluated for their cytotoxicity against MCF-7, HeLa and Hep G2 cell lines using MTT assay. G1-PETIM-m-PEG SP@Ag NPs, silver nitrate, G1-PETIM-m-PEG SP and a physical mixture of the latter two were evaluated for antibacterial activity against *S. aureus*, MRSA, *E. coli* and *P aeruginosa*. The synthesised G1-PETIM-m-PEG SP@Ag NPs were non-agglomerated, spherical and monodisperse, with an average particle size of 36.44 ± 2.51 nm, and found to be non-cytotoxic, even up to 100 µg/ml. The minimum inhibitory concentration values against *S. aureus* and MRSA (Gram-positive bacteria) were 18.5 and 74 µg/ml respectively, and against *E.coli* and *P. aeruginosa* (Gram-negative bacteria), the values were 9.25 and 74 µg/ml respectively. These low MIC values confirmed that Ag NPs retained their antibacterial potential even upon stabilisation by the G1-PETIM-m-PEG SP. The results obtained in this study suggest that the synthesised G1-PETIM-m-PEG SP is an attractive biocompatible star polymer for the stabilisation of Ag NPs.

Keywords: star-shaped · poly (ethylene glycol) · stabiliser · silver nanoparticles · antibacterial · *P. aeruginosa* · methicillin-resistant *S. aureus*

Introduction

Noble metal nanoparticles have attracted attention owing to their distinctive properties and potential applications in numerous areas for instance sensors, medicine, catalysts and electronics.[1-3] Various kinds of metal nanomaterials have been studied to date, however silver nanoparticles (Ag NPs) are reported as being the most effective and promising for biomedical and pharmaceutical use.[4] This can be ascribed to their good antimicrobial efficacy against not only bacteria, but also viruses and other eukaryotic micro-organisms.[4, 5] When compared to other antimicrobial agents, Ag is one of the most potent, displays a strong toxicity to a wide range of microorganisms and has a particularly low human toxicity.[6-8] While elemental silver and silver salts have been recognised as antimicrobial agents in preventive and curative health care since ancient times,[6] their use could cause unwanted adsorption of ions in the sweat glands and epidermal cells.[6, 9, 10] Ag NPs therefore appears to be a better candidate compared to silver cation salts and complexes.[6] Furthermore, the importance of Ag NPs as promising antibacterials lies in the fact that, unlike commercial antibiotics, they do not damage useful enzymes in the host.[6]

Ag NPs have unique physical and chemical properties, and are considered to be an alternate for developing novel antibacterial agents. Furthermore, they have varied medical applications, such as coatings for medical devices, wound dressings, textile fabrics etc.[4] Bacterial resistance has also not yet been detected with the use of Ag NPs, due apparently to the difference in the mechanism of the antibacterial actions of the diverse forms of Ag.[11-13] It is extremely unlikely that resistance to antimicrobial Ag might ever occur, since this would mean that an organism would have to undertake simultaneous mutations in each critical function in just a single generation to escape the compounds multiple actions.[7, 14]

Despite the fact that Ag NPs have displayed good performance as antibacterials, a significant challenge currently linked to their application is identifying strategies to provide the satisfactory stability of their dispersions to prevent aggregation of the NPs. The generation of spacious aggregates shows a noteworthy drop in activity of NPs, which results in inferior performance and a loss of antibacterial activity.[13, 15] Therefore, Ag NPs are frequently fabricated by the reduction of silver nitrate, and are then stabilised by capping agents to decrease the risk of aggregation, which arises from the high surface area of nanoparticles.[15] To prevent particle agglomeration, numerous polymers have been investigated to stabilise these metal colloids.[16] The polymers studied so far to stabilise Ag NPs include polyethylene glycols (PEG),[17] poly(vinylalcohols) (PVA),[18] poly(vinylpyrrolidones) (PVP),[19] polyacrylamides,[20] polyurethanes (PU),[21] as well as highly branched molecules, such as dendrimers,[22] hyperbranched polymers,[23] and star polymers.[2] As polymers act as a kind of matrix, they have been extensively utilised for trapping NPs.[24-26] The search for new polymers as stabilising agents is therefore of great importance to facilitate their application as antimicrobials.

Star polymers (SPs) are known as the simplest type of branched materials, wherein at least three linear polymer chains with essentially identical lengths are attached to only one branching point (core).[27-29] These polymers can contain chemically identical or different arms (miktoarm) linked to the core. They have enticed considerable attention owing to their unique topological structure and attractive physical and chemical properties, which are different from their linear counterparts.[29, 30] Usually, SPs have reduced hydrodynamic dimensions, lower solution and melt viscosities than their linear counterparts as well as equal molecular weights.[31, 32] Moreover, they also comprise a higher degree of end group functionalities that are fairly important in specialised applications.[33] Therefore, if the components of the SPs are biodegradable and/or biocompatible, these copolymers have potential biomedical applications,

such as drug/gene delivery, tissue engineering, diagnosis, medical devices, and antibacterial/antifouling biomaterials.[29, 30] These SPs could be a novel family of stabilising agent for preparing colloidal Ag NPs. Although an amphiphilic modified hyperbranched polyethyleneimine polymer has been used to stabilise Ag NPs and displayed certain attractive advantages, for instance, the quasi-spherical branched assembly with numerous inner cavities and nearly nonexistence of chain entanglements,[34] very few SPs, particularly water-soluble SPs, have been utilised as stabilising agents so far.[2, 23, 32]

PEG has been documented as fairly effective for attaining protein resistant surfaces.[35-37] Furthermore, PEG is non-toxic and non-immunogenic, which can be an added benefit for its use in biomedical and pharmaceutical applications.[36] Incorporating PEG into SPs is of recent interest in the literature.[37] Cross linked multi-arm star PEG systems have proven to be particularly successful at not only avoiding protein adsorption, but also in preserving the functional form of proteins, specially immobilised on surfaces coated with these polymers. Star PEG systems are attractive to confer protein resistance to surfaces because of their dense core structures. The high density of reactive groups on the surface of SPs can also be advantageous, for instance, in attaining a high binding capacity on surfaces giving immunoreactive groups to antibodies.[37-39] Although PEG proposes numerous advantages, its properties have not been widely reported for producing Ag NPs, and there are only a handful of papers reported to have used PEG as a stabilising and/or reducing agent to produce Ag NPs.[17, 40, 41] Finally, although PEG/dendrimer star polymers have been synthesised and applied previously,[42, 43] according to our knowledge, thus far they are yet to be utilised as stabilising agents for the preparation of metal NPs.

In this study, we therefore designed and synthesised a novel six-arm star-shaped PEG polymer using a generation 1 (G1) poly propyl ether imine (PETIM) dendrimer. In the present investigation, m-PEG, as well as the synthesised G1-PETIM-m-PEG SP, were evaluated as stabilising agents for producing Ag NPs. The particles were evaluated in terms of their DLS,TEM, UV visible spectroscopy, XRD, cytotoxicity assay and antimicrobial properties to assess their potential as stable and non-agglomerated NPs with effective antibacterial activity against Gram positive (susceptible and resistant) and Gram negative bacteria.

Experimental

Materials

Tert-butyl acrylate and 3-amino-1-propanol were purchased from Alfa Aesar, (Germany). 4-(dimethylamino) pyridine (DMAP), 1,3,5-benzenetricarbonyl trichloride, lithium aluminum hydride ($LiAlH_4$), poly (ethylene glycol) methyl ether (Mn = 5000g/mol) (m-PEG), sodium borohydride ($NaBH_4$), sodium hydride (NaH) (60% dispersion in mineral oil), sodium sulfate, silica gel and silver nitrate ($AgNO_3$) were purchased from Sigma-Aldrich (USA). Thionyl chloride and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Merck (Germany). All other reagents and solvents were of analytical grade and used without further purification. Purified water used throughout the studies was produced in the laboratory with a Milli-Q water purification system (Millipore corp., USA). Nutrient Broth, Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA) were obtained from Biolab (South Africa). The bacterial cultures used were *Staphylococcus aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus* (MRSA) [*Staphylococcus aureus* Rosenbach ATCC BAA 1683], *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

Methods

Fourier transmission infrared spectroscopy (FT-IR)

FT-IR spectra of all the compounds were recorded on a Bruker Alpha-p spectrometer with diamond ATR (Germany) as per standard protocols.

Nuclear magnetic resonance (NMR)

^{1}H NMR and ^{13}C NMR measurements were performed on a Bruker 400/600 Ultrashield™ (Germany).

Synthesis of G1 PETIM-ester 1

A mixture of 3-amino-1-propanol (5 g; 67 mmol) in methanol (20 ml) was added drop wise to a solution of *tert*-butyl acrylate (51.2 g; 399 mmol) in methanol (100 ml), and was stirred at room temperature for 8 h. Excess *tert*-butyl acrylate and solvent were removed in vacuo, with the crude product obtained being diluted with dichloromethane and washed with brine (75 ml). The organic layer was dried over anhydrous sodium sulfate and concentrated to yield clear colourless ester (21 g; 96%). A mixture of the ester (3 g; 9 mmol) and DMAP (3.3 g; 27 mmol) in toluene (60 ml) was refluxed for 3 h and cooled to room temperature. 1,3,5-benzenetricarbonyl trichloride (0.6 g; 2.3 mmol) was then added to the mixture and the reaction

was refluxed for 6 h. Toluene was removed in vacuo, and the crude product was purified by column chromatography (silica, mesh size 60-100; hexane/EtOAc, 4:6) to obtain G1 PETIM-ester **1** as a colourless oil (1.5 g; 60%). FT-IR (neat) ν: 2976, 1721, 1366, 1233, 1148, 950 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.43 (s, 54H), 1.96 (q, 6H), 2.38 (t, 12H), 2.57 (t, 6H), 2.77 (t, 12H), 4.42 (t, 6H), 8.83 (s, 3H).

Synthesis of G1 PETIM-alcohol 2

A solution of $LiAlH_4$ (1.32 g; 34.78 mmol) in dried THF (40 ml) was added to a 2 neck round bottomed flask equipped with a guard tube and then flushed with N_2 gas. A mixture of compound **1** (5 g; 4.34 mmol) in dried THF (20 ml) was cooled to 0°C and added drop wise to the round bottomed flask, which was maintained at 0°C. On completion of the addition, the reaction mixture was stirred overnight at room temperature and thereafter slowly added to ice, dried with sodium sulfate and filtered. The filtrate was concentrated under vacuum to yield compound **2** (2.12 g; 67%). FT-IR (neat) ν: 3294, 1593, 1135, 1050 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.55 (m, 12H), 2.41 (m, 6H), 2.50 (m, 18H), 3.43 (t, 12H), 3.36 (b, 6H), 4.47 (m, 6H), 8.50 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 29.79, 50.86, 59.54, 60.69, 62.97, 122.89, 142.0, 169.6.

Synthesis of G1-PETIM-chloride 3

Thionyl chloride (5.9 g; 49.59 mmol) was added drop wise to a mixture of compound **2** (2 g; 2.74 mmol) in dry dichloromethane (50 ml) and the reaction was refluxed overnight. Thionyl chloride was removed in vacuo using a NaOH trap and the crude product **3** was used for further reaction. FT-IR (neat) ν: 2960, 1248, 1730, 653 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d6) δ: 1.83 (m, 6H), 2.19 (m, 12H), 2.49 (bs, 12H), 2.53 (bs, 6H), 3.75 (t, 12H), 4.81 (bs, 6H), 9.65 (s, 3H).

Synthesis of G1-PETIM-m-PEG SP 4

A mixture of m-PEG (35.6 g; 7.12 mmol) in dried THF (60 ml) was added to a round bottomed flask, NaH (0.33g; 13.75 mmol) was added to it and stirred for 30 min. The reaction was then heated to 80°C and compound **3** (0.98g; 1.17 mmol) was added dropwise. On completion of the addition, the reaction was refluxed for 24 h. The reaction material was concentrated under vacuum to remove excess solvent and then recrystallised in acetone to yield compound **4**. FT-IR (neat) ν: 2881, 1466, 1359, 1146, 1099 cm^{-1}. ^{1}H NMR (400 MHz, D_2O) δ: 1.83 b (-O-CH_2-

C**H**$_2$-CH$_2$-N-), 3.30 s (-CH$_2$-O-C**H**$_3$), 3.45 b (-N-CH$_2$-CH$_2$-C**H**$_2$-O-), 3.62 b (-O-C**H**$_2$-C**H**$_2$-O-), 3.80 b (-C**H**$_2$-N-(C**H**$_2$)$_2$), 4.55 b (Ar-COO-C**H**$_2$-), 7.32 s (Ar-**H**). ^{13}C NMR (100 MHz, D_2O) δ: 46.66, 58.15, 60.48, 62.50, 65.37, 69.69, 72.51, 74.00, 76.88, 100.01, 127.85, 138.25.

Preparation of silver nanoparticles

10 ml of $AgNO_3$ (0.01M) was added drop wise to the G1 PETIM-m-PEG SP (0.17 g; 0.006 mmol) in water (25 ml) under magnetic stirring at room temperature. After completion of the addition, 1.5 ml of $NaBH_4$ (0.1 M) was added dropwise to the mixture under vigorous stirring. The reaction mixture was further stirred for 10 min. To synthesise m-PEG@Ag NPs, m-PEG was used instead of G1 PETIM-m-PEG, and for plain Ag NPs, this was done without any stabiliser following the same procedure.

UV analysis

The surface plasmon resonance (SPR) of the silver nanoparticles was monitored by UV-visible spectroscopy. To confirm the reduction of silver ions, the solution was scanned in the range of 200–700 nm (Shimadzu 1650PC, Japan) using a quartz cuvette with water as the reference. The solution was obtained by diluting 1 ml of silver nanoparticles in 30 ml of water.

Particle size (PS), polydispersity index (PDI) and zeta potential (ZP)

The average PS, PDI and ZP were determinedby Dynamic Light Scattering (DLS) using a NanoZS Zetasizer (Malvern Instruments Corp., UK) at 25 °C with a path length of 10mm in polystyrene cuvettes. 200 µl of each sample was diluted with 10 ml of distilled water. The analysis consisted of three measurements in triplicate for each sample and results were expressed as mean size ± S.D.

Transmission Electron Microscopy (TEM)

The size and morphology of the silver nanoparticles were examined using a transmission electron microscope (Jeol JEM-2100, Japan). The sample was prepared by placing a drop of Ag NPs on carbon-coated copper grid and thereafter allowed to dry in air before being transferred to the microscope operated at an accelerated voltage of 200 kV.

Powder X-ray diffraction (XRD)

Powder XRD patterns were obtained using a Bruker D8 Advance Diffractometer (Germany) equipped with a graphite monochromator operated at 40 kV and 40 mA. The radiation source was a CuKα X-ray source with λ = 1.5406 Å. Data was collected at a step of 0.021 ° and at a scanning speed of 0.454 ° s-1. The 2θ range covered was between 10 ° to 90 °.

In vitro cytotoxicity study

Cell culture: Human breast cancer (MCF-7), HeLa and Hepatocellular carcinoma (Hep G2) cell lines were cultured with complete medium (minimum essential medium, supplemented with 10 % bovine calf serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin). Cells were maintained at 37 ˚C in a humidified atmosphere of 5% CO_2 in air.

Solutions: The test compound was dissolved in distilled water as a stock solution,[44] and diluted in the culture medium at concentrations of 20, 40, 60, 80 and 100 $\mu g/ml^{-1}$ as working solutions.[45]

MTT assay: The cells harvested from the exponential phase were seeded equivalently into a 96-well plate (2.2×10^3) and incubated for 24 h to allow for adherence. Thereafter, the culture medium was removed and replaced with fresh medium (100 µl per well), with the sample being added to the wells to achieve final concentrations. The control wells were prepared by adding the culture medium only. The wells containing the culture medium without cells were used as blanks. All experiments were performed with six replicates. Upon completion of the incubation for 48 h, the culture medium and compounds were removed and replaced with fresh medium (100 µl) and 100 µl of MTT solution (5 mg/ml in PBS) in each well. After 4 h incubation, the media and MTT solution was removed and 100 µl of DMSO was added to each well to solubilize the MTT formazan. Optical density (OD) was measured using a Mindray MR-96A microplate spectrophotometer (China). The OD of each well was measured on a microplate spectrophotometer at a wavelength of 540 nm.[46] The percentage cell viability was calculated as follows:

% cell survival = [A540 nm treated cells] / [A540 nm untreated cells] X 100 (1)

(A540: absorbance at a wavelength of 540 nm)

Antimicrobial activity

Determining the minimum inhibitory concentrations (MICs): The MICs of the G1 PETIM-m-PEG SP, $AgNO_3$, G1 PETIM SP@Ag NPs and a mixture of SP and $AgNO_3$ were determined using the broth dilution method. The quantities were equivalent to the amount of individual components present in the final SP@Ag NP solution. The compounds were tested against *S. aureus*, MRSA, *E.coli* and *P. aeruginosa*. The bacterial cultures were grown overnight in Nutrient Broth at 37 °C and adjusted to 0.5 McFarlands standard with distilled water. Serial dilutions of the test materials were prepared in MHB from the stock solutions. The test bacteria were added to each dilution and incubated overnight at 37 °C. Thereafter, each dilution was spotted on MHA plates and incubated overnight at 37° C. After incubation, the MHA plates were examined for growth to determine the MICs.

Stability studies

The stability of the optimised G1 PETIM-m-PEG SP@Ag NPs was evaluated at room temperature (RT) and at 4 ºC over a period of three months. The physical appearance, PS, PDI and ZP were used as assessment parameters for stability.

Statistical analysis

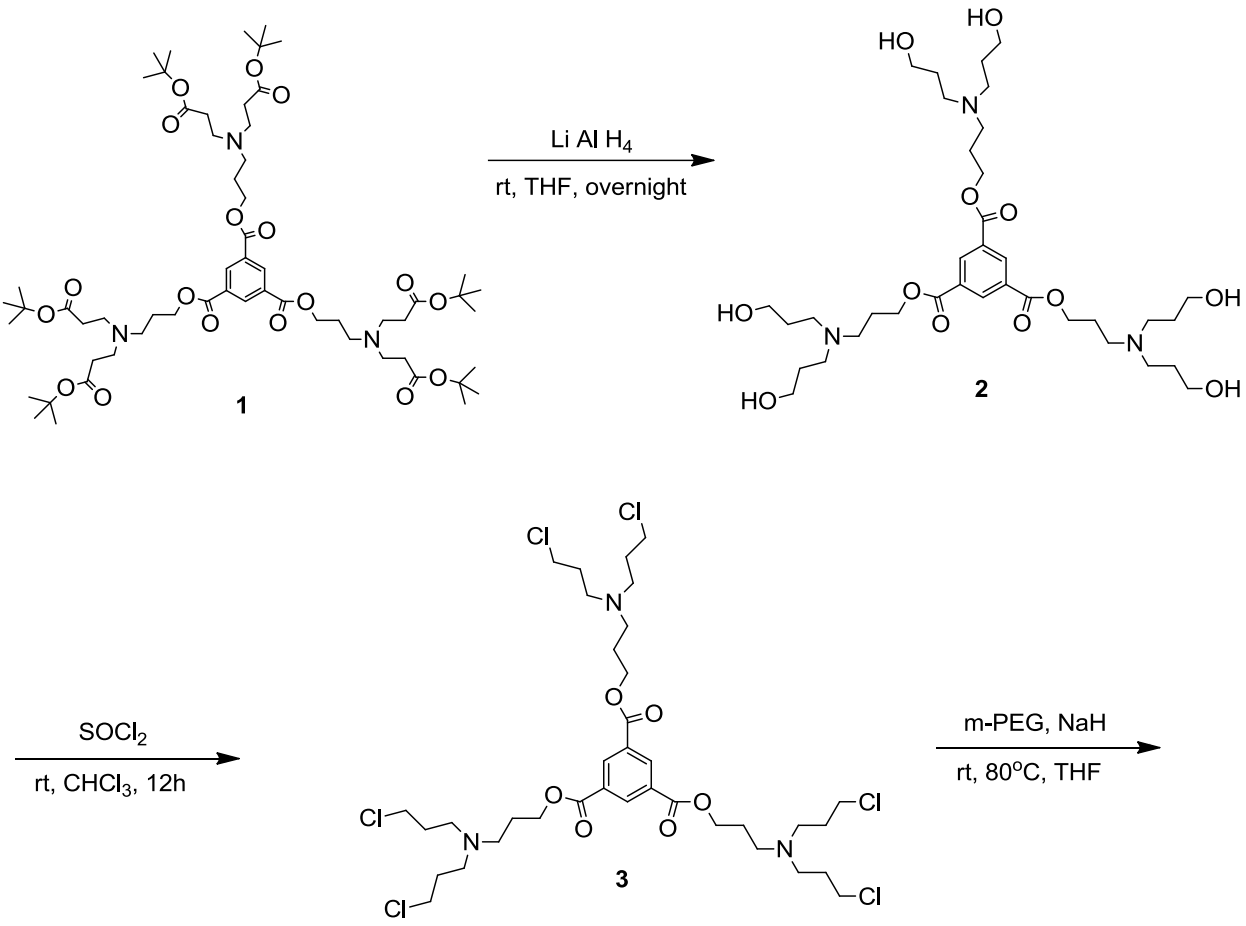
The results expressed as mean ± standard deviation (SD) were analysed using one-way analysis of variance (ANOVA), followed by the Mann-Whitney test using GraphPad Prism® (Graph Pad Software Inc. Version 5, San Diego, CA). A *p* value of less than 0.05 was considered statistically significant.

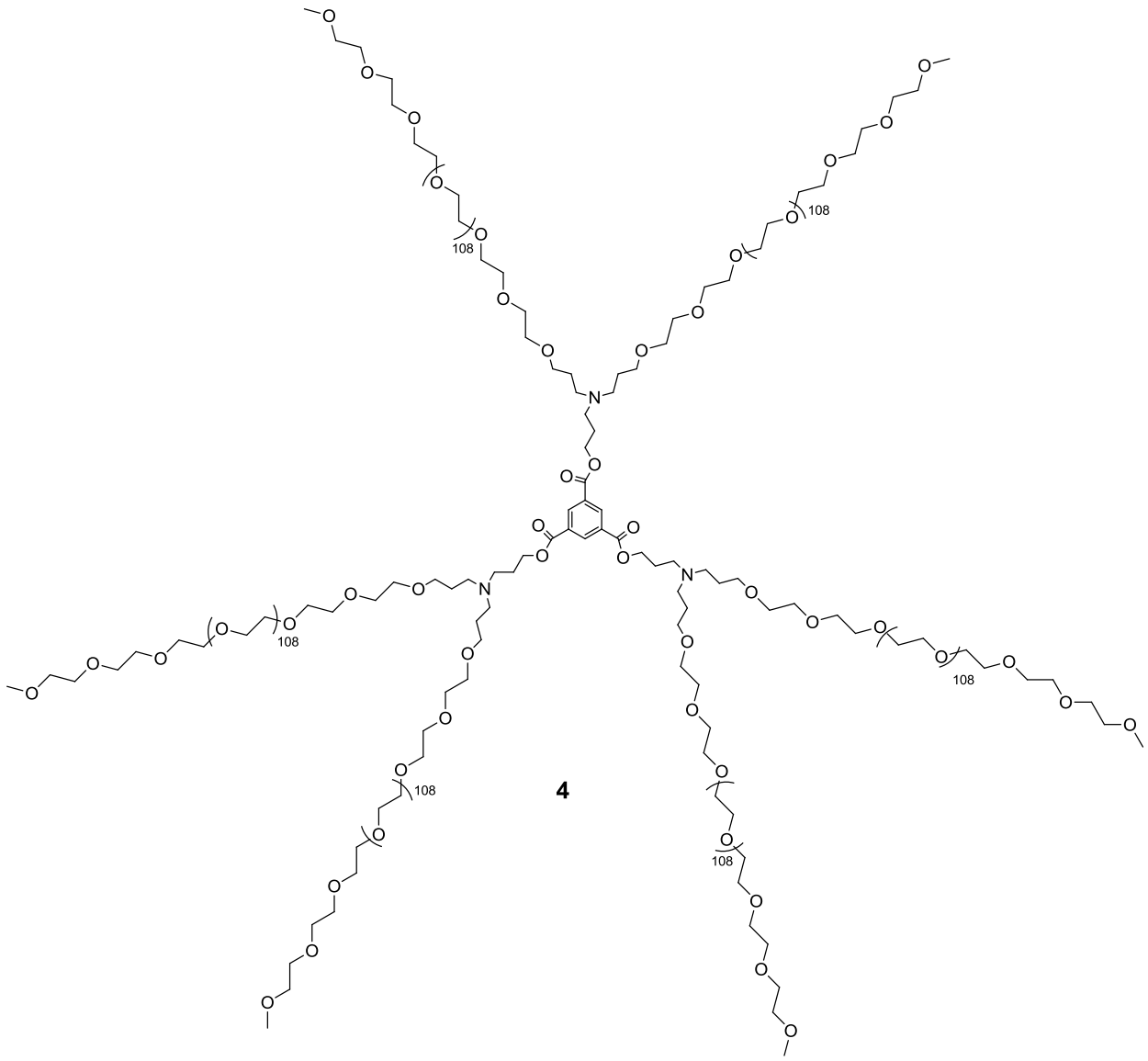
Results and discussion

Synthesis

The synthesis of G1 PETIM-ester **1** was accomplished by following the literature reported procedure.[14, 47] In short, dendron with primary alcohol as a focal functionality was prepared using a Michael addition reaction between 3-amino-1 propanol and *tert*-butyl acrylate. This dendron was then condensed with 1,3,5-benzenetricarbonyl trichloride in the presence of DMAP to obtain tert-butyl ester terminated G1 PETIM dendrimer **1.** The lithium aluminium hydride ($LiAlH_4$) mediated reduction of **1** was followed by a subsequent reaction of formed G1-PETIM dendrimer-OH **2** with thionyl chloride ($SOCl_2$) afforded G1 PETIM dendrimer-Cl

3. Finally, **3** was coupled to m-PEG in the presence of NaH to afford a six-arm G1 PETIM-m-PEG SP **4** (Scheme 1).





Scheme 1 Synthesis of G1 PETIM-m-PEG SP

Characterisation

The techniques used to characterise the intermediates and G1-PETIM-m-PEG SP were FT-IR and NMR (^{1}H and ^{13}C). The formation of G1 PETIM-ester 1 was confirmed by comparing the analytical data with literature data.[47] The conversion of *tert*-butyl ester of **1** to alcohol to form G1-PETIM-OH was confirmed by the disappearance of an ester peak at 1721 cm^{-1} and the appearance of an alcohol peak at 3294 cm^{-1} in FT-IR; disappearance of ester protons from 1.43 δ ppm and shift of adjacent –CH_2- peak from 2.57 δ ppm to 1.55 δ ppm in ^{1}H NMR due to shielding by formed alcohol function at the periphery. In the ^{13}C NMR, the ester carbons disappeared at 80.3 and 171.7 δ ppm, and the shift in –**C**H_2-C=O- carbon from 33.8δ ppm to

50.85 δ ppm was due to the formation of $–CH_2-CH_2-OH$ in ^{13}C NMR. The peak around 653 cm^{-1} in the FT-IR spectra of the G1-PETIM-chloride (Fig. S1, Supporting Information) is attributed to C-Cl stretching vibrations that appear in the range from 730-550 cm^{-1}.[48] The C-Cl stretch, along with the disappearance of the O-H stretching band in the 3400-3200 cm^{-1} region, confirmed the formation of the G1-PETIM-chloride. Finally, the formation of the G1 PETIM-m-PEG SP was confirmed by a disappearance of –C-Cl stretch at 653 cm^{-1} and the appearance of a strong –C-O- ether stretch at 1099 cm^{-1} in FT-IR (Fig. S2; Supporting Information). The characteristic peaks in ^1H NMR of G1 PETIM-m-PEG SP were of $–CH_2-O-$ at 3.56 δ ppm, $-CH_2-N-$ peak at 3.80 δ ppm and terminal $–O-CH_3$ at 3.23 δ ppm (Fig. S3; Supporting Information), whereas in ^{13}C NMR, they were terminal $–O-CH_3$ at 60.48 δ ppm, -C=O- ester at 138.25 δ ppm and aromatic carbons at 100 and 127.84 δ ppm (Fig. S4; Supporting Information).

Synthesis and characterisation of G1 PETIM-m-PEG SP@Ag NPs

Synthesis of plain Ag NPs, m-PEG Ag NPs and G1 PETIM-m-PEG SP@Ag NPs was accomplished via chemical reduction using sodium borohydride ($NaBH_4$). Upon complete addition of $NaBH_4$, the transparent solution formed aggregates (**Fig 2**) in the case of the plain Ag NPs and m-PEG Ag NPs. However, the transparent solution converted to a brown coloured dispersion for the SP coated NPs, indicating the formation of G1 PETIM-m-PEG SP@Ag NPs. For the plain Ag NPs and m-PEG Ag NPs, the intensity of the SPR peak was very low, which could be due to the incomplete formation of Ag NPs followed by aggregation (**Fig. 1a and 1b**). Aggregation of the plain and m-PEG Ag NPs can be clearly seen in **Fig. 2**. The peak at a wavelength of 394.30 nm in the UV-visible spectra due to surface plasmon resonance (SPR) of the electrons in the conduction band of Ag further confirmed the formation of G1 PETIM-m-PEG SP@Ag NPs.[49, 50] The symmetric and markedly narrow shape of the plasmon band (**Fig. 1c**) indicated that the G1 PETIM-m-PEG SP@Ag NPs were monodisperse and spherical in shape.[50, 51]

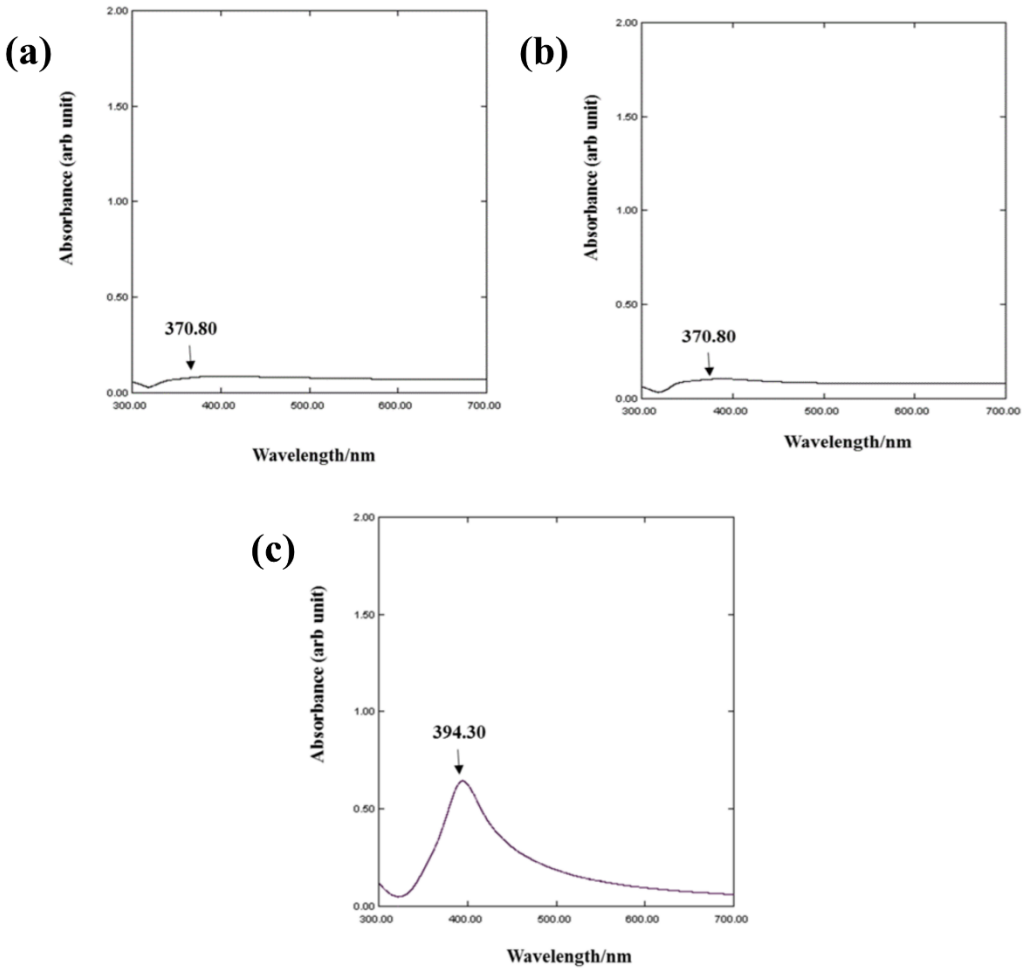


Fig. 1 (a) UV-visible absorption spectra of plain Ag NPs, (b) UV-visible absorption spectra of m-PEG Ag NPs and (c) UV-visible absorption spectra of G1 PETIM-m-PEG SP Ag NPs**.**

Fig. 2 Images of: (a) plain Ag NP, (b) G1 PETIM-m-PEG SP@Ag NP and m-PEG@Ag NP solutions.

Particle size, polydispersity index, zeta potential and morphology

To assess the size and morphology of the Ag NPs, DLS and transmission electron microscopy (TEM) studies were performed. Results from the DLS studies indicated extremely large particles sizes of 1870 nm ± 829.0 and 1025 nm ± 246.1, and PDIs of 0.864 ± 0.166 and 0.662 ± 0.120 for plain Ag and m-PEG@Ag NPs respectively. It was observed through TEM investigations that aggregates had formed in the plain and m-PEG@Ag NPs (**Fig 4** and **5).** This proved the inefficiency of m-PEG on its own to stabilise formed Ag NPs. However, for G1 PETIM-m-PEG SP@Ag NPs, a particle size of 36.44 nm ± 2.51 with a PDI of 0.414 ± 0.007 was observed. TEM studies then confirmed that the G1 PETIM-m-PEG SP@Ag NPs were non-agglomerated, and spherical in shape with uniform particle size in the range of 25-30 nm (**Fig. 3**). The ZP for G1 PETIM-m-PEG SP@Ag NPs was found to be -23.7 ± 2.47, this negative value for ZP showing good stability of the NPs.

Noble metal NPs have been synthesised in various sizes, compositions and shapes.[52] Although Ag NPs have been reported to be as small as 1 nm, and it has been shown that particle size as well as shape can have an effect on antimicrobial activity, [4, 53] Ag NPs in the range of 1 – 100 nm have demonstrated strong bactericidal activity against both Gram positive and Gram negative bacteria, with our NPs being well within this size range.[53, 54] Stabilised NPs have also been shown to have varying sizes and shapes, depending on the type of stabilising agent used.[13] Popa *et al* prepared PEG stabilised Ag NPs in sizes ranging from 4 – 50 nms,[17] whereas Huang et al prepared Ag NPs stabilised by an amphiphilic star-shaped copolymer with a size range of between 10 – 20 nms.[2] The literature shows that considerably enhanced activity has been noted for stabilised Ag NPs when compared to their unmodified counterparts.[13] Therefore, our G1 PETIM-m-PEG SP@Ag NPs is within the range of effective NP sizes according to literature.[53, 54]

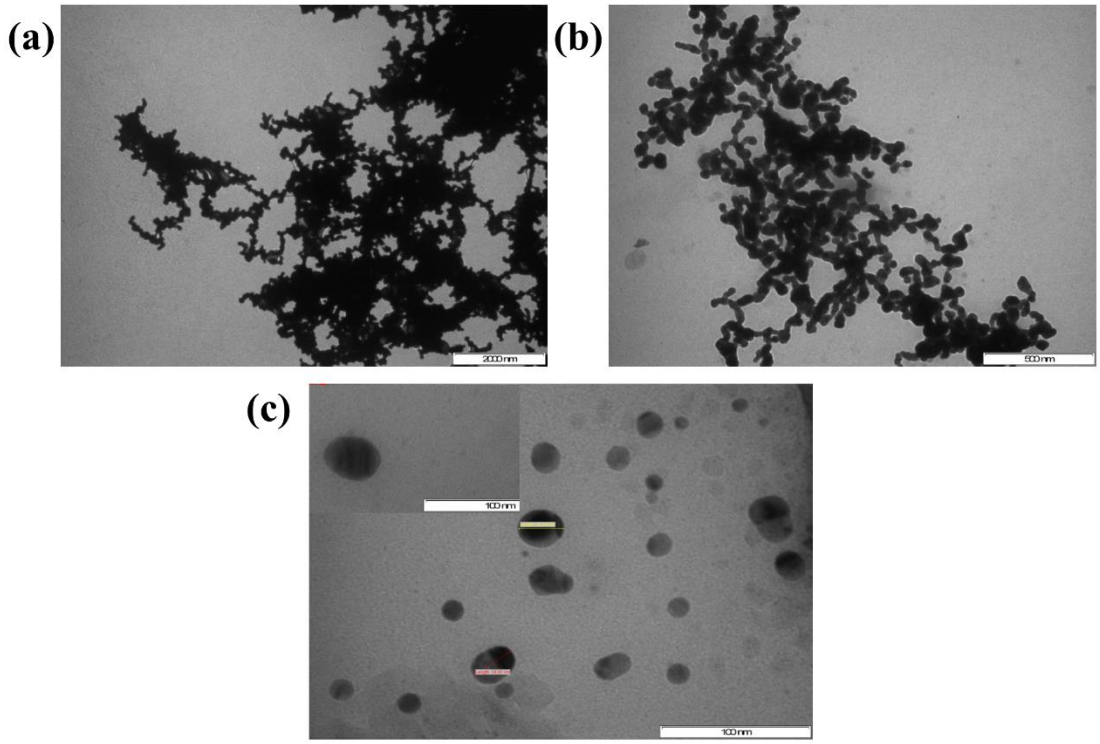


Fig. 3 TEM images of: (a) plain Ag NPs, (b) m-PEG@Ag NPs and (c) G1 PETIM-m-PEG SP@Ag NPs; inset shows a single G1 PETIM-m-PEG SP@Ag NP.

XRD analysis

The XRD pattern of lypophilised G1 PETIM-m-PEG SP@Ag showed peaks at 2θ values of 38.3, 44,4, 64,4, 77.6, 81.6^{o} which were characteristic peaks of Ag NPs, representing the 111, 200, 220, 311 and 222 Braggs reflection of the face-centered cubic (fcc) structure of silver. The 2θ values were in good agreement with previously reported values for Ag NPs.[55, 56] The presence of two prominent peaks of G1-PETIM-m-PEG SP at 2θ values of about 19.3 and 23.4 confirmed that G1 PETIM-m-PEG SP@Ag was stabilised by the G1 PETIM-m-PEG SP (Fig. 4a-b).

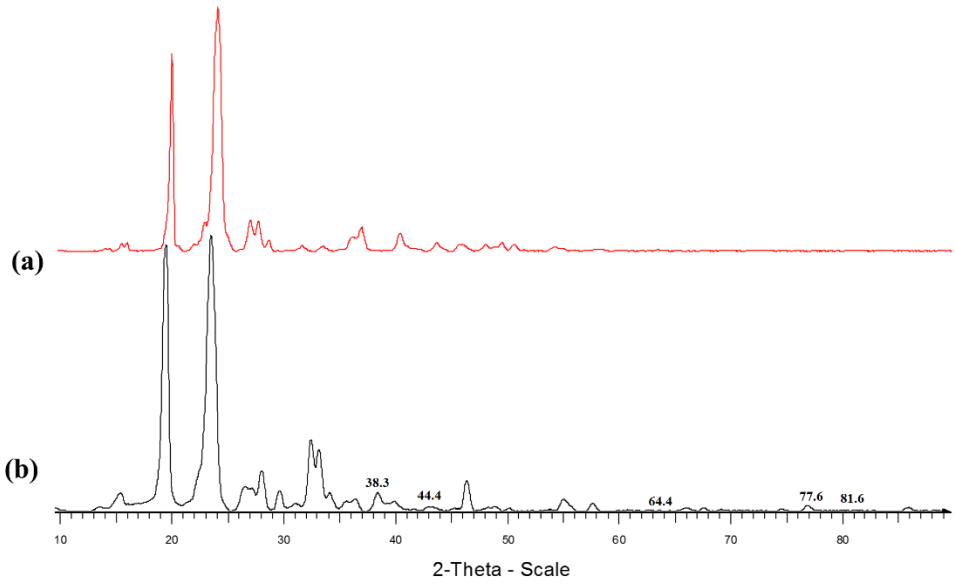


Fig. 4 XRD patterns of (a) G1 PETIM-m-PEG SP and (b) G1 PETIM-m-PEG SP@Ag NPs.

***In vitro* cytotoxicity studies**

The cellular response to a compound can be determined via cytotoxicity assays that deliver information about cell death and their metabolic activities.[57] MCF-7, HeLa and Hep G2 cell lines are widely used to assess the biocompatibility of newly synthesised materials for diverse applications.[58-60] The safety of the G1 PETIM-m-PEG SP and G1 PETIM-m-PEG SP@Ag NPs in biological studies was therefore established by an *in vitro* cytotoxicity study on MCF-7, HeLa and Hep G2 cell lines using the MTT assay. The cytotoxicities of $AgNO_3$, G1 PETIM-m-PEG SP, and G1 PETIM-m-PEG SP@Ag NPs were based on the biochemical reduction of MTT by viable cells.[61] $AgNO_3$ displayed a cell viability in the range of 59.91% to 30.97% against all the studied cell lines, indicating its toxicity to mammalian cells in a concentration dependant manner. There was a statistically significant decrease ($p < 0.05$) in cell viability by $AgNO_3$ across all the cell lines studied when compared to their respective controls. The results were supportive of previous findings, where $AgNO_3$ had significantly reduced cell viability with increasing concentration[62], which further supports the fact that silver is known to be non-toxic to human cells in minor concentrations.[4, 63] Cell viability across all the cell lines was 98.37% to 75% and 87.39% to 70.47% for the G1 PETIM-m-PEG SP and G1 PETIM-m-PEG SP@Ag NPs respectively, within the concentration range studied (**Fig. 5**). The results further indicated that the effects of the synthesised compounds on the cell lines were not dose dependent, as no dose dependent trends were observed for either G1 PETIM-m-PEG SP or G1 PETIM-m-PEG SP@Ag NPs (**Fig. 5**) compared to that of $AgNO_3$. However, with the exception

of the G1 PETIM-m-PEG SP at 80µg/ml and the G1 PETIM-m-PEG SP@Ag NPs at 40µg/ml against HeLa cells, there were no other statistically significant differences observed for the remaining cell lines at any of the concentration studied. The results displayed statistically significant differences for the G1 PETIM-m-PEG SP and G1 PETIM-m-PEG SP@Ag NPs, however, the cell viability was greater than 75%. This data suggests that the G1 PETIM-m-PEG SP and G1 PETIM-m-PEG SP@Ag NPs could be considered safe.[64, 65] The range of cell viability attained in this study also specifies that the G1 PETIM-m-PEG SP and G1 PETIM-m-PEG SP@Ag NPs exhibited a low toxicity level on all chosen cell lines.[14, 64] These findings therefore demonstrate the biocompatibility of the synthesised G1 PETIM-m-PEG and biosafety of G1 PETIM-m-PEG SP@Ag NPs.

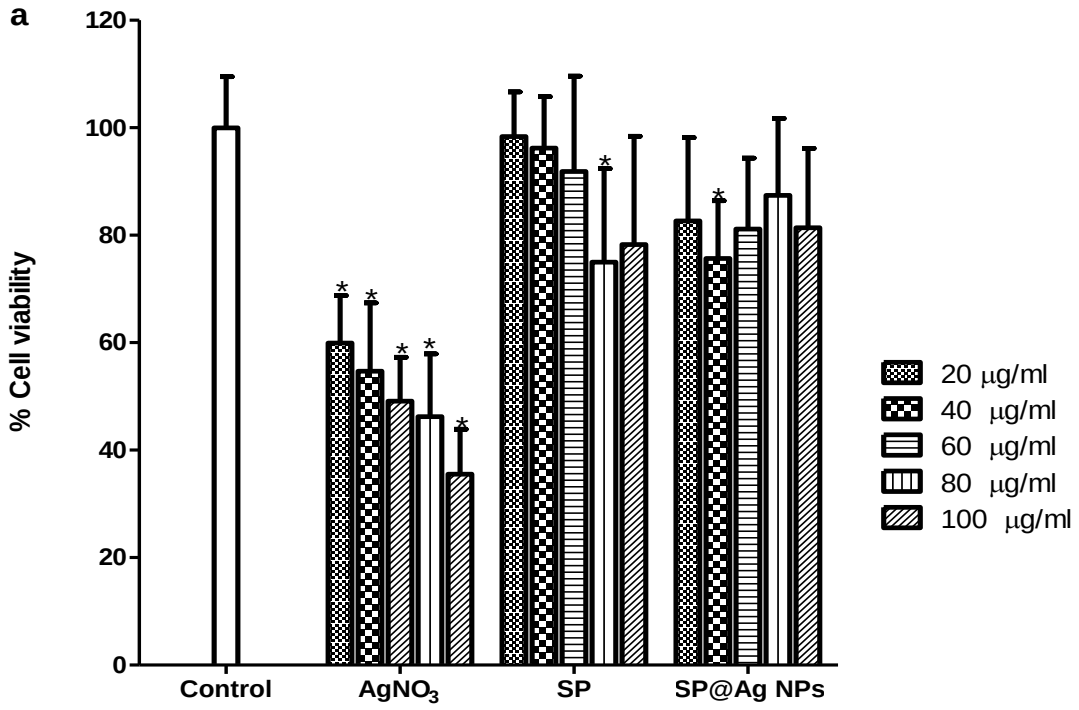
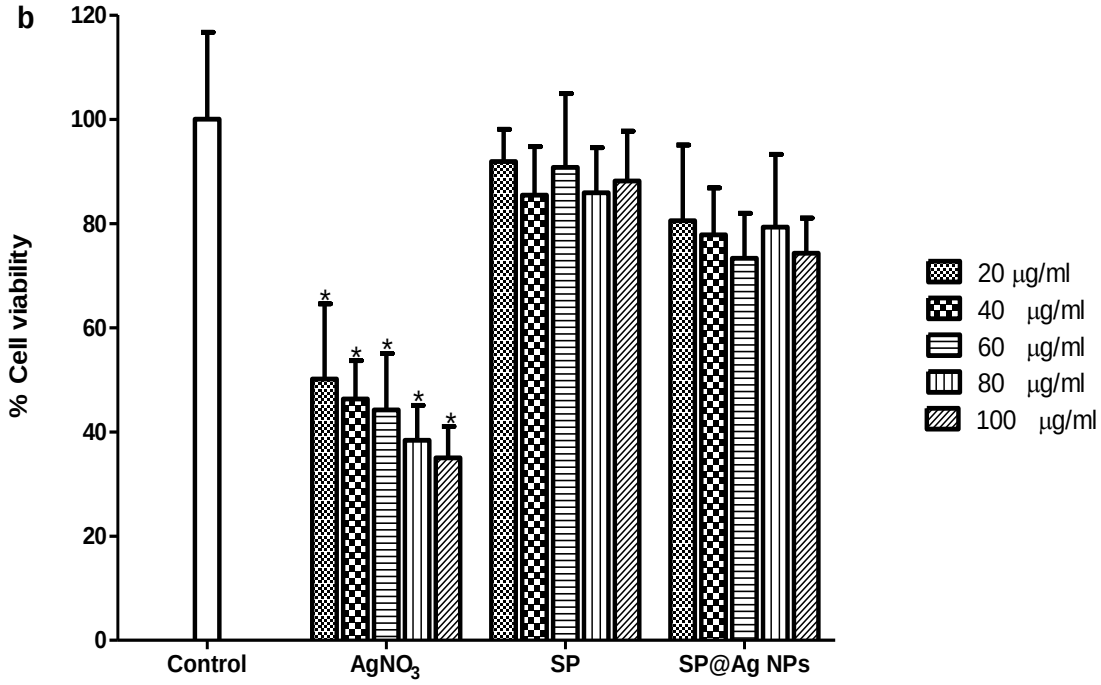


Fig. 5 (a) Cytotoxicity assay on MCF-7 cell lines displaying percentage cell viability after exposure to $AgNO_3$ and various concentrations of the G1 PETIM-m-PEG SP and G1 PETIM-m-PEG SP@Ag NPs. The results are presented as mean ± SD. (n = 6). *denotes significant difference compared to the untreated control ($p < 0.05$).



(b) Cytotoxicity assay on HeLa cell lines displaying percentage cell viability after exposure to $AgNO_3$ and various concentrations of the G1 PETIM-m-PEG SP and G1 PETIM-m-PEG SP@Ag NPs. The results are presented as mean ± SD. (n = 6). * denotes significant difference compared to the untreated control ($p < 0.05$).

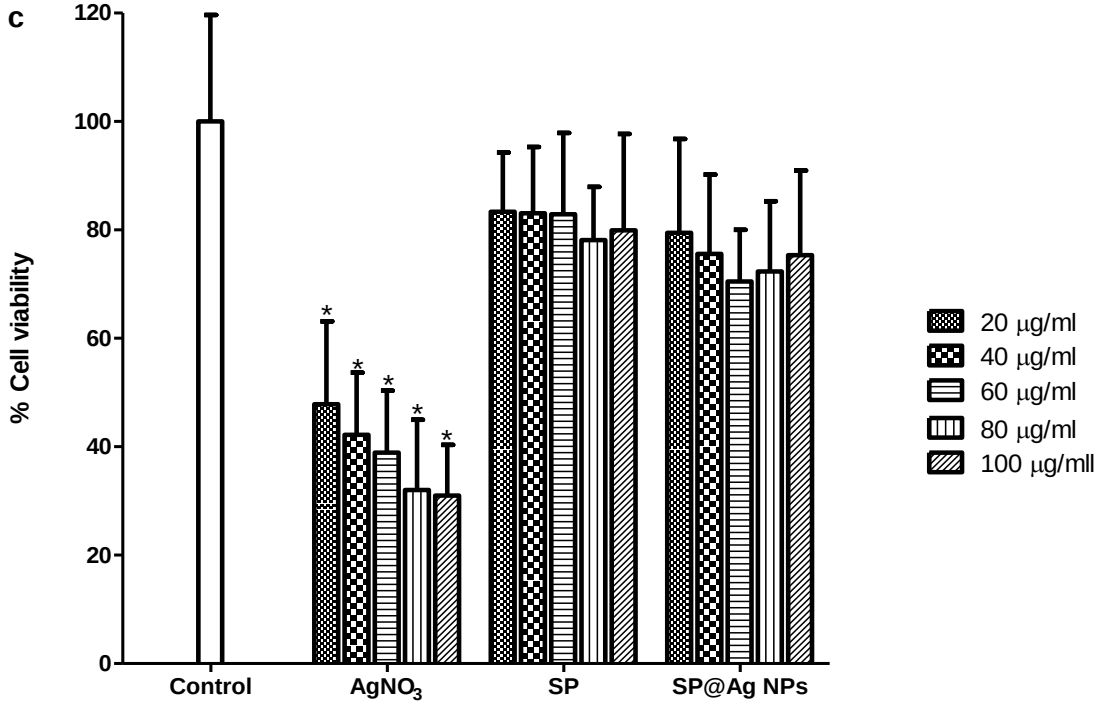


Fig. 5 (c) Cytotoxicity assay on Hep G2 cell lines displaying percentage cell viability after exposure to $AgNO_3$ and various concentrations of the G1 PETIM-m-PEG SP and G1 PETIM-m-PEG SP@Ag NPs. The results are presented as mean ± SD. (n = 6). * denotes significant difference compared to the untreated control ($p < 0.05$).

In vitro antimicrobial studies

The antimicrobial activity of $AgNO_3$, G1 PETIM-m-PEG SP, a mixture of $AgNO_3$ and G1 PETIM-m-PEG SP, and G1 PETIM SP@Ag, NPs were investigated against *S. aureus*, methicillin-resistant *S. aureus* (MRSA) [Gram positive], and *E. coli* and *P. aeruginosa* (Gram negative). A summary of the results for the minimum inhibitory concentration (MIC) values for *in vitro* antimicrobial activity is presented in Table 1.

Table 1 MIC results for *in vitro* antimicrobial activity of G1 PETIM-m-PEG SP, $AgNO_3$, a mixture of the G1 PETIM-m-PEG SP and $AgNO_3$, and G1 PETIM-m-PEG SP@Ag NPs against *S. aureus*, MRSA, *E.coli* and *P aeruginosa*.

Sample	**MIC (µg/ml)**			
	Organism			
	S. aureus	**MRSA**	***E. coli***	***P. aeruginosa***
G1 PETIM-m-PEG SP	-	-	-	-
Ag NO_3	0.58	18.5	9.25	18.5
Mixture of G1 PETIM-m-PEG SP and Ag NO_3	37	18.5	4.62	18.5
G1 PETIM-m-PEG SP@Ag NPs	18.5	74	9.25	74

The G1 PETIM-m-PEG SP displayed no antimicrobial activity against all the bacterial strains used in this study. $AgNO_3$ was found to be the most effective amongst all other materials tested, having the best activity against *S. aureus* (0.58 µg/ml), followed by the physical mixture of $AgNO_3$ and the G1 PETIM-m-PEG SP. This is due to the fact that ionised silver induces structural changes in the bacterial cell walls and nuclear membranes, as it is extremely reactive once it binds to tissue proteins. Consequently, it leads to cell distortion and ultimately cell death. Additionally, Ag ions can bind to bacterial DNA and RNA, and can hence inhibit bacterial replication. However, these antimicrobial properties of Ag are reliant on the amount and rate at which Ag is released.[4, 66] Despite the potent antimicrobial activity of Ag, it is vital

to note that silver is non-toxic to human cells only in minute concentrations,[63] which clearly restricts the use of Ag ions in the form of $AgNO_3$, or any other salt as antibacterial agents.[14]

When compared to $AgNO_3$, although the MICs of the G1 PETIM-m-PEG SP@Ag NPs were higher, they were within the ranges of previously reported MIC values for Ag NPs against *S. aureus*,[67] MRSA,[68] *E. coli* [53] and *P. aeruginosa*.[69] The G1 PETIM-m-PEG SP@Ag NPs therefore enjoys the advantage of being less toxic than $AgNO_3$, and is more stable than the uncapped Ag NPs.

The MIC values of the G1 PETIM-m-PEG SP@Ag NPs against *S. aureus* and MRSA were 18.5 and 74 µg/ml respectively, whereas against *E.coli* and *P. aeruginosa*, the values were 9.25 and 74 µg/ml respectively. The lowest MIC against *E. coli* displayed a considerable difference when compared to the other bacterial strains tested. This may be attributable to the cell wall composition of *E. coli*. Gram-negative bacteria having just a thin peptidoglycan layer (~2–3 nm) between the cytoplasmic membrane and the outer membrane,[70] whereas the Gram-positive bacteria lack the outer membrane and have a much thicker peptidoglycan layer of approximately 30 nm.[71] The thick peptidoglycan layer of Gram-positive bacteria could have prevented an uptake of silver ions in the cytoplasm, while the less thick wall of the Gram-negative bacteria could have facilitated the fast internalisation of G1 PETIM-m-PEG SP@Ag NPs in the cell wall, subsequently changing the DNA into a condensed form. The internalised G1 PETIM-m-PEG SP@Ag NPs would then have interacted with the thiol groups in the proteins, leading to structural changes in the protein and eventually cell death.[4, 72] Ag NPs are reported to have excellent antibacterial activity against *E. coli*.[73] When bacteria such as *E. coli* are treated with metal NPs, the bacterial membrane displays a noteworthy increase in permeability, leaving the bacterial cells unable to properly regulate transport through the plasma membrane, inevitably results in cell death. It is commonly known that the outer membrane of *E. coli* cells are principally fabricated from tightly packed lipopolysaccharide molecules, which proposes an effective permeability barrier.[73, 74] Metal depletion can result in the development of irregular shaped pits in the outer membrane and altered membrane permeability, which is a result of the progressive release of liposaccharide molecules and membrane proteins.[75] We can therefore speculate this as being the reason the G1 PETIM-m-PEG SP@Ag NPs having the best activity against *E. coli*.[73]

However, *P. aeruginosa*, although a Gram-negative bacteria, displayed an MIC value greater (low potency) than the Gram-positive *S. aureus*. This could be due to the differences between the cell walls of *E. coli* and *P. aeruginosa*. A general outer membrane permeability

of *P. aeruginosa* is 12 – 100 fold lower than *E. coli*. [76] This low outer membrane permeability, along with a secondary resistance mechanism such as efflux, could be the reasons for the high MIC value of G1 PETIM-m-PEG SP@Ag NPs against *P. aeruginosa.* [76]

Finally, the MIC value of 74 µg/ml against MRSA states/proves that the synthesised G1 PETIM-m-PEG SP@Ag NPs are efficient enough to inhibit the growth of drug resistant bacteria, and could therefore be an attractive delivery system to treat infections by MRSA.

Stability studies

The results from stability studies on G1 PETIM-m-PEG SP@Ag NPs are presented in Table 2. There were no statistically significant changes ($p > 0.05$) in PS, PDI and ZP between NPs stored at 4 °C and RT over a period of three months. In addition, no change in physical appearance and colour was noticed throughout the study period. The results confirmed the stability of G1 PETIM-m-PEG SP@Ag NPs under all specified storage conditions.

Table 2 Effect of storage condition on particle size, PDI and ZP.

Storage conditions	**Particle size (nm)**		**PDI**		**ZP**	
Time (days)	**4 °C**	**RT**	**4 °C**	**RT**	**4 °C**	**RT**
0	36.44 ± 2.51	36.44 ± 2.51	0.414 ± 0.007	0.414 ± 0.007	-23.7 ± 2.47	-23.7 ± 2.47
30	42.28 ± 5.06	45.84 ± 6.90	0.371 ± 0.039	0.337 ± 0.068	-22.1 ± 1.73	-23.1 ± 1.79
60	43.00 ± 5.53	46.46 ± 6.95	0.353 ± 0.062	0.336 ± 0.073	22.3 ± 4.61	-23.0 ± 3.79
90	43.35 ± 5.13	45.73 ± 6.81	0.344 ± 0.059	0.336 ± 0.064	-23.1 ± 5.24	-24.1 ± 2.78

*PS, PDI and ZP are expressed at mean, n=3.

Conclusion

G1 PETIM-m-PEG SP is a novel and biocompatible material which proved to be an efficient stabilising agent for the synthesis of Ag NPs. The results obtained in this study revealed that synthesised G1 PETIM-m-PEG SP@Ag NPs was an effective antimicrobial agent against Gram-positive, Gram-negative and drug-resistant bacteria with low toxicity to eukaryotic cells.

The applicability of the six-arm star-shaped G1 PETIM-m-PEG star polymer in drug delivery systems can further be exploited by its use in the preparation of surface modified nanoparticulate drug delivery systems, which can bypass phagocytic blood clearance, and ultimately increase the blood circulation time.

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Notes and References

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CHAPTER 5. REVIEW ARTICLE

5.1 Introduction

The following review paper was published in an international ISI journal:

Kalhapure, R.S., Suleman, N., Mocktar, C., Seedat, N., Govender, T., 2014. Nanoengineered Drug Delivery Systems for Enhancing Antibiotic Therapy. Journal of Pharmaceutical Sciences, 3, 872-905. (IF = 2.59)

Ms. N Suleman contributed to the literature searches as well as the collection and compilation of review papers and all research papers with regard to nano antibiotic delivery systems. Ms N Suleman also contributed to writing part of the introduction.

5.2 Published paper

The published article (DOI: 10.1002/jps.24298) can be found below.

REVIEW

Nanoengineered Drug Delivery Systems for Enhancing Antibiotic Therapy

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ABSTRACT: Formulation scientists are recognizing nanoengineered drug delivery systems as an effective strategy to overcome limitations associated with antibiotic drug therapy. Antibiotics encapsulated into nanodelivery systems will contribute to improved management of patients with various infectious diseases and to overcoming the serious global burden of antibiotic resistance. An extensive review of several antibiotic-loaded nanocarriers that have been formulated to target drugs to infectious sites, achieve controlled drug release profiles, and address formulation challenges, such as low-drug entrapment efficiencies, poor solubility and stability is presented in this paper. The physicochemical properties and the *in vitro*/*in vivo* performances of various antibiotic-loaded delivery systems, such as polymeric nanoparticles, micelles, dendrimers, liposomes, solid lipid nanoparticles, lipid–polymer hybrid nanoparticles, nanohybirds, nanofibers/scaffolds, nanosheets, nanoplexes, and nanotubes/horn/rods and nanoemulsions, are highlighted and evaluated. Future studies that will be essential to optimize formulation and commercialization of these antibiotic-loaded nanosystems are also identified. The review presented emphasizes the significant formulation progress achieved and potential that novel nanoengineered antibiotic drug delivery systems have for enhancing the treatment of patients with a range of infections. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: infectious diseases; nanoantibiotics; antibiotic resistance; nanodrug delivery systems; nanotechnology; polymeric drug carrier; polymeric drug delivery systems; controlled release; targeted drug delivery

INTRODUCTION

Infectious diseases continue to be one of the main reasons for death globally for both adults and children, and is recognized as a signi cant public health challenge.[1] Africa and South Africa in particular have a high burden of infectious diseases, including speci cally a large portion that is of bacterial origin. As a result of this, gastrointestinal, respiratory, sexually transmitted, and hospital acquired infections are leading causes of death in the developing world.[2] In addition, emerging and re-emerging infectious diseases,[3] together with issues such as the growing global trade and international travel and the probability of bioterrorist attacks in several countries, have compounded the seriousness of infectious diseases. Importantly, there is a recent growing acknowledgement that infections also play an important role in facilitating the occurrence of noncommunicable diseases. For example, diseases such as certain cardiovascular disorders, cancers, asthma, and gastrointestinal diseases have been reported to be linked to infectious diseases (including bacterial infections) as an underlying cause/risk factor.[4] The consequent adverse economic, social, and political impact of the global burden of infectious diseases therefore warrants novel and effective treatment strategies to overcome these challenges.

The advent of antibiotics, which was initiated with the introduction of penicillin more than 70 years ago and the more advanced compounds in later years, revolutionized the treatment of infectious diseases, and contributed signi cantly to decreasing the associated morbidity and mortality.[3] Antibiotics are considered pivotal in virtually all critical therapeutic areas, for example, general surgery including organ transplant procedures, treatment of premature babies, and chemotherapy in cancer patients cannot be achieved without effectively treating and preventing bacterial infections.[5] However, there are numerous limitations associated with the current antibiotic drug therapies. Several available dosage forms of antibiotics are compromised by inadequate drug concentrations at target infection sites, severe side effects, increased frequency of administration, and poor patient compliance that compromise drug therapy.[3,6] These limitations, together with the widespread use and abuse of antibiotics, have led to their most serious limitation, resistance to bacterial microorganisms. Microbial resistance nulli es the use of even the most potent antibiotics, which leads to patient suffering and/or mortality because of infection control failure and escalated health care costs.[3] Among these resistant pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA),[7] vancomycin-resistant *Enterococcus* (VRE),[8] vancomycin-resistant *S. aureus* (VRSA),[9] and penicillin-resistant *Streptococcus pneumonia*[10] have become major clinical threats. The antibiotic resistance crisis has also been further aggravated by pan drug-resistant and extensively drug-resistant organisms to antibiotics, which has reached alarming levels globally.[5,11]

According to a recent report released by the WHO on April 30, 2014, antibiotic resistance can no longer be regarded as an issue for the future but rather a current crisis that requires urgent interventions.[12] Although new antibiotics are being investigated to overcome antibiotic resistance, a steady and gradual decline in the introduction of new drugs have been reported by the US Food and Drug Administration (FDA).[13] This is because of exorbitant costs and lengthy times for eventual regulatory approval of new compounds, as well as low returns on

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investment, which compounds the current crisis.[14] Two systemic antibacterial agents were approved for use in humans by the US FDA from 2008 to 2012, compared with 16 approved from 1983 to 1987.[15] It is clear that the pace of drug development and registration has not been timeously responsive to the rapid development of resistance by microbial pathogens. This escalating emergence of antibiotic resistance to currently used antibiotics and decline in introduction of new antibiotic drugs is clearly a threat to human health globally. The search for new and effective strategies to enhance drug therapy with current antibiotics is therefore recognized globally as a major focus area of research priority.

The signi cant bene ts of using nanotechnology for treating various diseases such as cancer,[16 19] AIDS,[20 24] in ammation,[25 27] and hypertension[28 30] by improving the solubility, bioavailability, ef cacy, and speci city of drugs are widely documented in the literature. Nanotechnology, which refers to the design, production, and application of nanosized materials, is regarded as a new paradigm for optimizing the outcomes in infectious diseases treatment.[3]

Novel nanosized drug delivery systems could be a promising strategy to overcome the current challenges associated with antibiotic therapy because of their unique physicochemical properties. These include their large ratio of surface area to mass, small size, and unique interactions with microorganisms and cells of the host, as well as their ability to be structurally and functionally modi ed.[31,32] The advantages of a nanosized antibiotic drug delivery system include targeted delivery, relatively uniform distribution in the identi ed tissue, enhanced cellular internalization and solubility, sustained drug release and minimized side effects, and improved patient compliance.[33,34] Furthermore, nanosystems themselves have been found to inherently overcome existing speci c drug-resistance mechanisms by microbes.[35] In addition, the codelivery of multiple antibiotics into these nanosystems that are capable of having antimicrobial activity and overcoming resistance mechanisms themselves can promote synergistic activities and resistance overcoming effects.[31] These advantages are recognized as major contributors to overcoming bacterial resistance associated with poor delivery of antibiotics.[36]

Nanodrug delivery systems therefore offer an advanced and superior approach to overcoming several limitations associated with antibiotic drug therapy, including the serious global threat of antibiotic resistance. Compared with cancer and cardiovascular disease conditions, use of nanodrug delivery systems for speci cally encapsulating and delivering antibiotic drugs is still in its infancy.[3] Because of its potential advantages, there has been a surge of data in the literature on a range of differently engineered antibiotics-loaded nanodrug delivery systems. A perusal of the literature highlights the need for a review paper that speci cally focuses on the various reported nanodrug delivery systems to date that have been used for antibiotics. A comprehensive review of the various nanoengineered drug delivery systems that have emerged for antibiotic drugs is presented. The paper will therefore identify the technological progress that has been achieved regarding the development of these delivery systems and their potential for addressing the various formulation and therapeutic challenges with current antibiotic therapy. Future studies that need to be conducted for optimization and commercialization of these antibiotic-loaded nanosystems will be identi ed.

NANOENGINEERED ANTIBIOTIC DELIVERY SYSTEMS

The development of nanomedicines has facilitated an increase in the therapeutic index of many components. With changes in size from tens of micrometers to tens or hundreds of nanometers having been a signi cant technological and medical breakthrough.[37] A comprehensive literature search on several databases from 1960 to 2014 identi ed a range of nanodelivery systems for antibiotics that include liposomes, polymeric nanoparticles (PNPs), solid lipid nanoparticles (SLNs), lipid polymer hybrid nanoparticles (LPHNs), dendrimers, nanoemulsions (NEs), micellar systems, nanostructures made of pure carbon [carbon nanotubes (CNTs), nanosheets, and nanorods], nanohybrids, and others. As the 10 main nanodelivery systems that are used for antibiotic delivery, these will be discussed and evaluated in detail.

Liposomes

Liposomes, the rst closed bilayer systems, were described in 1965 and were soon proposed as drug delivery systems[38] using natural or synthetic lipids. Phosphatidylcholine (PC), which is a neutral phospholipid that contains fatty acyl chains, is one of the most commonly used lipids in liposome preparation. Adjustment of membrane rigidity and stability can be achieved by incorporating cholesterol into the preparation.[39] The two main classes of liposomes are multilamellar vesicles that comprise multiple phospholipid bilayer membranes, and unilamellar vesicles (ULVs) comprising a single lipid bilayer. ULVs can be further divided into large ULVs and small ULVs.[40] Since their inception, the most commonly applied methods used for preparing liposomes include thin- lm hydration,[41] reversed-phase evaporation,[42] solvent injection techniques,[43,44] and detergent dialysis.[45] Materials used for preparation, classi cation, and different techniques for the preparation of liposomes can be found elsewhere in the literature.[40,46 57]

Liposomes, consisting of phospholipid bilayers, are spherical lipid vesicles that can provide an improvement in the solubility of compounds and promote fusion with biological membranes and the subsequent release of their entrapped compounds into the target site.[58 60] In addition, it is possible to incorporate both hydrophilic and hydrophobic antimicrobial drugs in the aqueous core and in phospholipid bilayer, respectively.[33,61] Liposomes appear to be the earliest reported nanodrug delivery systems studied for antibiotic delivery in the literature, and clearly provided a platform for conceptualizing and developing other antibiotic nanodelivery systems. They have emerged as nanodelivery vehicles for antibacterial therapy, speci cally as they promote targeted delivery to the infection site, improve pharmacokinetics, reduce toxicity, and enhance antibacterial activity of antibiotics.[62]

Historically, the use of liposomes for antibiotic entrapment can be traced back to the early 1970s, after which this eld has expanded signi cantly to include various antibiotics in liposomes to effectively treat infections. A summary of various reported liposomal systems for antibiotic therapy with their rationale for formulation development is provided chronologically in Table 1. This overview clearly shows that liposomes have diverse applications for addressing various challenges with antibiotic therapy. Their potential for treating numerous disease conditions, being effective against a wide range of microorganisms, reducing toxicity, enhancing stability, and achieving sustained drug release and activity have been con rmed. More

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Table 1. A Chronological Overview of Antibiotic Liposomal Development

Formulation	Active Ingredient	Targeted Microorganism	Rationale for Formulation	Reference
PC, diacetyl phosphate, and cholesterol	Filipin	None	Removal of the haemolytic activity of Filipin.	Ref. 63
Egg lecithin, cholesterol, phosphatidic acid, dipalmitoyl lecithin, and stearylamine	Potassium benzyl penicillin	None	Lysosomal localization of liposome-entrapped drugs in the liver and spleen.	Ref. 64
Egg PC, cholesterol, and phosphatidic acid	Dihydrostreptomycin	*S. aureus*	Killing of intraphagocytic *S. aureus*.	Ref. 65
Egg PC, cholesterol, diacetylphosphate, and total lipid extract of rat intestinal mucosa	Ampicillin, amoxicillin, cephalexin, sodium cephazolin, sodium ceftezol, sodium cephalothin, cephaloridine, and cephradine	None	Study of the liposomal membrane permeability to antibiotics.	Ref. 66
PC, cholesterol, and phosphatidylserine	Cephalothin	*S. typhimurium*	Intracellular killing of the microorganisms.	Ref. 67
PC, cholesterol, and phosphatidylserine	Penicillin-G	*Listeria monocytogenes*	Treatment of intracellular infections	Ref. 68
Proprietary formulation prepared by Fountain Pharmaceuticals, Inc. (Knoxville, Tennessee)	Tobramycin and silver sulfadiazine	*P. aeruginosa*	Topical delivery for treatment of soft tissue wounds.	Ref. 69
Egg PC, cholesterol, and diacetylphosphate	Vancomycin and teicoplanin	MRSA	Treatment of intracellular MRSA.	Ref. 70
Egg lecithin and cholesterol	Amikacin, netilmicin, tobramycin	*P. aeruginosa*, *Xanthomonasmaltophilia E. Coli*, *Streptococcus faecalis*, and *S. aureus*	Enhancement of antibacterial activity.	Ref. 71
Soybean PC and cholesterol	Ampicillin	*Micrococcus Luteus*	Improvement of drug stability and retention of antibacterial activity.	Ref. 72
Hydrogenated PC and cholesterol	Gentamycin	None	Modi cation of drug release pro le to achieve sustained drug release.	Ref. 73
Proprietary formulation prepared by Bristol	Amikacin	*Mycobacterium Avium*	Prolongation of antibacterial activity in *in vivo* studies.	Ref. 74
Dipalmitoyl-DL-α-phosphatidyl-L-serine, cholesterol, lipopolysaccharide, L-α-phosphatidyl-DL-glycerol, dihexadecyl hydrogen phosphate, dihexadecyl hydrogen phosphate, dihexadecyl hydrogen phosphate, and 1,2-dipalmitoyl-*sn*-glycero-phosphatidic acid sodium salt	O oxacin	*Enterococcus faecalis*, *Escherichia Coli*, *S. aureus*, and *P. aeruginosa*	Enhancement of the activity of uoroquinolone antibacterials.	Ref. 75
Dipalmitoylphosphatidylcholine, cholesterol, and dimethylammonium ethane carbamoyl cholesterol	Penicillin-G	*S. aureus*	Enhancement of the effectiveness of penicillin-G at low concentration and short exposure time.	Ref. 76
Cationic, anionic, and neutral liposomes lecithin (egg PC), stearylamine and cholesterol, L-α-phosphatidyl-DL,-glycerol and cholesterol, and lecithin and cholesterol	Cipro oxacin and vancomycin	*S. aureus*	• Treatment of chronic *staphylococcal osteomyelitis* by combination therapy • Reduction in nephrotoxicity, enhancement of antibacterial activity depending on charge and sonication time.	Ref. 77

Continued

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Table 1. Continued

Formulation	Active Ingredient	Targeted Microorganism	Rationale for Formulation	Reference
1,2-Dioleoyloxy-3-trimethylammonium-propane, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, PC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-distearoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine	Meropenem	*P. aeruginosa*	Enhancement of antibiotic activity against sensitive and resistant strains.	Ref. 78
1,2-Dimyristoyl-*sn*-glycero-3 phosphocholine and cholesterol	Gentamicin	*P. aeruginosa*	Improvement of killing time and prolongation of antimicrobial activity to treat chronic respiratory infections associated with cystic brosis.	Ref. 79
PC, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-3000] (ammonium salt), L-α-phosphatidyl ethanolamine-*N*-(lissaminerhodamine B sulfonyl) (ammonium salt) and 1,2-distearoyl-3-trimethylammonium-propane (chloride salt), poly(ethylene glycol)-α-disteroyl)phosphatidyl-ethanolamine,-ω-benzotriazole carbonate MW 3400	Rifampicin	*S. epidermidis*	Development of an antimicrobial barrier on polymer surface of interest for medical applications.	Ref. 80
1,2-Distearoyl-*sn*-glycero-3-phosphocholine, methylpolyethyleneglycol 1, 2-distearoyl-phosphatidyl ethanolamine conjugate	Vancomycin	None	Increasing lung tissue concentration of vancomycin for effective treatment of pneumonia caused by MRSA by surface modi cation of liposomes with PEG.	Ref. 81
Egg PC and cholesterol	Vancomycin	MRSA	Selective delivery of antimicrobials to the sites of bacterial infections by utilizing bacterial toxins to activate drug release from gold nanoparticle-stabilized phospholipid liposomes.	Ref. 82

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recent studies are focusing on exploiting the bene ts of surface modi cation and responsive drug delivery to further enhance the effectiveness of liposomal systems. Some of these studies are brie y discussed further.

One of the rst applications for liposomes in antibiotic drug delivery is reported for lipin, a polyene macrolide antibiotic known for its haemolytic activity.[63] It should however be noted that the liposomes in this study were not explored as a carrier, but rather as a model to test the sterol receptor hypothesis of polyene action. Similarly, a few years later, liposomes were also used as a model to investigate the intestinal absorption mechanisms of several antibiotics.[66] Although not used as a delivery system itself, liposomes have proved useful in providing the necessary information for optimizing therapy with polyene macrolide antibiotics.

The potential of liposomes as an antibiotic carrier probably began with Gregoriadis.[64] He entrapped potassium benzyl penicillin in liposomes composed of egg lecithin, cholesterol, phosphatidic acid, dipalmitoyl lecithin, and stearylamine to overcome the failure of penicillin to penetrate cells of the reticuloendothelial system (RES). These *in vivo* studies with rats showed lysozymal localization of penicillin-entrapped liposomes into the liver and spleen.[64] This early study did not focus on antibacterial activity against microorganisms, as researchers at that stage were attempting to prove its targeting potential. The intracellular residence of bacteria may complicate effective treatment of bacterial infections. In subsequent studies, other research expanded this area, and reported specifically on intracellular killing of various classes of sensitive and resistant bacteria by liposomal formulations using drugs such as dihydrostreptomycin,[65] cephalothin,[67] penicillin-G,[68] vancomycin, and teicoplanin.[70] In addition to intracellular targeting, liposomes have been studied for topical applications, with reports indicating that topical infections of soft tissues by *Pseudomonas aeruginosa* can be effectively treated by liposomal tobramycin silver sulfadiazine.[69]

Several other liposome-based antimicrobial drug delivery systems have also been recently developed for various applications and for reducing antibiotic toxicity,[58] and have found applications in vaccine technology. Zhao et al.[83] genetically linked the urease linear epitope with cholera toxin B subunit to obtain a novel fusion peptide CtUBE and expressed it in *Escherichia coli*, and formulated an oral liposome vaccine against *H. pylori*. The sizes of the liposomes were between 100 and 500 nm, and almost 71.4% CtUBE was entrapped in liposomes. The study demonstrated that after oral immunization, liposomal CtUBE was able to protect BALB/c mice from *H. pylori* infections.[83] Another unique study emphasized the diverse applications of liposomal antibiotic formulations. Surface coating of polystyrene by cationic rifampicin-loaded liposomes was performed in order to develop an antimicrobial barrier on a polymer surface to be exploited for medical uses.[80] The rifampicin-loaded liposomes as an antimicrobial barrier reduced bacterial growth on polystyrene, with activity being dependent on the charge of the liposomes with the polystyrene surface. Effective activity against various organisms for other disease conditions, such as gentamicin liposomes[79] and meropenem liposomes[78] against *P. aeruginosa*, ampicillin liposomes against *Micrococcus luteus*,[72] and penicillin liposomes against *S. aureus*[76] have also been reported.

Another research goal by liposomal researchers has been to achieve prolonged release and/or enhanced activity of antibiotics. In early studies, Omri and Ravaoarinoro[71] entrapped various antibiotics (amikacin, netilmicin, and tobramycin) into liposomes. Although netilmicin had lower liposomal encapsulation ef ciencies than tobramycin and amikacin, it had reduced minimum inhibitory concentrations (MICs) against Gram-positive and Gram-negative bacteria compared with free drug, whereas liposomal tobramycin and amikacin antibacterial activity was not improved as compared with the free solution. In this study, only encapsulation ef ciencies and antimicrobial activities were reported. Being initial liposomal antibiotic formulation studies in this eld, other critical data such as size, polydispersity index, surface charge, morphology, and stability were not reported, unlike more recent papers where this is essential. Prolonged and/or enhanced activity has also been reported for liposomal formulations, such as gentamycin,[73] amikacin,[74] o oxacin,[75] penicillin-G,[76] meropenem,[78] and gentamicin[79] against a wide range of microorganisms. The prolonged antibacterial activity has been attributed to the sustained release of drugs from liposomes, which have also been shown to enhance the stability of antibiotics. For example, it has been shown that free ampicillin lost 50% initial activity after 5 weeks of storage at 4°C, whereas some of the liposomal ampicillin formulations lost only 17% activity.[72] On the basis of the differences between liposomal formulations, it would be useful in future to investigate how variables such as drug encapsulation ef ciencies and lipid content affect stability as well as antimicrobial activity.

Liposome size and surface charge can be modi ed and optimized depending on its therapeutic application.[84] Liposomes encompassing surface modi cation with materials such as glycolipids or sialic acid have been prepared.[85] Thus, cationic or anionic liposomes can be prepared by using cationic or anionic ingredients in the liposomal formulations. In one such study, to establish a new antibiotic therapeutic approach against chronic staphylococcal osteomyelitis infections presenting in rabbits, two antibiotics, namely, cipro oxacin and vancomycin were encapsulated alone and in combination in liposomes. The study was undertaken to: (1) lower nephrotoxicity, (2) overcome poor antibiotic accumulation in bone tissue, (3) completely sterilize bone tissue by combination therapy, and (4) most importantly to facilitate optimal liposome bacterium interaction via evaluation of cationic, anionic, and neutral liposomes.[77] The results showed a greater percentage of drugs being entrapped in charged liposomes than neutral, and among all the three formulations, enhanced antibacterial activity against *S. aureus* was observed for cationic liposomal formulation. This proved the concept that interaction between the cationic liposomes and negatively charged bacterial cell surface can occur.[77,86] Reduction in nephrotoxicity was also reported with animal studies using rabbits.

Another active area of research is surface modi cation of liposomes, which is used for various purposes, such as stabilizing liposomes against fusion[87] and controlling liposome blood clearance.[88] The incorporation of poly-(ethylene glycol) (PEG) in the liposome composition represented a major step in the development of liposomes with increased circulation and half-life.[85] Pneumonia caused by MRSA is dif cult to treat with vancomycin because of low lung tissue and intracellular penetration of vancomycin, leading to MRSA evading phagocytic killing. Muppidi et al.[81] proved that MRSA pneumonia can be effectively treated by using PEGylated liposomal vancomycin as compared with conventional and non-PEGylated

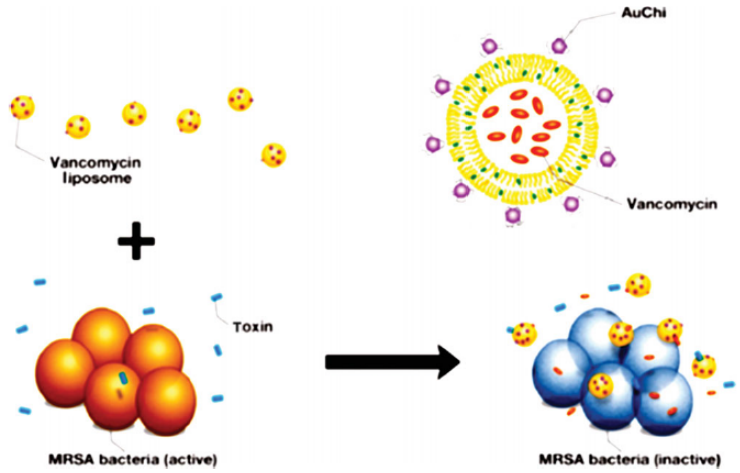

Figure 1. Schematic principle of bacterial toxin-triggered antibiotic release from gold nanoparticle-stabilized liposomes to treat toxin-secreting bacteria. Reproduced from Pornpattananangkul et al.[82] with permission from American Chemical Society.

preparations. This was possible because of the ability of PEGylated liposomal vancomycin to signi cantly extend circulation time in the blood, and increase lung, liver, and spleen deposition while also reducing accumulation in the kidney tissue. It has been suggested that administration of PEGylated liposomal vancomycin may enhance the effective treatment of MRSA pneumonia and simultaneously reduce the nephrotoxicity risk. This study was purely an *in vivo* study, and the promising results with these surface modi cation studies should be followed up with formulation optimization and characterization investigations to con rm stability and activity. It would also be interesting to investigate how the PEGylation affects antibacterial activity in terms of interaction with bacterial cell membranes.

In a recent paper, surface modi cation of liposomal surface was explored not only for altering distribution, but also for achieving triggered drug release at an infection site. A new approach to differentially release vancomycin to the site of infection to inhibit the growth of *S. aureus* for topical treatment of skin bacterial infections was developed by attaching chitosan-modi ed gold nanoparticles (AuChi) onto the surface of negatively charged liposomes.[82] This strategy was based on the fact that few bacteria release a toxin, and this toxin can be used to activate drug release from AuChi-stabilized liposomes. In nature, *S. aureus* secretes alpha haemolysin (α-toxin) as a water-soluble 34 kDa protein monomer.[89] A heptameric structure with a central 2 nm size pore is formed when the α-toxin spontaneously incorporates into the lipid membranes and self-oligomerizes. This pore permits the passive diffusion of small molecules of up to 3 kDa through the membranes.[90,91] Figure 1 illustrates the principle involved in the selective release of vancomycin at the site of infection.[82] The mechanism involves binding of AuChi to the negatively charged surface of liposomes via electrostatic attractions, which stabilizes liposomes by preventing fusion with one another and also prevents unwanted drug leakage. When the stabilized liposomes have reached the vicinity of *S. aureus*, the α-toxin secreted by bacteria inserts into the liposome membrane and forms pores that allow the encapsulated vancomycin to be released. The vancomycin that has been released close to the bacteria will then be allowed to exert its rapid and local antibacterial activity.

Incubation studies with MRSA con rmed 48% and 100% release within 0.5 and 24 h, respectively, and no drug release in the absence of MRSA. Vancomycin release in the presence of MRSA therefore con rmed the drug release in the presence of the bacterial toxin only. The study did not report release data on unmodi ed vancomycin liposomes, which could have provided additional supportive con rmation of the principle of triggered release with the AuChi modi cation. Antibacterial studies showed that the AuChi vancomycin liposomes inhibited microbial growth to the same level as vancomycin liposomes. Therefore, the triggered release only on exposure to the toxin with retention of antibacterial activity was considered an improved approach for enhancing therapy with vancomycin. This approach will certainly provide a new paradigm for the treatment of infections, by speci cally releasing antibiotics at infection target sites while minimizing possible nontarget adverse effects.[82]

The overview in Table 1 indicates a decrease in the last few years of the use of liposomes for antibiotic delivery. This could be because of the already extensive body of literature available for its application in other disease states, as well as to some disadvantages that are being overcome by newer technologically advanced systems, as discussed later in this paper. In the present scenario, liposome nanotechnology has nevertheless advanced to such an extent that it is possible to modify their surface, attach other nanoparticles (NPs) or targeting moieties on their surface in order to obtain site-speci c/targeted delivery and to control the release of antibiotics. Ongoing research regarding the delivery of antibiotics via liposomes using advanced nanotechnological aspects will certainly be fruitful if some challenges such as stability (*in vitro* and *in vivo*) are addressed, which will expedite several potential liposome-based antibiotic clinical products in the 21st century.

Polymeric Nanoparticles

Polymeric nanoparticles are solid colloidal particles, ranging in size from 1 to 1000 nm. They comprise several biocompatible polymeric matrices in which the therapeutic moiety is either entrapped, adsorbed, or covalently attached.[92] Because of their polymeric composition, PNPs may have greater stability than liposomes in biological uids and under storage.[93] The main aim of preparing NPs using polymers is to increase therapeutic bene ts, minimize side effects of conventional drugs, and to ef ciently deliver drug to a target site.[94,95] Natural polymers, such as chitosan, gelatin, and alginate as well as synthetic polymers, such as poly(lactic-co-glycolic)acid (PLGA), poly-*n*-(cyanoacrylate), and polycaprolactone (PCL) are widely used to fabricate PNPs.[96]

Poor therapeutic ef cacy because of rapid clearance by RES, the initial drawback of PNPs, has been overcome using strategies such as modi cation with hydrophilic excipients.[97] PNPs have been widely studied for various disease states, such as in ammatory bowel diseases,[98] cancer,[99] hypertension and angina,[100] airway in ammatory diseases,[101] diabetes,[102] and AIDS.[103] Although nanotechnology for antibiotics is still in its infancy, PNPs appear to be one of the most extensively studied nanosystems for antibiotic delivery. Their unique characteristics for antibiotic delivery include: (1) structural stability; (2) possibility of synthesis with a sharper size distribution; (3) precise tuning of properties such as particle size, surface charge, and drug release pro les via selection of appropriate polymers, surfactants, and organic solvents during preparation; and (4) the option of modifying the functional groups at the surface of PNPs by either drug moieties or targeting ligands.[104] The active moiety can be encapsulated, entrapped, dissolved, or attached to a polymeric matrix to generate either NPs, nanospheres, or nanocapsules depending on the method of preparation employed. Dispersion of preformed polymers and polymerization of the monomers have been mainly used for the preparation of NPs.[105] Other methods of PNP preparation can be found elsewhere.[106 108]

Polymeric nanoparticles have been explored for delivering a wide range of antibiotics for the treatment of diverse infections caused by different bacteria and have shown enhanced therapeutic ef cacy. Table 2 depicts a chronological summary of antibiotic-loaded PNP systems reported in the literature. The polymers and antibiotics used, method of PNPs preparation, characterization study performed, and the main ndings achieved are extracted, summarized, and presented. As can be seen in Table 3, in initial studies, polyalkylcyanoacrylates (PACA) were the materials of choice for preparing antibiotic-loaded PNPs.[109 111] To address the problem of resistance of intracellular infections to chemotherapy because of low intracellular uptake of commonly used antibiotics or their decreased activity at the acidic pH of lysosomes,[110] several studies have been conducted to deliver antibiotic intracellularly using PNPs. In early studies, ampicillin was bound to polyisohexylcyanoacrylate (PIHCA) to form PNPs, with an average size of 187 ± 13 nm for intracellular targeting of antibiotic. *In vivo* studies in experimentally infected C57BL/6 mice revealed that the therapeutic index of ampicillin against *Salmonella typhimurium* was increased by 120-fold when bound to PIHCA NPs.[109] Furthermore, in *in vivo* studies on PIHCA, bound ampicillin PNPs showed that 0.8 mg of ampicillin incorporated into NPs had a greater therapeutic effect as compared with 48 mg of free ampicillin against *S. typhimurium*. Furthermore, the ampicillin NPs were rapidly taken up by the liver and spleen, leading to a subsequent higher concentration of the drug in these organs.[110]

Formulation development of polyethylcyanoacrylate (PECA) NPs containing pe oxacin and o oxacin quinolone antibiotics using the incorporation or adsorption method was reported by Fresta et al.[111] These PECA NPs exhibited twofold to 50-fold more antimicrobial activity against *P. aeruginosa*, *S. aureus*, *E. coli*, and *Enterococcus faecalis*, with *in vivo* proof that the delivery system was preferentially captured by the mononuclear phagocyte system. In another experiment using PACA, cipro oxacin-loaded polyethylbutylcyanoacrylate (PEBCA) nanoparticlulate formulation with adequate drug loading and release properties was developed by an emulsion polymerization technique. It should be noted that MIC or minimum bactericidal concentration (MBC) against *S. Typhimurium* was not changed by the binding of cipro oxacin to PEBCA NPs. MIC and MBC values were same (0.062 and 0.5 μg/mL, respectively), irrespective of the form used.[112] Several years later in 2007, *N*-thiolated and acrylated β-lactam antibiotics were also loaded onto polyacrylate nanoparticles by conjugation onto its framework to protect it from the β-lactamase enzyme.[117,118] NP formulations of *N*-acrylated β-lactam antibiotic were found to be more potent compared with NP formulations of *N*-thiolated one. It should be noted that these early studies were mainly focused on studying the antimicrobial activity (*in vitro* and *in vivo*) of antibiotic-loaded PACA NPs, with few attempts only at formulation optimization, in depth characterization of PNPs, and surface modi cation for targeted delivery.

Table 2 also reveals a recent decrease in the use of PACAs for synthesizing PNPs. As from the 21st century scientists are clearly switching to more biocompatible and biodegradable natural and synthetic polymers, such as PLGA, chitosan, lecithin, and PCL. Furthermore, the synthesis of novel biocompatible and biodegradable materials to formulate nanosystems for infection control is also an emerging research area in the literature,[132 134] and polymers with multifunctional properties for antibiotic delivery is no exception to this trend. These studies are described in the section hereunder.

Poly(lactic-co-glycolic)acid appears to be one of the most widely studied polymers for antibiotic delivery. Initially, Dillen et al.[114] attempted the formulation development of cipro oxacin PLGA NPs using a factorial design to study the effect of different parameters on particle size, zeta potential, drug entrapment, and release. Their ndings showed that homogenization had a marked effect on particle size, release rate, and entrapment ef ciency. Homogenization decreased the particle size and drug release, but also increased the drug entrapment ef ciencies. In this study, antibacterial activity of the PNPs was found to be comparable to free drugs against *P. aeruginosa* and *S. aureus*.[114] However, it should be noted that although 100% of the drug was not released after 24 h, it nevertheless had equivalent activity. These researchers recognized that PLGA, being negative, might have low interactions with the anionic mucus for ocular infections. They then extended this study and incorporated cationic polymers into this PLGA formulation. In a subsequent study, they investigated the effect of including cationic polymers, namely, Eudragit® RS100 or RL100 on physicochemical properties, the release pro le, and antibacterial activity of cipro oxacin-loaded PLGA-containing PNPs.[115] They found that the zeta potential of all formulations

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Table 2. Polymeric Nanoparticulate Systems Used for Antibiotic Therapy

Polymer	Active Ingredient	Preparation Method	*In Vitro*/*In Vivo* Characterization Studies	Main Findings	Reference
Polyisohexylcyanoacrylate	Ampicillin	Emulsion polymerization	• Laser light scattering • *In vivo* antibacterial activity	Increased potency of ampicillin-bound NPs than free ampicillin assessed by *in vivo* treatment of salmonellosis infection of *S. typhimurium*.	Ref. 109
Polyisohexylcyanoacrylate	Ampicillin	Emulsion polymerization	• *In vitro* drug release studies in fetal calf serum • *In vitro* antibacterial activity using *B. subtilis* spores • *In vivo* experiments on *S. typhimurium*- and *L. monocytogenes*-infected mice	Greater therapeutic efficacy of ampicillin-bound NPs than free ampicillin confirmed by experimental *Listeria monocytogenes* infection.	Ref. 110
Polyethylcyanoacrylate	Pefloxacin mesilate and norfloxacin	Incorporation or adsorption method	• Size and molecular weight • Morphology • MIC by broth dilution • Drug accumulation studies in bacteria	Enhancement of antimicrobial activity against *P. aeruginosa*, *E. coli*, *S. aureus*, and *E. faecalis* from twofold to 50-fold.	Ref. 111
Polyethylbutylcyanoacrylate	Ciprofloxacin	Emulsion polymerization	• Size by light scattering • Zeta potential by zeta sizer • Molecular weight by gel permeation • Loading efficiency using HPLC and agar diffusion method • Release kinetics • *In vitro* antibacterial activity using microdilution method	• Efficient loading of drug, controlled release, and suitable size PNPs for intravenous administration. • The presence of ciprofloxacin in polymerization medium strongly influenced the NP size and molecular weight because of the formation of tight chemical bond between ciprofloxacin and ethyl cyanoacrylate.	Ref. 112
Lectin and gliadin	Acetohydroxamic acid	Desolvation method	• Size and zeta potential • Morphology by SEM • Drug entrapment • Drug release by dialysis cell membrane method • *In vitro* activity on pig gastric mucin • NP binding to *H. pylori* using agglutination assay • *In vitro* growth inhibition assay • *In situ* adherence assay on adult human esophagus, stomach, and duodenum	Targeted antibiotic delivery onto carbohydrate receptors of *H. pylori* bacteria, enhanced antibacterial activity as compared with free drug.	Ref. 113

Continued

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Table 2. Continued

Polymer	Active Ingredient	Preparation Method	*In Vitro/In Vivo* Characterization Studies	Main Findings	Reference
PLGA	Cipro oxacin	Emulsi cation solvent evaporation method	• Size and zeta potential • Drug loading • *In vitro* release • Differential scanning calorimetry (DSC) • X-ray diffraction (XRD) • *In vitro* antibacterial activity	• Enhanced drug entrapment • Decreased particle size and release rate of cipro oxacin • Faster drug release after gamma sterilization of PNPs	Ref. 114
PLGA, Eudragit RS® 100, or RL 100	Cipro oxacin	Emulsi cation solvent evaporation method	• Size and zeta potential • DSC • Drug loading • *In vitro* release • *In vitro* antimicrobial activity • Evaluation of NP adhesion to *P. aeruginosa* and *S. aureus*	Prolonged drug release, positively charged NPs for prolonged residence time in anionic mucus for effective management of *P. aeruginosa*, and *S. aureus* infections.	Refs. 115 and 116.
Butyl acrylate and styrene	Acrylated penicillins	Free radical emulsion polymerization	• Size, zeta potential, and morphology using DLS, TEM, and atomic force microscopy (AFM) • Stability • *In vitro* antibacterial activity	Enhanced activity against β-lactamase producing MRSA.	Ref. 117
Butyl acrylate and styrene	N-thiolated β-lactam derivatives	Free radical emulsion polymerization	• Size, zeta potential, and morphology using DLS, SEM, TEM, and AFM • *In vitro* cytotoxicity • *In vitro* antibacterial activity	Novel β-lactam antibiotics and polymeric NPs thereof for enhanced anti-MRSA activity.	Ref. 118
PLGA	Cipro oxacin	Multiple emulsion solvent evaporation method	• Drug content and loading ef ciency • XRD • TEM • Size • Drug release studies • *In vitro* and *in vivo* susceptibility testing of NPs • *In vitro* and *in vivo* antibacterial activity	Effective *in vivo* growth inhibition of pathogenic *E. coli* because of sustained-release characteristics of NPs.	Ref. 119

Continued

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Table 2. Continued

Polymer	Active Ingredient	Preparation Method	*In Vitro/In Vivo* Characterization Studies	Main Findings	Reference
PLGA and PCL	Doxycycline	Solvent evaporation	• Size and zeta potential • SEM • Fourier transform infrared spectra (FT-IR) • DSC • Entrapment ef ciency • *In vitro* release • *In vitro* antibacterial activity	Increased entrapment of drug, sustained release with enhanced activity against DH5α strain of *E. coli*.	Ref. 120
PLGA	Azithromycin	Nanoprecipitation	• Size and zeta potential • SEM • Encapsulation ef ciency • DSC • FT-IR • *In vitro* dissolution study • *In vitro* antibacterial activity	Ef cient drug loading, sustained release, increased ef ciency against *S. typhi* than free drug with the targeting of drug to phagocytic cells.	Ref. 121
PLGA	Levo oxacin	Modi ed standard methods	• Size and zeta potential • SEM • In vitro drug release • *In vitro* antibacterial activity • Drug encapsulation and loading	Enhanced encapsulation of highly water-soluble antibiotic by modi cation of preparation method.	Ref. 122
Chitosan tagged with folic acid	Vancomycin	Emulsi cation	• FT-IR • Size • TEM • *In vitro* cytotoxicity • *In vitro* antibacterial activity	Effective drug delivery system for VRSA. Transport of drug-loaded NPs through endocytosis across the plasma membrane into cytoplasm.	Ref. 123
PLGA	Rifampin and azithromycin	Emulsion solvent evaporation	• Size and zeta potential • Drug loading and encapsulation ef ciency • *In vitro* release • Study of NP traf cking to infection	Enhanced effectiveness of the antibiotics in microbial burden in chlamydia infections by intracellular targeting.	Ref. 124
α-ω-Functionalized poly(ethylene oxide)	Gentamicin	Ring-opening metathesis copolymerization	• Size • *In vitro* cytotoxicity • *In vitro* antibacterial activity	pH-sensitive NPs for local delivery of antibiotics	Ref. 125

Continued

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Table 2. Continued

Polymer	Active Ingredient	Preparation Method	*In Vitro*/*In Vivo* Characterization Studies	Main Findings	Reference
O-carboxymethyl chitosan	Tetracycline	Ionic cross-linking	• Size • SEM • FT-IR • *In vitro* drug release • Bacterial binding study • *In vitro* antibacterial activity • *In vitro* cytotoxicity • Hemolysis assay • Coagulation assay • Platelet aggregation assay • Confocal microscopy	Sustained release, improved bioavailability, and intracellular targeting of *S. aureus*.	Ref. 126
PLGA, PVA, chitosan, and alginate	Tobramycin	Modi ed emulsion/solvent diffusion technique	• Size and zeta potential • TEM • Drug encapsulation • *In vitro* assessment of NP interaction with mucus • *In vitro* release kinetics • *In vitro* antimicrobial susceptibility testing	• PVA and chitosan optimize the size and modulate the surface properties of NPs. • Ef cient entrapment of antibiotic into NPs because of alginate. • Good *in vitro* antibacterial activity of NP formulation against *P. aeruginosa* planktonic cells.	Ref. 127
PLGA PLH PEG	Vancomycin	Double emulsion/solvent evaporation method	• Size and zeta potential • TEM • pH-dependent characterization of NPs • NP bacterium binding using ow cytometry and uorescence confocal imaging • Drug encapsulation and release • *In vitro* antibacterial study	PLGA PLH PEG NPs as systemically administered drug carriers that can target and potentially treat Gram-positive, Gram-negative, or polymicrobial infections associated with acidity	Ref. 128

Continued

Table 2. Continued

Polymer	Active Ingredient	Preparation Method	*In Vitro*/*In Vivo* Characterization Studies	Main Findings	Reference
Chitosan and heparin	Amoxicillin	Emulsi cation	• Size and zeta potential • TEM • Encapsulation and loading capacity • Drug release • *In vitro* cellular uptake and confocal laser scanning microscopy • Western blotting and immuno uorescence staining • *In vitro* cytotoxicity study • *In vitro* and *in vivo* antibacterial activity • Histological examinations and immunohistochemistry staining analysis • *In vitro* and *in vivo*	A multifunctional NP system for targeting *H. pylori*	Ref. 129
PLGA	Vancomycin	Modi ed solvent evaporation method	• Size and zeta potential • Drug loading and loading ef ciency • FT-IR • DSC • XRD • *In vitro* release • *In situ* intestinal permeation	Oral biodegradable vancomycin NPs with improved intestinal permeability	Ref. 130
PCL	Roxithromycin	Emulsion solvent evaporation technique	• Size and zeta potential • SEM • Encapsulation ef ciency and drug loading • Short-term stability study • *In vitro* drug release • *Ex vivo* human skin penetration study	Development of organogel containing roxithromycin NPs for delivery to hair follicles	Ref. 131

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Table 3. Summary of SLNs Investigated for Antibiotic Delivery

Lipid	Antibacterial Agent	Size (nm)	Zeta Potential (mV)	Targeted Microorganism	Main Findings	Reference
Stearic acid	Tobramycin	85 ± 5	−20.3	None	Gastrointestinal absorption of tobramycin, prolonged circulation time than i.v.-administered tobramycin solution.	Ref. 170
Stearic acid	Tobramycin	85 ± 5	−20.3	None	Increased passive transport of tobramycin incorporated in SLN to cross the BBB.	Ref. 171
Stearic acid	Cipro oxacin	73 ± 2 to 98 ± 44	−28 ± 1	None	Prolonged antibiotic release in a controlled manner.	Ref. 172
Tetradecanoic acid	Enro oxacin	116.7 ± 15.5	−29.03 ± 0.64	*S. aureus*	Sustained and prolonged drug release, increased bioavailability, and extended mean residence time in combination with fatty acid.	Ref. 173
Palmitic acid		111 ± 7.2	−31.57 ± 3.76			
Stearic acid		217.3 ± 20.1	−40.03 ± 0.67			
Hydrogenated castor oil	Tilmicosin	343 ± 26	−7.9 ± 0.4	*S. aureus*	Sustained drug release, sustained and enhanced antibacterial activity, and decreased degree of in ammation.	Ref. 174
Stearic acid	Nor oxacin	250 ± 5	−31.1 ± 1.85	*E. coli*	Sustained drug release and enhanced antibacterial activity.	Ref. 175
Compritol 888® ATO	Vancomycin	102.7 ± 1.01	−38.8 ± 2.1	*S. aureus*, MRSA	Ion pairing of vancomycin with antibacterial fatty acid (linoleic acid) enhanced encapsulation ef ciency and antibacterial activity of vancomycin in SLNs.	Ref. 135

containing Eudragit was positive and sustained release of cipro oxacin was achieved. All formulations were comparable to the free drug solution, con rming no loss of activity on encapsulation into a sustained-release formulation. It was also noted that drugs in this formulation were less active in killing *S. aureus* compared with *P. aeruginosa*. To understand the activity demonstrated, a further paper with ow cytometry studies on these PNPs presented the nding that Eudragit NPs showed more bacterial adhesion with test organisms (*P. aeruginosa* and *S. aureus*) compared with PLGA-only NPs, and can thus reside for prolonged time in anionic mucus membrane to effectively manage infections.[116] This opened a new research area of targeted delivery of antibiotics based on surface charge difference between bacteria and PNP formulation. The ndings of this study also emphasized the importance of polymer choice, not only for NP formation, but also for antibacterial activity.

Poly(lactic-co-glycolic)acid NPs containing cipro oxacin with particle sizes of 100 300 nm were also formulated and evaluated for their antibacterial potential (*in vitro* and *in vivo*) against pathogenic *E. coli* by Jeong et al.[119] These NPs displayed lower *in vitro* antibacterial activity as compared with free cipro oxacin and was attributed to their sustained drug release pro les. Cipro oxacin was released from NPs over a period of 14 days. However, *in vivo* antibacterial activity of NPs was greater than the free drug, showing the superiority of the formulation. Although these authors did not explain the differences in *in vitro* and *in vivo* behavior of the PNPs against the free solution, this may clearly be because of the fact that the *in vitro* studies were carried out after 24 h and for a single time period only, whereas in the *in vivo* study, mice were sacri ced after 3 days.[119] This suggests that sustained-release antibiotic formulations should undertake *in vitro* activity studies over a prolonged period, as has been performed is several studies for nanoantibiotic formulations other than PNPs.[135,136] In other studies, NPs formulated using PLGA polymer have been shown to enhance the delivery of azithromycin and rifampicin to intracellular chlamydial infections caused by chlamydia trachomatis and chlamydia pneumonia.[124] Using detailed micrometric, crystallographic (Fourier transform infrared, X-ray diffraction, and differential scanning calorimetry), mathematical modeling of drug release data, and *in situ* permeability evaluations, an improvement in intestinal permeability of vancomycin in male Wistar rats was observed by delivering it via PLGA NPs.[130] The researchers attributed this nding from less than 500 nm size NPs to the large surface area, improved paracellular passage, and their endocytic uptake.

Poor incorporation ef ciencies of the drug into NPs are a well-recognized challenge, especially with water soluble drugs. To this end, several groups working with PLGA polymers have investigated varying approaches to enhance

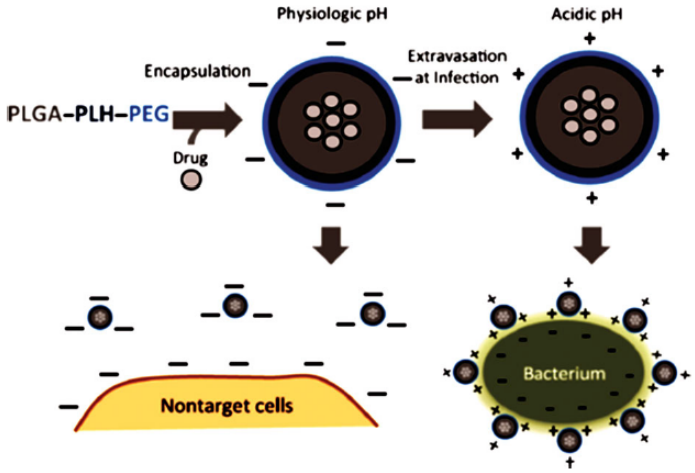

Figure 2. Schematic representation of the designed surface charge-switching PNPs-mediated drug targeting to bacterial cell walls. Reproduced from Radovic-Moreno et al.[128] with permission from American Chemical Society.

encapsulation ef ciencies.[120 122,127,137] Cheow and Hadinoto,[122] in their study with levo oxacin, modi ed the standard NP preparation techniques, single- and double-emulsi cation solvent evaporation, and nanoprecipitation. They found that encapsulation ef ciency of highly water-soluble drugs in PLGA NPs can be enhanced by these modi ed methods by taking levo oxacin as a model drug.[122] The inclusion of lecithin into the aqueous phase, and modifying the water miscibility level of the oil phase, were found to be particularly useful. In another study, the drug and polymer ratio was particularly investigated to prepare azithromycin PLGA NPs for optimum encapsulation and biological properties. A drug to polymer ratio of 1:3 was found to be optimal in enhancing encapsulation ef ciency to 78.5%. The optimized formulation was more effective against *S. typhi* by displaying equivalent antibacterial effect at 1/8th the concentration of the free drug,[121] As combining PCL with PLGA was found to increase the doxycycline entrapment ef ciency, selecting appropriate polymeric core composition can be a useful strategy for enhancing drug encapsulation. A PLGA PCL ratio of 80:20 was found to be optimal to increase entrapment ef ciency to 32% from 25% at a PLGA PCL ratio of 60:40. Altering the aqueous phase pH from 7.4 to 4 additionally increased entrapment to 70%.[120] A study by Ungaro et al.,[127] who formulated a PLGA NP dry powder formulation as a pulmonary delivery system or tobramycin, also highlighted the importance of helper hydrophilic polymers, for example, chitosan, alginate, and polyvinyl alcohol (PVA) for achieving optimal drug entrapment, size, and release pro les.

A recent development in the eld of PLGA NPs for antibiotic delivery has been its modi cation to synthesize a polymer that is particularly responsive to infection sites. Vancomycin-encapsulated, pH-responsive, surface charge-switching PLGA-*b*-poly(L-histidine)-*b*-poly(ethylene glycol) (PLGA-PLH-PEG) NPs have been synthesized (mean size = 196.0 ± 7.8 nm). A lack of interaction of NPs with bacteria at pH 7.4 and at acidic pH strong af nity of NPs toward bacteria was observed. PLH gets protonated because of the acidic pH at the infection site and activates a surface charge-switching mechanism that leads to binding of the NPs to the negatively charged bacteria (Fig. 2).[128] This was con rmed by NP-binding studies using confocal imaging and ow cytometry. Studies demonstrated pH-sensitive NP binding to bacteria, that is, a 3.5 ± 0.2- to 5.8 ± 0.1-fold increase in bacterial binding at pH 6.0 as compared with 7.4 was reported. It was also observed that upon reduction in pH, the PLGA-PLH-PEG NPs switched their surface charge from a negative zeta potential at pH 7.4 (−3.9 ± 0.4 mV) to a positive one. They also showed that the surface charge transition occurred, as early as pH 7.0 (2.3 ± 1.0). The results obtained using PLGA-PLH-PEG NPs are promising, and pave the way for synthesizing other responsive PLGA-based polymers. These studies have therefore clearly con rmed PLGA as a suitable material for antibiotic-loaded PNP formulations.

Among the natural polymers, chitosan has attracted considerable interest for the use against microbial growth because of its antimicrobial and antifungal activity.[138 140] Its antimicrobial action may be because of ef cient binding to negatively charged bacterial cell walls that destabilizes the cell envelope altering permeability, followed by attachment to DNA and inhibition of its replication.[141] Several approaches have been used to exploit chitosan as a polymer for antibiotic delivery. Folic acid tagged noncytotoxic chitosan NPs have been employed as Trojan horses to target vancomycin into the bacterial cell by synthesizing a new carboxymethyl chitosan-2,2′-(ethylenedioxy)-bis-(ethylamine)-folic acid (CMC-EDBE-FA) polymer. This experiment was performed to address the problem associated with VRSA treatment, which is a serious issue in medical practice.[123] FA, an essential nutrient required for nucleotide synthesis for bacteria helps to transport the NPs loaded with drug through endocytosis, across the plasma membrane, and into the cytoplasm.[142,143] The prepared nanoconjugated vancomycin decreased both the MIC and MBC values of VRSA to a signi cant level (Fig. 3).

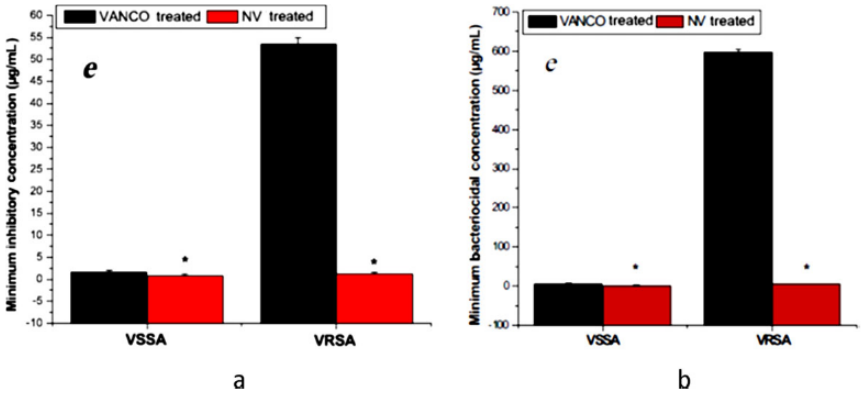

Figure 3. (a) Minimum inhibitory concentration and (b) MBC of vancomycin (vanco) and nanoconjugated vancomycin (NV) against vancomycin susceptible and resistant S. *aureus* (VSSA and VRSA). Reproduced from Chakraborty et al.[123] with permission from IOP Publishing.

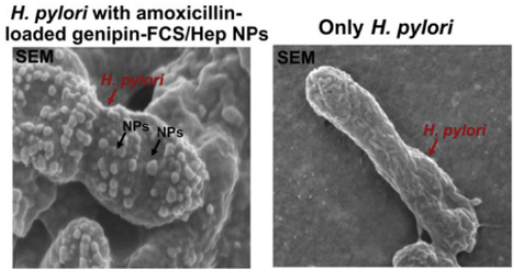

Figure 4. Scanning electron microscope images showing strategy and observation for eradicating H. *pylori* by amoxicillin-loaded genipin-FCS/Hep NPs. Reproduced from Lin et al.[129] with permission from Elsevier Science Ltd.

Using ionic cross-gelation technique, biocompatible, 200 nm-sized tetracycline (TC) encapsulated *O*-carboxymethyl chitosan NPs have also been prepared to eradicate intracellular *S. aureus* infections effectively.[126] Recently, amoxicillin entrapped genipin cross-linked fucose chitosan/heparin NPs (genipin FCS/Hep NPs) in the size range of 150 210 nm have been shown to eradicate *H. pylori*, a Gram-negative microorganism causing gastric infections. Via in-depth studies on this multifunctional responsive polymeric PNP including encapsulation, release, *in vitro* cellular uptake and confocal laser scanning microscopy, *in vitro* growth inhibition, *in vivo* animal studies, histology and immunochemistry, and uorescent bacteria binding, this formulation was shown to decrease drug release at gastric acids and increased release at an *H. Pylori* survival situation (Fig. 4). In addition, a more complete *H. pylori* clearance effect and ability in decreasing gastric in ammation associated with *H. pylori* was reported.[129]

Other polymers have also been randomly used in the literature to encapsulate antibiotics, and are highlighted hereunder. As NPs may accumulate in hair follicle openings, drug delivery through this mechanism, with the use of NPs, is gaining more importance. Roxithromycin NPs (size 300 nm), using PCL as a polymer, were prepared using an emulsion solvent evaporation method and were embedded in pluronic-lecithin organogel (PLO). *In vitro* human skin penetration studies revealed that it is possible to preferentially target the pilosebaceous unit with the polymeric NPs, whereas the PLO formulation did not promote follicular penetration more ef ciently than suspension of NPs.[131] Therefore, antibiotic-loaded PNPs can now also be entrapped into a gel for facilitating transdermal delivery.

The synthesis of pH-sensitive functionalized NPs by ring-opening metathesis copolymerization (ROMP) has also been disclosed by Pichavant et al.[125] For this purpose, a pH-sensitive α-norbornenyl-poly(ethylene oxide) macromonomer was used to synthesize different polymeric derivatives. The plurifunctionalization of NPs containing prodrugs and reactive chemical groups as carboxylic acids was explored in the study using macromonomer route. Gentamycin was linked via a pH-sensitive imine bond to a polymer, and the NPs prepared using ROMP were found to be noncytotoxic by neutral red and MTT assays. The MIC measurements performed at different pH values (4 7) on *S. epidermidis* revealed that for gentamycin-functionalized macromonomer, there was no signi cant inhibition of growth at pH 7, whereas a decrease at conditions of pH 4 and 5 was observed.[125] For targeted delivery, lectin-conjugated gliadin NPs speci cally binding to carbohydrate receptors on *H. pylori* cell walls with release of the antimicrobial agents into the bacteria were found to have an inhibitory effect twofold higher than gliadin NPs.[113]

Thus, the section on PNPs can be summarized as: rst, PNPs are extensively studied nanodelivery systems for antibiotics and have advantages over liposomes; second, it is possible to achieve site-speci c and targeted delivery of antibiotics by surface modi cation of PNPs with targeting moieties, and by using pH-responsive materials for synthesis or by formation of covalent bonds, which can be degraded at acidic environment at infection site. Third, the eld of antibiotic PNPs seems to be growing, and there are opportunities for scientists to develop novel-biocompatible and biodegradable-responsive polymers for antibiotic PNPs formulation, as conventionally used natural and synthetic polymers have been exploited extensively and have some limitations. Lastly, the literature indicates that

Table 4. Antibacterial Activity of VCM-HCl, VCM-LA2, VCM-HCl_SLNs, and VCM-LA2_SLNs

Formulation	MIC (μg/mL)[a]							
Bacteria	*S. Aureus*				MRSA			
Time (h)	18	36	54	72	18	36	54	72
Blank SLNs	NA	NA	NA	NA	NA	NA	NA	NA
VCM-HCl	15.62	NA	NA	NA	3.91	NA	NA	NA
VCM-LA2	218.75	437.5	109.35	218.75	1750	850	1750	1750
VCM-HCl_SLNs	15.62	250	500	NA	15.62	500	500	NA
VCM-LA2_SLNs	62.5	31.25	31.25	31.25	15.62	15.62	15.62	15.62

[a] $n = 3$.
NA, no activity.
Reproduced from Kalhapure et al.[135] with permission from Elsevier Science Ltd.

most of the antibacterial studies are carried out *in vitro* and therefore, in future, there is a need to focus studies on *in vivo* performance of reported and newly developed antibiotic PNPs.

Solid Lipid Nanoparticles

Solid lipid nanoparticles, introduced in the early 1990s, have gained signi cant popularity as an alternative drug delivery colloidal system[144] because of their advantages. These include using biocompatible materials, being easy to scale up preparation techniques, stability during storage,[145,146] high entrapment of lipophilic drugs into their lipophilic core,[147,148] protection of labile drugs against degradation,[149 152] improved body/tissue tolerance, and less stringent regulatory requirements because of utilization of physiologically acceptable lipids.[145,146] SLNs typically have mean diameters ranging in size from 50 to 1000 nm[148] and can be delivered by almost all routes for various disease conditions.[153] Avoiding organic solvents and the feasibility of production on a larger scale are two main advantages of SLNs. They are uniquely attractive in that they display the advantages of conventional NPs while simultaneously eliminating some of their reported drawbacks, such as the high cost of polymers and phospholipids used for producing PNPs and liposomes, the need to maintain drug bioactivity throughout the conjugation scheme if the drug is being conjugated to PNPs,[154] rapid leakage of water-soluble drugs, and poor storage stability.[105]

A high melting point lipid composition forms the core of SLNs. The core remains in the solid state at room and body temperature and is coated with amphiphilic surfactants that form the outer shell.[148] Many solid lipids, such as stearic acid,[155] palmitic acid,[156] glycerol behenate (Compritol 888 ATO),[157] and glyceryl monostearate[158] have been used in preparing SLNs. Similarly, various surfactants, such as poloxamer 188, 182, 407, 908,[159 161] tween 20, 80,[162,163] and solutol HS 15[164] have been reported to stabilize the SLN formulation. Recently, novel surfactants, such as polyhydroxy surfactants[165] and an oleic acid based bicephalous dianionic surfactant,[166] have also been found as potential stabilizers for SLN preparations. A comprehensive list of lipids and surfactants used in SLN formulation development can be found elsewhere in the literature.[167,168] High-pressure homogenization and microemulsion technique are the two main techniques employed for the production of SLNs. However, many other methods such as the ultrasound and solvent-based techniques have been used to promote cost-effective and simpler ways of production.[169]

Although SLNs have shown great therapeutic potential for delivering drugs with diverse pharmacological activities, the development history of their antibiotic delivery system is shorter. A literature search for this paper revealed that there are fewer SLN-based antibiotic delivery systems compared with other drug classes.[135] SLN-based antibiotic formulations with their properties (size and zeta potential), microorganism/s used to assess antibacterial activity, and main outcomes of the study are summarized chronologically in Table 4. The data indicate that SLNs are being exploited for overcoming absorption inhibitors, facilitating transport across membrane barriers, modifying drug release pro les, increasing bioavailability, and enhancing and prolonging antibacterial activity.

Tobramycin, which is administered via the oral route, is used against *P. aeruginosa* infections.[176] Its poor absorption rate is because of active exportation of the drug from the cells via P-glycoproteins (P-gp) and ATP-dependent drug ef ux pumps. This poor intestinal absorption was overcome by formulating tobramycin-loaded SLNs, which signi cantly suppressed the P-gp ef ux pump by penetrating the intestinal linings through endocytosis rather than passive diffusion. SLNs removed from drug ef ux pumps released the drug inside the cells after being internalized through endocytosis. Achievements of tobramycin-loaded SLNs were modi ed pharmacokinetics, low amounts taken up by the kidneys and high lung concentration following intravenous administration by the duodenal and intravenous route.[170] They reported that aminoglycosides have low permeability across the blood brain barrier (BBB) when administered via the parenteral route. In a subsequent paper, these authors showed that in tissue distribution studies, no tobramycin could be detected in the brain after an i.v. solution, whereas it was detected in the brain, with SLN indicating passage through the BBB.[171] This important study with an antibiotic, although not having antibacterial activity studies, con rmed the use of SLNs to overcome the P-gp ef ux pump and pass through the BBB when loaded with an antibiotic.

Other studies have con rmed their abilities to provide sustained drug release and prolonged antibacterial activity. Jain and Banerjee[172] developed a SLN-based single dose nanodelivery system for cipro oxacin that provided a prolonged release of the antibiotic in a controlled manner. Their study revealed that SLNs of cipro oxacin were more promising than other cipro oxacin nanodelivery systems that have been formulated.[172] Similarly, enhancement of *in vitro* and *in vivo* antimicrobial activity of tilmicosin against *S. aureus* was achieved by encapsulating it into SLNs that were formulated

using hydrogenated castor oil.[174] This research group also prepared nor oxacin-loaded SLNs as a novel formulation and studied different aspects of the formulation such as stability, *in vitro* release, *in vitro* antibacterial activity, and *in vivo* ef cacy in mice against *E. coli*. SLNs were found to be stable for up to 9 months at 4°C, and the drug release was slower, lasting for 48 h. Although the SLN formulation was initially less effective within 24 h, it was interestingly much more effective than the bare nor oxacin during *in vitro* antibacterial evaluations at all other time points up to 144 h, con rming sustained drug release. For *in vivo* therapeutic ef cacy, treatment was performed 2 h postintraperitoneal infection of mice with *E. coli*. Enhanced ef cacy was observed for SLNs, which was indicated by decreased bacteria in the spleen and kidney homogenates and a high proportion of survivors, which was probably because of the high bioavailability of drugs.[175]

The role of fatty acids in enhancing SLN preparations with antibiotics is being increasingly recognized. Saturated carbon fatty acids are commonly used as a lipid matrix to prepare SLNs. As they vary in terms of carbon chain length and properties, Xie and coworkers[173] investigated the in uence of fatty acids on the properties and pharmacokinetics of enro oxacin-loaded SLNs. It was found that stearic acid produced SLNs with the highest encapsulation and had a greater zeta potential but larger particle size and polydispersity index than palmitic acid and tetradecanoic acid. Although in *in vitro* studies the three developed formulations exhibited similar antibacterial activity as that of native enro oxacin, in *in vivo* studies, it was found that the bioavailability of tetradecanoic, palmitic, and stearic acid SLNs increased 6.79-, 3.56-, and 2.39-fold, whereas the mean residence time of the drug was extended from 10.60 to 180.36, 46.26, and 19.09 h, respectively.[173] This study therefore highlighted the signi cant effects of the fatty acid properties as the lipid matrix on the performance of SLNs. In a more recent study, our group exploited the diverse advantages of fatty acids by including them as a counter ion to form an ion pair with vancomycin, instead of being the lipid core itself, as was performed in the previous study. A Compritol-based SLN formulation (VCM-LA2_SLNs) of vancomycin and linoleic acid using an ion pairing mechanism[135] was prepared. Our goal was to develop a nanoantibiotic system acting by multiple simultaneous mechanisms of actions, as it would be dif cult for bacteria to develop resistance to such a system, this requiring multiple simultaneous mutations in the same microbial cell.[35,177] Linoleic acid served two purposes in the formulation; (1) it acted as a contra ion for vancomycin to form an ion pair, and (2) being an antibacterial, it served as a nondrug antibacterial agent in the formulation. The particle size and polydispersity index of the formulated VCM-LA2_SLNs were 102.7 ± 1.01 nm and 0.225 ± 0.02, respectively. Zeta potential was -38.8 ± 2.1 mV, con rming the high stability of VCM-LA2_SLNs. The study revealed greater encapsulation of vancomycin in SLNs, and enhanced and extended period of antibacterial activity of the novel formulation against MRSA and *S. aureus*. Encapsulation ef ciencies were 16.81 ± 3.64 and 70.73 ± 5.96 for vancomycin SLN and the developed VCM-LA2_SLNs, respectively. Although at the initial 18 h testing time, bare vancomycin showed highest activity (low MIC) against both *S. Aureus* and MRSA (15.62 and 3.91 μg/mL, respectively), at subsequent time intervals (36, 54, and 72 h), VCM-LA2_SLNs was the only active formulation against both the strains exhibiting MICs of 31.25 and 15.62 μg/mL, respectively, against *S. aureus* and MRSA (Table 5).[135] The strategy of coencapsulation of a fatty acid with an antibiotic in SLNs therefore proved successful in enhancing activity against sensitive and resistant strains. Investigating the effect of other fatty acids of different carbon chain lengths on drug loading and antibacterial activity, as well as on molecular modeling to explain their association with the SLN, will be an interesting study to guide their selection for future optimal formulations.

Although SLNs are emerging as a lipidic delivery system of choice for nanodrug delivery, this review shows that despite its advantages, this nanodelivery system has not been exploited to a great extent for antibiotics. One of the reasons might be the hydrophilic nature of most antibiotics used clinically, which will have low entrapment ef ciency and loading capacity in the hydrophobic lipids. Recent studies do indicate that this problem could be surpassed by the use of techniques such as ion pairing and/or conjugation mechanisms. Detailed characterization using techniques such as atomic force microscopy, confocal laser scanning microscopy, and ow cytometry to elucidate the mechanisms involved in antibacterial activity with these systems should also be considered.

Lipid–Polymer Hybrid Nanoparticles

Liposomes and PNPs appear to be the most explored nanoparticulate system for antibiotics thus far. To overcome some of the reported limitations associated with these systems though, LPHNs have been more recently introduced.[32] LPHNs are novel integrated systems in which the structural and architectural advantages of a polymer core and the biomimetic properties of lipids are combined to generate a delivery system that is superior. LPHNs are therefore solid, nanosized particles composed of at least two components: lipid and polymer.[178] In a well-designed LPHN, the polymeric core serves to entrap either water- or oil-soluble drugs and to provide a robust structure, whereas the external lipid coat serves as a biocompatible shield. The latter also functions as a template for surface modi cation and further acts as a barrier to minimize the burst release of water-soluble drugs.[179]

A number of methods have been reported to produce LPHNs, namely, multiple step procedure involving coincubation of separately prepared NPs and lipid vesicles[180,181]; a single-step nanoprecipitation technique[32,182]; a method using emulsi cation with lipids replacing traditional surfactants[183]; a sonication method[182]; and a double-emulsi cation-solvent-evaporation technique.[184] A recent review on LPHNs provides details on materials and methods used for preparing, identifying the physicochemical characteristics, immunocompatibility, and their applications in drug delivery. LPHNs have to date been studied most extensively for delivering anticancer drugs.[178] It is only recently since 2011 that these LPHNs possessing characteristics of both liposomes and PNPs being explored for their bene ts in antibiotic delivery.

Table 5 provides a summary of research undertaken so far on the preparation of antibiotic-loaded LPHNs, with four of the ve papers emanate from the same research group. In the earliest reported antibiotic-loaded LPHN study, three uoroquinolone antibiotics, cipro oxacin, levo oxacin, and o oxacin were entrapped in LPHNs using PLGA as a polymer and PC as a lipid component by a double-emulsi cation-solvent-evaporation method in pursuit of developing nanodrug delivery system for treating pulmonary infections. The study also explored the factors affecting encapsulation ef ciency and

Table 5. Summary of Studies Undertaken to Date with LPHNs and Antibiotics

Antibiotic	Nature of Antibiotic	Polymer and Lipid	Main Findings	Characterization Studies	Reference
Levo oxacin O oxacin Cipro oxacin Tobramycin	Hydrophobic Hydrophobic Hydrophilic Hydrophilic	PLGA and PC	• Ionicity of the drug and lipid is important with regard to LPHNs preparation. • Drug lipophilicity and aqueous solubility affect drug loading and drug release; more lipophilic drug has higher drug loading and sustained release pro le. • LPHNs are larger in size, zeta potential, encapsulation, and drug loading compared with its nonhybrid counterpart. • Incorporation of D-α-tocopheryl polyethylene glycol 1000 succinate stabilized the formulation. • Sizes between 120 and 420 nm with the highest encapsulation of 25% with o oxacin.	• Particle size • Zeta potential • Entrapment ef ciency • Drug loading • *In vitro* drug release • SEM	Ref. 179
Levo oxacin	Hydrophobic	PLGA and PC	• Particle size of LPHNs ranged from 240 to 420 nm with a zeta potential of approximately 26 mV, encapsulation ef ciency ranging from 19% to 21% and drug loading of 2.3% 2.4% (w/w). • LPHNs exhibited a higher antibacterial ef cacy against *P. aeruginosa* bio lm cells, however, not against planktonic cells. • Possibly, the presence of lipid may have enhanced the antibiotic diffusion into the bio lm matrix resulting in more effective bio lm cell eradication.	• Particle size and zeta potential • Entrapment ef ciency • Drug loading • *In vitro* release studies • SEM • Bio lm susceptibility testing	Ref. 184
Levo oxacin Cipro oxacin O oxacin Calcein	Hydrophobic Hydrophilic Hydrophobic Hydrophilic	PLGA, rhamnolipid and PC	• Particle size ranged from 280 to 400 nm with a zeta potential range of (−)30 (+)10 mV and a drug loading of 0.5% 2.3% (w/w) • Encapsulation ranged from 5% to 55% depending on the BCS class of the drug. • A rhamnolipid-triggered release was observed with calcein, however, not with BCS class I drugs because of their high lipid membrane permeability. • The rhamnolipid-triggered release capability of LPHNs will enable targeted drug release in the vicinity of bio lm colonies and therefore improved antibacterial ef cacy is expected.	• Particle size • Zeta potential • Entrapment ef ciency • *In vitro* drug release • SEM	Ref. 185
Levo oxacin	Hydrophobic	PLGA and lecithin	• LPHNs exhibited a size of ≈420 ± 30 nm with zeta potential in the range of (−) 25 30 mV, encapsulation ef ciency of ≈19% and drug loading of ≈2.0% (w/w). • Spray drying produced dimpled hollow spherical nano-aggregates whereas spray freeze drying produced large spherical porous nano-aggregates. • PVA was better than mannitol in facilitating nano-aggregate reconstitution. • Nano-aggregates produced by spray freeze drying were superior to those produced by spray drying.	• Particle Size and distribution • Zeta potential • Entrapment ef ciency • Drug loading • Powder characterizations	Ref. 186
Clindamycin phosphate	Hydrophilic	Stearic acid, dextran sulfate and sodium alginate	• LPHNs ranged from 400 to 900 nm. • Particle size was not affected by polymer type or the amount of drug, polymer, and surfactant. • Polymer dextran sulfate had higher degree loading and drug release than sodium alginate.	• Particle size and distribution • Entrapment Ef ciency • Drug loading • *In vitro* drug release studies • SEM	Ref. 187

 DOI 10.1002/jps.24298

stability of LPHNs.[179] This paper clearly formed the foundation for subsequent antibiotic-loaded LPHN systems, as it highlighted the importance of lipid and drug ionicity for forming the NPs and drug lipophilicity, as well as aqueous solubility on drug entrapment and release pro les. The poor stability of the LPHNs in this study was overcome by the addition of d-α-tocopheryl PEG 1000 succinate as a solubilizer. The low drug encapsulation and inadequate stability reported in this paper re ect the challenges with this delivery system during their preparation. Strategies such as choice of solvents, pH of aqueous phase, and counter ion complexation can be considered for enhancing drug incorporation, whereas other hydrophilic substances can be considered to modify the surface to promote stability during storage and *in vivo*. Having established critical factors for successfully forming LPHNs, these authors then proceeded to investigate the antibio lm ef cacy of the LPHNs against *P. aeruginosa* preparing LPHNs containing PLGA, PC, and levo oxacin. LPHNs, both in suspension and powder form, displayed higher antimicrobial activity against 1-day-old *P. aeruginosa* bio lm cells than nonhybrid NPs, but were less effective against planktonic cells.[184] To further enhance the performance of these LPHNs as antibio lm drug carriers, the target release of the encapsulated drug at bio lm colonies needed to be demonstrated. In another study, they investigated the trigger release properties of the LPHNs in response to rhamnolipids that are present in bio lm colonies of *P. aeruginosa* by using various biopharmaceutical classi cation system (BCS) antibiotic drugs as a model.[185] In the absence of the triggering agent (rhamnolipid), both levo oxacin and o oxacin (BCS class I model drugs) were readily released from the LPHNs at rapid rates. The percentage of levo oxacin and o oxacin released in 6 h were 70% and 90%, respectively. These fast release rates were attributed to their free solubility in water and high lipid membrane permeabilities, con rming that the presence of the lipid coat did not deter their outward diffusion. In the absence of the triggering agent, calcein (BCS class III model drug) was eventually released, but only in minimal amount from the LPHNs, which was indicated by a 20% release of the encapsulated calcein after 2.5 h. This initial calcein release was likely because of the dissolution of nonencapsulated calcein present on the NP surfaces. Upon the addition of rhamnolipid, calcein was immediately released, with almost 60% being released within the rst 5 min. This study therefore showed that rhamnolipid-triggered release may enable targeted release in the vicinity of bio lm colonies. Although previous studies mainly focused on formulation variables, the focus of another paper by this group was on optimizing manufacturing technologies for these LPHNs. They compared spray-drying (hollow dimpled spherical nanoaggregates) and spray freeze-drying (large spherical porous nanoaggregates) techniques to produce inhalable dry powder forms of LPHNs. It was found that both methods were able to produce inhalable dry powders of the LPHNs in the form of microscale aggregates.[186] Nanoaggregates produced by the spray freeze-drying technique was superior to those produced by spray drying.

The most recent paper by Abbaspour et al.[187] used sodium alginate and dextran sulfate as polymers and stearic acid as the lipid to prepare clindamycin-loaded LPHNs. They used a multilevel factorial design to nd a mathematical relationship between the amount of polymers and the amount of surfactants on drug-loading ef ciencies. They attributed higher drug-loading ef ciencies with dextran sulfate, rather than to sodium alginate to ionic interactions between the anion in dextran sulfate and the cationic clindamycin. Although it is clearly useful to use an experimental design, this study could have been strengthened if the generated mathematical model had been validated. Furthermore, although the authors indicate the undertaking of scanning electron microscope (SEM) analysis of the LPHNs, which con rmed their morphology, no SEM images were provided in the paper.

These studies with antibiotic-loaded LPHNs clearly con rm their potential as an effective nanosystem for antibiotics. Table 5 shows that to date, PLGAs have been mainly used as the polymer, with the basic characterization in terms of size, polydispersity index, *in vitro* release, and surface morphology having been studied. Only antibacterial activity for bio lm susceptibility testing has been assessed. In-depth physicochemical/mechanical characterization studies, including *in vitro* and *in vivo* bacterial activities against a range of organisms, are therefore essential for formulation optimization. The reported advantages of this delivery system necessitate investigating various classes of antibiotics with different polymers and lipids to identify optimal formulation excipients. In addition to antibio lm therapy, other applications that can be studied include antibacterial activity against sensitive and resistant bacterial strains for infections as well as macrophages infection studies. Mechanistic studies to understand the complex self-assembly of the drug, lipid, and polymer into these LPHN constructs will also be useful. These studies, together with tuning the lipid and polymer composition and employing surface strategies, will certainly result in LPHNs emerging as novel effective hybrid nanodelivery systems. This will provide new platform for developing nanoantibiotics with enhanced performance in terms of high drug (both hydrophilic and lipophilic) loading, targeted delivery, as well as sustained and prolonged activity.

Dendrimeric Nanostructures

Dendrimers are homogenous, well-de ned monodisperse structures. They consists of tree-like structures in nanosized form and are radially symmetric molecules.[188] These monodisperse nanosized polymers are shaped like the head of a tree, and exploit two traits, that is, globular structure and polyvalency, which is found in many naturally occurring systems.[189 194] Tomalia et al.[195] disclosed the synthesis of rst family of dendrimers, known as poly(amido amine) (PAMAM), resulting in PAMAM becoming one of the most popular dendrimers. Since their disclosure, a variety of dendrimers have been synthesized and evaluated for various applications in chemistry, nanotechnology, biomedicine, and pharmaceutical sciences.[17,196 201] Depending on the chemical moieties and types of linkages present, dendrimers are classi ed into four types: glycodendrimers,[202] peptide dendrimers,[203] janus dendrimers,[204,205] and metallodendrimers.[206] Dendrimers have gained increasing interest among drug delivery scientists because of their nanosize, globular shape, derivatizable peripheral functionality, multivalency, tunable inner cavities, and physicochemical properties that resemble those of biomolecules. Their applications in drug delivery technology include: as vehicles,[207] solubility enhancers for poorly soluble drugs,[208] controlled release,[209] targeted delivery,[210,211] prodrug preparation,[212 214] HIV prophylaxis,[215] gene therapy,[216,217] as vaccines,[218] in diagnostics,[219] and as drugs.[220]

Table 6. Dendrimers with Their Role in Antibiotic Drug Delivery

Dendrimer	Drug	Role of Dendrimer	Reference
PAMAM	Nadi oxacin and pruli oxacin	Drug carrier to enhance solubility without affecting antibacterial activity.	Ref. 224
PPO-PAMAM	Triclosan	Micellar carrier with high drug loading and controlled release for hydrophobic drug.	Ref. 225
PAMAM	Sulfamethoxazole	Solubility enhancer to obtain increased antimicrobial activity with sustained release.	Ref. 226
PAMAM	Erythromycin	Conjugation with a drug to act as a carrier for sustained and targeted intracellular delivery in periprosthetic in ammation.	Ref. 227
PAMAM	Azithromycin	Conjugation with a drug to act as a carrier for ef cient intracellular delivery to address chlamydia infections.	Ref. 228
PAMAM	Erythromycin and tobramycin	No speci c role. Study was conducted to investigate effect of dendrimers on antibacterial activity of two drugs with different solubility pro le.	Ref. 229
PAMAM	Silver sulfadiazine	Solubility enhancer forming a NP system with enhanced antimicrobial properties for the topical treatment of burn-wound infections.	Ref. 230
PAMAM	Vancomycin	Scaffold for vancomycin to form drug dendrimer conjugate with high-binding avidity to bacterial cell wall.	Ref. 231
PPI	Nadi oxacin	Coadministration with antibiotic for enhancement of antibacterial activity.	Ref. 232
PPI	Cipro oxacin	Coadministration with antibiotic for reducing the required dose of drug for antibacterial activity.	Ref. 233
HPO hexadentate-based dendrimeric chelators	Nor oxacin	Combination agent with antibiotic for synergistic bactericidal effect.	Ref. 234

The literature reveals that dendrimers themselves have been found to be effective antibacterials, which prompted many scientists to focus on synthesizing antibacterial dendrimers. The details of these antibacterial dendrimers are out of the scope of this review and can be found elsewhere.[221 223] The following sections, therefore, only highlights the use of dendrimers to enhance the properties of antibiotics via nanostructures. Table 6 is a chronological summary of studies where dendrimeric materials have been used to prepare antibiotic-loaded nanostructures. These antibiotic-loaded dendrimeric nanostructures have been exploited for enhancing drug solubility and antibacterial activity, for prolonging sustained drug release, and to prepare various nanostructures, such as micelles and conjugates, for antibiotic delivery.

Because of poor aqueous solubility of quinolone antibacterials, there are dif culties in formulating their liquid dosage forms, consequently restricting their use in topical formulations. To overcome this problem, Cheng et al.[224] investigated the potential of G3-G5 PAMAM dendrimers as biocompatible carriers for an improvement in the aqueous solubility of nadi oxacin and pruli oxacin. They observed that the solubility of quinolones was greater in higher generation dendrimers than in lower ones. Encapsulation/complexation of quinolones into/with dendrimers resulted in excellent solubility enhancement and a similar antibacterial activity as that of pure drugs.[224] Similarly, sulfamethoxazole, which causes problems in its clinical applications because of its poor solubility, has been investigated for its solubility, *in vitro* drug release, and antibacterial activity using PAMAM dendrimers with ethylenediamine core.[226] The results of this investigation revealed that there was a 40-fold solubility increase in G3 PAMAM dendrimer solutions (10 mg/mL) as compared with the solubility in double-distilled water. The release of drug from dendrimer was also sustained, with the dendrimer drug being more potent against *E. coli* than free sulfamethoxazole (almost fourfold to eightfold increase in antibacterial activity).[226] A recent study indicated that PAMAM dendrimer complexes with silver sulfadiazine, a poorly soluble drug, and silver could be employed to achieve a bottom-up approach to synthesize and enhance the solubility of highly soluble silver sulfadiazine NPs and create a nanosystem with enhanced antimicrobial properties.[230]

The amphiphilic linear dendritic block copolymer composed of poly(propylene oxide) (hydrophobic core), and PAMAM dendrimer (outer corona), was prepared and triclosan, a hydrophobic drug, encapsulated in layer-by-layer lms formed from micelles of the dendritic polymer showed release times over a period of several weeks. Furthermore, a Kirby Bauer test on *S. aureus* con rmed that the released drug was still active to ensure growth inhibition of *S. aureus*.[225]

Targeted intracellular delivery has also been a goal for dendrimeric nanostructures of antibiotics, with erythromycin, a macrolide antibiotic, being conjugated with bifunctional PAMAM dendrimer (G4-OH-Link-NH_2), which resulted in its sustained release. This study further focused on intracellular delivery studies for erythromycin as an anti-in ammatory agent to manage periprosthetic in ammation. It has been also observed that the synthesized conjugate retained its antibacterial activity, its antibacterial activity being similar to free erythromycin against *S. aureus* at different concentrations.[227] The lack of detailed studies on antibacterial activity of conjugate was

addressed in 2011 by Mishra et al.,[228] who synthesized conjugate of azithromycin, a macrolide antibiotic, with G4-PAMAM dendrimer, to obtain dendrimer drug conjugate nanodevice for treating *Chlamydia trachomatis* infections. This study explored the potential of G4 PAMAM dendrimers as intracellular drug delivery vehicles into chlamydial inclusions. Approximately 90% of the drug was released from the azithromycin PAMAM conjugate over a 16 h period and azithromycin readily entered the *Chlamydia*-infected HEp-2 cells and inclusions. When added at the time of infection, the conjugate was significantly superior to free drugs in the prevention of productive infections in cells. In addition, the conjugate was found to be better in decreasing the size and number of inclusions after adding the conjugate at either 24 or 48 h post infection. This study emphasized the nding that even if the organism is in the persistent form, dendrimers can ef ciently deliver drugs to growing intracellular *C. trachomatis*.[228]

Recent ndings suggest that even coadministration of antibiotic with a dendrimer results in lowering the dose of drug required for antibacterial action.[232,233] This was proved by coadministering nadi oxacin[232] and cipro oxacin[233] with G4 PPI dendrimer. G4 PPI dendrimers and their maltose-modi ed derivatives exhibited enhanced antibacterial activity of nadi oxacin against Gram-negative *E. coli* ATCC 25922, *P. aeruginosa* ATCC, 15442 and *Proteus hauseri* ATCC 13315 without any harmful effect on eukaryotic cells.[232] Similarly, coadministration of cipro oxacin with PPI dendrimers resulted in a formulation with improved antibacterial properties of a cipro oxacin at lower concentrations against Gram-positive *S. aureus* ATCC 6538 and Gram-negative *E. coli* ATCC 25922. These ndings are signi cant because of drug resistance as a result of the extensive use of antibiotics.[233] However, a study on the effect of G2 and G3 PAMAM dendrimers on the antibacterial activity of poorly water-soluble erythromycin and freely water-soluble tobramycin disclosed that though solubility of erythromycin was increased by seven to eightfold in PAMAM dendrimers, there was only a minimal effect on its antimicrobial activity.[229] A twofold and fourfold decrease in MBC values of erythromycin was observed for hydroxyl-terminated and amine-terminated G3 PAMAM, respectively. Furthermore, it was found that there was no in uence of PAMAM on the antimicrobial activity of tobramycin. Antibacterial activity studies in this investigation were performed on *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Klebsiella pneumonia* ATCC 700603, *E. cloacae* ATCC 700323, *Acinetobacter baumannii* LMG 1025, and clinical strains of *S. aureus* and *E. Faecalis*. [229] The differences among these studies show the in uence of dendrimer type in terms of core, branching element, and dendrimer generation on antibiotic activity.

A dendrimer was recently used to conjugate vancomycin to increase the drug cell wall avidity,[231] this being active against Gram-positive bacteria because of its strong attraction to a cell wall precursor terminated with a $_{(D)}$-Ala-$_{(D)}$-Ala peptide residue (Ala-alanine).[235 237] However, it is not active against VRE, as it displays a weak af nity for the $_{(D)}$-Ala-$_{(D)}$-Lac (Lac-lactate) residue present on its surface.[238] Vancomycin-conjugated G5 PAMAM dendrimer series have been synthesized and their avidity to $_{(D)}$-Ala-$_{(D)}$-Ala or $_{(D)}$-Ala-$_{(D)}$-Lac cell wall precursor was established using surface plasmon resonance studies. The nanoconjugates exhibited signi cant enhancement in avidity in the tested cell wall models. As compared with free vancomycin, the nanoconjugate showed a greater increase in binding by four to ve orders of magnitude. As a synthetic polymer, NP, with a size of 5.4 nm G5 PAMAM dendrimer, served as a platform for conjugating multiple copies of vancomycin on its structure, resulting in high-avidity binding on the bacterial surface. Iron oxide magnetic nanodevices were prepared using the conjugates with high af nity to the bacterial surface to investigate the possibility of combining the bacteria-targeting strategy with the speed and convenience delivered by magnetic isolation technology. These dendrimer-covered iron oxide magnetic NPs demonstrated a more rapid sequesteration of bacterial cell walls compared with iron oxide NPs. The study proved the concept that bacteria-targeted dendrimers might be used for fabrication of magnetic NPs, with the resulting formulation opening a convenient route for bacterial magnetic isolation and enumeration.[231]

Most recently, synergistic *in vitro* bactericidal effect against Gram-positive (*B. subtilis* and *S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria has been reported for nor- oxacin in combination with 3-hydroxypyridin-4-one (HPO) hexadentate-based dendrimeric chelator. Owing to their large molecular weight, dendrimeric chelators penetrate membranes slowly and have the bene t of low toxicity compared to smaller molecules. The authors therefore proposed that a combined formulation of HPO hexadentate-based dendrimeric chelator and quinolone antibiotic can have medical potential, principally in treating external infections including wounds and ulcers.[234]

The studies on dendrimer-mediated nanodelivery of antibiotics are limited, although drugs from several therapeutic categories have been studied for their delivery, either by conjugation, entrapment, or encapsulation to enhance their performance in terms of release pattern, solubility, and pharmacological action. This lack in dendrimer-mediated delivery of antibiotics may be attributed to the fact that the research focused mainly on inventing new dendrimers with their antibacterial activity. Although it is interesting to obtain novel dendritic antibacterial dendrimers that may evolve as potential drug candidates in future, it should be noted that US FDA approval of these new chemical entities as antibiotics is a long process. In the present situation, there is an urgent need developing novel nanoformulations using currently existing biocompatible dendrimers and antibiotic drugs in order to combat emerging resistant strains. The review also revealed that PAMAMs are the mostly studied dendrimers for antibiotic delivery, and that most of the studies have focused on *in vitro* antibacterial activity. Therefore, other novel biocompatible dendrimers that have already been reported in the literature should also be exploited for effective nanodelivery of antibiotics, and more emphasis should be given to *in vivo* performances of these nanosystems in order to introduce a dendrimeric nanoantibiotic in clinical trials.

Nanoemulsions

Nanoemulsions can be described as heterogeneous systems comprising dispersed oil droplets stabilized by surfactant molecules in an aqueous media. Their nanometer size makes them kinetically stable during storage over long-term periods.[239,240] NEs display many attractive biological and pharmaceutical characteristics including biodegradability, biocompatibility, ease of preparation, and physical stability.[241] Because of their interesting properties,

recently, increasing attention has been focused on NE-based drug delivery systems.[242] NEs can be effectively produced by high-pressure homogenization,[243] micro uidization,[244] ultrasonication,[245] and phase inversion.[246]

Nanoemulsions containing antibiotics have been investigated by several researchers for their bactericidal activity, with Penicillin G containing injectable NE being developed and studied for its properties.[241] NE has been proven to be a stable formulation for intravenous delivering rifampicin.[247] A water-in-oil emulsion technique has been established for preparing NE particles of chitosan/heparin with better encapsulation of amoxicillin. The formulated amoxicillin NE showed controlled release and localization at intracellular spaces and in the cell cytoplasm to the site of *H. pylori* infections, with a signi cant increase in the growth inhibition.[248] An oil-in-water submicron emulsion, with globule size of 278 ± 12 nm and prepared by incorporating hydrophobic ion-pair complexes of cipro oxacin with sodium deoxycholate in the core, showed high entrapment ef ciency, noncytotoxicity to J774 macrophage cells, and enhancement in antimicrobial ef cacy against *E. coli*, *S. aureus*, and *P. aeruginosa in vitro*.[249] Studies so far have focused on the role of NEs to enhance antibiotic activity, indicating that their applications as a delivery system to site-speci c delivery, sustained, and prolonged release could be further exploited. Besides these, NEs that have been formulated using different oils and are devoid of any antibiotic drug have also been found to be effective antibacterials, for example, peppermint oil NE,[250] cinnamon oil NE,[245] eucalyptus oil NE.[251] Overall, results of these studies suggest that antibacterial activity of bio-based oils could be enhanced by dispensing them into nano form.

Polymeric Micelles

Self-assembling colloidal systems possessing a core/shell structure (size < 200 nm) formed by assembly of block or graft amphiphilic block copolymers are known as polymeric micelles (PMs)[252,253] and are frequently based on copolymers having an AB diblock structure.[254,255] The hydrophobic core facilitates the solubilization of hydrophobic drugs via hydrogen bonding and/or hydrophobic interaction and the hydrophilic shell remains exposed to the external environment. This kind of arrangement helps in protecting the bioactive against degradation and also facilitates escape from the RES, thereby exhibiting prolonged systemic circulation.[256,257]

A few studies have been reported so far for antibiotic delivery via PMs. In one such report, cloxacillin sodium, an anionic drug, was incorporated into a protonated polyvinyl pyridine (PVP) block of polystyrene-*b*-2-vinyl pyridine-*b*-ethylene oxide (PS-PVP-PEO) micelles. The experiment was designed to investigate the possibility of the micelle being an antibiotic drug carrier. This study used zeta potential measurements, dynamic light scattering, and uorescence spectroscopy speci cally, and proved that cloxacillin could be ef ciently incorporated into 69 nm-sized micelles prepared from PS-PVP-PEO because of electrostatic interaction between the protonated PVP block and anionic drug.[258] Although the release kinetics were identi ed, this study would have been strengthened by including at least transmission electron microscope image to con rm the appearance and morphology of the micelles, drug encapsulation ef- ciencies, as well as antibacterial activity, as encapsulation of the drug molecule was not unexpected. PMs appear to be very promising ocular drug delivery systems because of their properties, such as high kinetic and thermodynamic stability, sustained drug release pro les, and the ability to act as an absorption promoter in order to enhance drug permeability across ocular epithelia.[253,259] Considering this fact, ocular delivery of netilmicin sulfate was studied by three copolymers of polyhydroxyethyl aspartamide. *In vitro* permeability studies with primary cultured rabbit conjuctival and corneal epithelial cells demonstrated that micelles of two of the polymers provided greater drug permeation across the latter compared with a simple drug solution or suspension.[260] Dif culty in transporting antibiotics through the BBB has also been overcome by PMs prepared from cholesterol-conjugated PEG and anchored with transcript or activator TAT peptide (TAT-PEG-b-Col). The cipro oxacin-loaded TAT-PEG-*b*-Col micelles smaller than 180 nm showed sustained antibacterial activity against *B. Subtilis* and *E. Coli*, and *in vivo* animal tests con rmed that the formulation can pass the BBB. This study therefore highlighted the applicability of these micelles for developing nanodelivery systems to treat brain infections.[261] The extensive *in vitro* and *in vivo* characterization of this PM formulation, in terms of size, zeta potential, morphology, *in vitro* release, antibacterial activity, cellular uptake, cytoxicity, and *in vivo* animal studies with male rats, is in contrast to the inadequately characterized system of PS-PVP-PEO micelles[258] mentioned earlier.

Increasing attention is being focused on polymers that are inherently antimicrobial because of their wide applications in the health care of both humans and animals.[262 265] The advantages of antimicrobial polymers are their effective inhibition of bacterial growth without the low-molecular-weight toxic chemicals being released to the environment,[265] as well as no resistance development by common bacterial strains such as *E. coli* and *S. aureus*.[266] This has stimulated researchers to develop PMs devoid of any drug as antibacterial agents, such as PMs containing quaternary ammonium compound poly[2-(tert-butylamino)ethyl methacrylate] (PTBAEMA or PTA).[267] On the basis of these ndings about PTA, Yuan et al.[265] reported synthesis of two triblock antibacterial polymers consisting of poly(ethylene oxide (PEO)-PCL **1** and PTA (PEO-*b*-PCL-*b*-PTA) **2** polymers. PEO was used to enhance the biocompatibilty and colloidal stability of the self-assembled micelles in aqueous solution, whereas PTA was used for interacting with the microbial cell wall/membrane. Both these polymers were able to form micelles in THF/water, with a mean diameter of 18 ± 3 nm for polymer **1** and 25 ± 4 nm for polymer **2**. The MBC for polymer **1** was 0.30 mM and 0.15 mM against *E. Coli* and *S. aureus*, respectively, whereas for polymer **2,** it was reported to be 0.20 mM and 0.08 mM in micellar form.[265] Thus, it can be concluded that these PEO-*b*-PCL-*b*-PTA polymers can be used as promising sterilizing agents or as antimicrobial drugs in future. The promising properties of the drug-loaded and drug-free antimicrobial PMs highlighted in this section indicates an opportunity for researchers to encapsulate current antibiotic drugs into the antimicrobial PMs to achieve a multifunctional delivery system with synergistic antibiotic effects.

CNTs, Nanohorns, and Nanorods

Carbon nanotubes, nanohorns, and nanorods have also been reported as nanosystems for antibiotics. Cylindrical nanostructures of pure carbon atoms covalently bonded in a hexagonal array are called CNTs,[268] produced either by arc

discharge, chemical vapor deposition, or laser ablation methods. The details on the methods of CNT production can be found elsewhere.[269] CNTs with a single pipe (1 5 nm diameter) are single-walled CNTs (SWCNTs), and those having many nested tubes (lengths from 100 nm to micrometers) are known as multiwalled CNTs (MWCNTs).[270] Both SWCNTs and MWCNTs possess antimicrobial activity, with the former exhibiting much stronger antimicrobial properties[271] than the latter. Although ease of functionalization together with its good chemical stability makes SWCNTs additionally attractive as antimicrobial biomaterials,[272] its synthesis cost are high.[273] Qi et al.,[274] in an attempt to exploit the lower costs with MWCNT and to overcome its reduced antibacterial activity, used covalent immobilization of cefalexin on MWCNTs via PEG as a linker to enhance the antimicrobial and antiadhesive characteristics of MWCNTs against *S. aureus* and *B. Subtilis* (Gram positive), and *E. Coli* and *P. aeruginosa* (Gram negative). Confocal laser scanning microscopy studies of attached MWCNTs and MWCNT cefalexin revealed that most of the *P. aeruginosa* and *S. aureus* cells were stained with propidium iodide dye (dead cells) on MWCNT cefalexin deposited lm, and with SYTO 9 dye (live cells) on the MWCNT deposited lm. This nding revealed that MWCNT cefalexin deposited lm has superior antimicrobial property than the drug-free MWCNTs deposited lm.[274]

Kang et al.[271] prepared low metal content, narrowly distributed and highly puri ed SWCNT with strong antibacterial activity. As with the study by Qi et al.,[274] such a SWCNT system could be used for encapsulating an antibiotic drug for enhanced activity. Aslan et al.[272] reported an interesting strategy to overcome the high cost and limited range of material properties with SWCNTs. They investigated the concept of combining SWCNTs (as a minority component) with a biomedical polymer, that is, PLGA, to obtain a material that would be antimicrobial and provide a broad range of structural, mechanical, and degradation properties. The SWCNT PLGA polymer was found to be far superior in antibacterial activity than the PLGA only. The possibility of antibiotic loading into biomedical polymers containing SWCNT being an effective strategy for a superior antimicrobial nonintegrated implant needs to be investigated further.

Although antimicrobial activity of CNTs has been reported, cytotoxicity associated with them is a major concern, as reported by a number of studies.[275 277] Future studies with drug-free and drug-loaded CNTs should therefore also focus on approaches to overcome the cytoxicity of these promising delivery systems.

Nanohorns are similar to fullerenes and SWCNTs, and consist of a seamlessly closed one-atom-thick wall of carbon that separates the exterior from the hollow interior. The body of a nanohorn is more or less tubular, with an irregularly varying diameter along its length. Representative nanohorn diameters are between 2 and 5 nm with one end being cone-shaped, the horn, whereas the opposite end is at or rounded.[278 280] Unlike nanotubes, nanohorns assembling into cylindrical bundles with their long axes parallel to each other form spherical aggregates.[278 281] A new type of graphene tubules with a diameter of 2 5 nm and a length of 40 50 nm is known as a single wall nanohorn (SWNH). A spherical aggregate with a narrow diameter distribution of 80 100 nm is formed by an assembly of approximately 2000 SWNHs.[280] The potential of nanohorns in drug delivery has been demonstrated.[281 283] SWNH aggregates have been reported as potential promising drug carriers having some advantages over other carriers. Oxidized SWNH (SWNHox) have been reported for providing controlled release of vancomycin hydrochloride (Fig. 5) to address the problems associated with the drug, such as severe side effects while blood concentration is too high. Controlled release was obtained by exploring the bene t of interaction between vancomycin hydrochloride and SWNHox. Additionally, to improve the dispersibility of this carrier system in aqueous systems, the

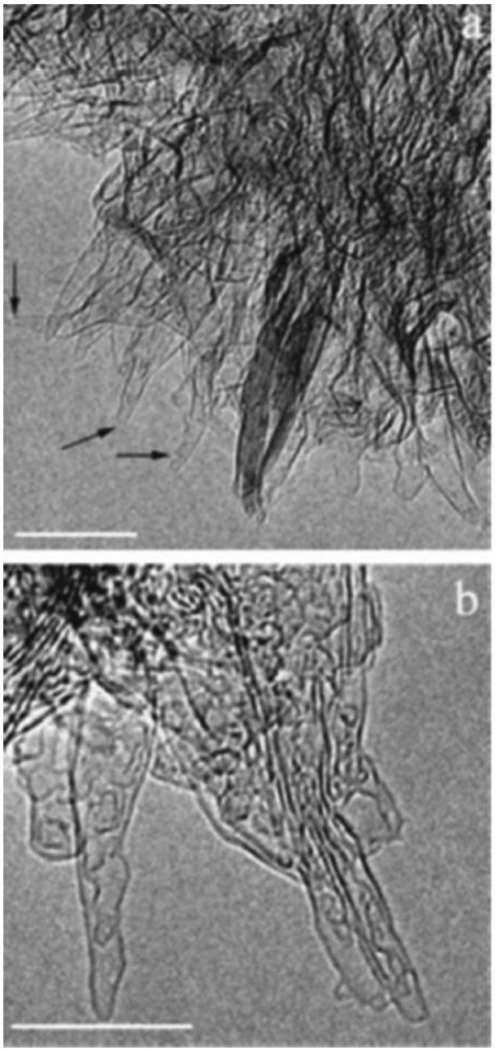

Figure 5. Transmission electron microscopy images of (a) SWNHox (scale bar = 20 nm) and (b) VCM SWNHox (scale bar = 10 nm). Reproduced from Xu et al.[284] with permission from Elsevier Science Ltd.

hydrophobic surface of SWNHox was modi ed by phospholipid PEG.[284]

Nanorods are rod-shaped NPs, with different kinds having been reported in the literature depending on the material used, for example, silver,[285] zinc oxide,[286] stannous oxide,[287] barium carbonate,[288] and gold,[289] the latter being an attractive vehicle for drug delivery applications.[290 292] Nanorods of lanthanum hydroxyapatite have been used for sustained amoxicillin release, speci cally those that showed antimicrobial activity against *bacillus*, *pseudomonas*, *E. coli*, and *S. aureus*. In addition to the antimicrobial and drug release studies, this nanorod system was extensively characterized for its physical properties. The increased surface area and suitable hardness, crystallinity, and crystallite size led the authors to propose this nanorod system as potential implants in the biomedical eld.[293]

Nanohybrids

Bioactive molecules incorporated in layered double hydroxide (LDH) forming nanohybrids (NHs) have gained attention in drug delivery, being normally referred to as hydrotalcites or anionic clays.[294] LDHs represent a family of synthetic or natural materials designated by the formula $[M_{(1-x)}^{II} M_x^{III}(OH)_2][A^{n-}]_{x/n}$. $2H_2O$, where M^{II} and M^{III} are divalent and trivalent metal, respectively, and A^{n-} is the interlayer anion.[295] The rst delivery system based on magnesium aluminum LDHs was reported in 2005.[296] LDHs form successive positively charged metal hydroxide layers and negatively charged anionic layers. Amid the various properties, the anion-exchange property of LDHs provides a simple method enabling replacement of the interlayer anion, thus permitting the synthesis of a various layered materials.[297] Using this ion-exchange reaction, bioactives have been incorporated/intercalated into LDHs to generate NHs with a slow release of the active.[298,299] Intercalation of two hydrophobic drugs, namely, gramicidin and amphotericin B and two hydrophilic drugs, namely, ampicillin and nalidixic acid, with LDHs was studied using a simple ion-exchange reaction. All four drugs intercalated successfully and the release studies showed that the synthesized NHs can function as controlled-release drug delivery systems for various antibiotics.[294] A new polymeric composite material has been prepared and characterized by incorporating chloramphenicol succinate-NH into a biocompatible, biodegradable polymer matrix, PCL. In the NH consisting of a LDH of Mg Al hydrotalcite type, simple ion-exchange reaction was used to replace the nitrate anions present in the host galleries with chloramphenicol succinate anions. The objective of the study was to develop a controlled-release formulation for topical application.[298] From the unique biphasic release pro les of chloramphenicol, the authors concluded that the structural design of this hybrid offers several ways to modify drug release properties. These consist of the ionic force present in the outside solution, drug concentration inside the inorganic lamellae, inorganic component concentrations into the polymer matrix, type of polymeric matrix, and the sample form and thickness. LDH NHs intercalated with amoxicillin by coprecipitation method have also been encapsulated into PCL electrospun bers. This NH-integrated system provided sustained release of the drug, although initial rapid release was found.[300] This study highlights the applicability of this NH system to be integrated into other novel delivery systems for further enhancing drug therapy.

The decoration of MWCNTs with metal NPs, such as Fe_3O_4, results in the formation of MWCNTs NHs. This exercise of decorating MWCNTs with metal NPs is executed to overcome toxic effects and dispersibility problems associated with MWCNTs, and confer unique features to the NH system. They have a proli c effect on microbicidal and bio lm inhibition activity, biocompatibility, and drug targeting.[301] Hyperbranched polyurethane (HBPU) is a well-known wound healing material and potent drug carrier.[301,302] Its application, along with Fe_3O_4 MWCNT NH to form Fe_3O_4 MWCNT NH/HBPU nanocomposites (NNCs), has been explored in the development of effective wound healing material. *In vitro* antibacterial activity of gentamicin sulfate-loaded NNCs against *K. pneumonia* and *S. aureus* MTCC96, using the agar well diffusion method, showed best performance along with good hemo compatibility and nonimmunogenicity because of controlled-release pro les. *In vivo* wound healing experiments performed on albino mice showed signi cant acceleration in wound healing process. Furthermore, the uid handling capacity and moisture vapor permeability of these NNCs suggested its immense potential to provide an optimal moist environment to accelerate the wound healing process. The ndings of this study prove that this novel Fe_3O_4 MWCNT NH/HBPU NNC is a potential wound healing material with the ability to deliver antibiotics to the wound site.[301] The incorporation of antibiotics either into NHs alone, intercalated with NHs for coencapsulation into bers, or loaded into NNCs comprising metal-coated CNT NHs and wound healing material, is evident of the diverse potential of NHs for antibiotic delivery.

Other Nanosystems for Antibiotic Delivery

In addition to the aforementioned more widely published nanoantibiotic systems, researchers have reported on a number of other nanodelivery systems for antibiotics, which are reviewed below.

Nanofibers

Nano bers are de ned as bers with a diameter of 100 nm or less, but in general, all bers with a diameter below 1 μm are considered as nano bers.[303] Nano bers are being studied for wound healing purposes in antibacterial therapy. Electrospun nano bers have shown great ability for wound dressing as a result of properties, such as their high-surface area that enables them to effectively absorb exudates and adjust the wound moisture.[304]

Electrospun drug-loaded nano brous membranes are advantageous over conventional nano bers. Electrospun sandwitch-structured PLGA/collagen nano brous membranes containing vancomycin and gentamicin were found to be effective wound dressing materials.[305] These authors successfully con rmed the antibacterial ef cacy, cytocompatibility, and sustained drug release properties of these antibiotic-loaded nano bers. Kataria et al.[306] recently reported the development of cipro oxacin-loaded transdermal patch prepared from PVA and sodium alginate (NaAlg) electrospun composite nano bers for local delivery of antibiotic. In their experiments, they prepared PVA, PVA NaAlg, cipro oxacin loaded PVA, and cipro oxacin-loaded PVA NaAlg nano bers, and performed comparative studies in terms of morphology, drug release, and *in vivo* wound healing ef cacy. All nano bers with average diameter in the range of 300 400 nm showed nonwoven mat-like structures

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and smooth surfaces. In *in vitro* drug release experiments, the drug release from PVA NaAlg nano bers was slower compared with PVA nano bers. Furthermore, higher hydroxyproline content in animal studies with cipro oxacin-loaded PVA NaAlg nano bers indicated their superior wound healing capability compared with the drug-loaded PVA nano bers, and in less time.[306] This study opens the opportunity of nano brous transdermal patches as an alternative and superior delivery system for local delivery of antibiotics and even other classes of drugs.

Nanofibrous Scaffolds

Regeneration of natural bone tissue or the creation of biological substitutes for defective bone tissues is possible through the use of scaffolds.[307] Nano brous scaffolds, as the terminology suggests, refers to scaffolds composed of nano bers. The advantages of a nano brous scaffold are its high surface-to-volume ratio, high porosity, changeable pore-size distribution, and similarity to the natural extracellular matrix in terms of morphology.[308] Nano brous scaffolds fall under the category of polymer-based drug carriers that are of synthetic origin, are biodegradable,[309] and are mainly used for tissue engineering purposes.[310] The advantages of electrospun nano brous scaffolds can be summarized as: (1) they can be used as carriers for both hydrophilic and lipophilic drugs, (2) ne control over the drug release pro le can be achieved by controlling the scaffold s porosity, morphology and composition, and (3) it is possible to achieve site-speci c delivery into the body for any number of drugs from the scaffold.[309] As a result of these advantages, nano brous scaffolds are being studied for delivering antibiotics such as (1) novel nano brous scaffolds of doxicycline to obtain high local bioavailability, low systemic side effects, and controlled delivery to treat dental, periodontal and bone infections[311]; (2) gentamicin-loaded novel PLGA/lecithin scaffolds for bone-repairing therapeutics[312]; (3) PLGA-based nano brous scaffolds with lidocaine, an anesthetic and mupirocin, an antibiotic having controlled-release mechanism for wound dressing[313]; and (4) cefoxitin sodium-incorporated PLGA-based nano brous scaffolds with sustained drug release for preventing postsurgical adhesion and infections.[309] Although one of the earliest antibiotic-loaded nano brous scaffold appears to have been reported 10 years ago in 2004, there have been very few studies since then addressing the necessity of surgery for implantation.

Nanosheets

Recent developments in nanotechnology have made it possible to fabricate quasi, two-dimensional, freestanding polymeric ultrathin lms (polymer nanosheets or simply nanosheets) with remarkable properties, such as high exibility, minimum surface roughness, and noncovalent adhesive properties.[314 319] The polysaccharide nanosheet forms a stable platform for facilitating drug loading, with nanosheets loaded with TC for treating gastrointestinal defects, such as gastric peritonitis and other surgical defects, having been reported in the literature.[319] TC was compressed between polyvinyl acetate (PVAc) and polysaccharide nanosheet to form a PVAc TC nanosheet of 177 nm thickness. *In vivo* studies on mice revealed that therapy with the PVAc TC nanosheet signi cantly increased survival rate of mice after cecal puncture, and an increase in intraperitoneal bacterial and leukocyte count was also suppressed.[319] In a separate paper, these authors found the same nanosheet to be an effective nanoantibiotic system to treat full-thickness burn wound infections by *P. aeruginosa in vivo*.[320] It would have been interesting for the researchers to have included bioadhesivity and textural analysis, as optimal bioadhesion and mechanical properties are critical aspects of this delivery system. These are preliminary studies on nanosheets, and formulation optimization and characterization appear to be in its infancy.

Nanoplexes

Nanoplexes are complexes of a drug and oppositely charged polyelectrolyte forming stable amorphous NPs, and are manufactured by mixing two aqueous salt solutions, one containing the former and the other the latter.[321] Cheow and Hadinoto[321] recognized that the amphiphilicity and solubility in acid or basic solutions of antibiotics can be exploited for preparing antibiotic NPs via a process known as self-assembly amphiphilie polyelectrolyte complexation. Higher drug-loading capabilities can therefore be achieved compared with conventional NPs. The authors synthesized drug polyelectrolyte complexes (nanoplexes) of o oxacin and levo oxacin by self-assembly complexation within dextran sulfate with an antibiotic loading of 60% 80% (w/w) and sizes less than 400 nm. The optimal preparation conditions based on its size, stability, and drug loading by varying the pH, polyelectrolyte charge ratio, drug, and salt concentration were identi ed. These nanoplexes were examined *in vitro* against *P. aeruginosa* planktonic cells and the activities were found to be comparable to native antibiotics. The main advantages of these nanoplexes were salt-promoted drug release and rapid antibiotic release, rendering it suitable for antibio lm treatment, which needs high doses of antibiotic in order to eliminate the appearance of antibiotic-resistant strains.[322] Nanoplexes certainly have promising potential for diverse applications and growth as it can facilitate high drug encapsulation, unlike polymeric and liposomal nanosystems, offers greener and simpler methods of preparation for various antibiotics, and the charged surface makes them readily functionalized.

CONCLUSIONS AND FUTURE PERSPECTIVES

Factors such as poor targeting of antibiotics to infection sites, increased dosing frequencies and side effects, the spread of resistance to currently used antibiotic medicines, slow development rate of newer antibacterials, and the possibility of resistance to future new antimicrobial drugs all highlight the need to follow novel approaches for managing microbial infections. In the last four to ve decades, considerable research has been undertaken on nanodelivery systems, resulting in revolutionary changes to drug delivery technology for various disease conditions. More recently, an explosion of interest in the use of nanotechnology to overcome the signi cant challenges associated with antibiotic drug therapy is evident in the literature.

This review indicated that a range of diverse nanoengineered drug delivery systems, such as liposomes, PNPs, SLNs, dendrimers, NEs, LPHNs, PMs, CNTs, nanorods, nanohorns, NHs, nano bers, nano brous scaffolds, nanosheets, and nanoplexes are being investigated for antibiotic delivery. Studies on these antibiotic-loaded nanosystems have con rmed enhanced activity against sensitive and resistant bacteria. The ability of these

nanosystems to improve solubility, stability, and drug entrapment provides sustained drug release, target infection sites, penetrate the BBB, improve antibio lm therapy, and overcome bacterial resistance have been amply demonstrated. It is also clear that researchers are moving toward antibiotic nanosystems with multifunctional properties and multiple mechanisms of action to enhance antimicrobial action and prevent drug resistance.

Although signi cant progress has been achieved in the eld of nanoantibiotics, much remains to be accomplished to optimize these systems for eventual regulatory approval and commercialization. This review has speci cally identi ed a number of areas that need to be investigated and prioritized. Formulation optimization technologies and in-depth physicochemical/mechanical characterization for newly emerging and promising antibiotic nanosystems, such as LPHNs, PMs, SLNs, nanorods/plexes/sheets, and dendrimers need to be prioritized, as these are less investigated in the literature compared with liposomes and PNPs. Several lipid- and polymer-based nanosystems can be enhanced by identifying and synthesizing new lipidic and polymeric materials with responsive properties to promote targeting to infection sites. For example, lipids and polymers responsive to speci c pH, bacterial toxin, and enzymatic changes at infection sites can be considered. Identifying these novel materials will widen the pool of superior materials for developing nanoantibiotics. The coencapsulation of antibiotics with other antibiotics, as well as nondrug antimicrobial agents, offers the opportunity of developing nanosystems with multiple mechanisms of action against bacteria that can enhance activity and also overcome resistance mechanisms. A goal should therefore be nanosystems comprising responsive antimicrobial materials with multiple antimicrobial agents. Such a multidimensional integrative nanodelivery system will give rise to a generation of smart nanoantibiotics. There is also a lack of data that offers a mechanistic and molecular understanding of these nanosystems in terms of their antimicrobial activity against various organisms, drug entrapment, and drug release properties. Such studies will guide formulation scientists in designing optimal antimicrobial materials and nanosystems. More formulation studies also need to focus on *in vivo* antimicrobial investigations for both widely and less studied antibiotic nanosystems. Scale-up and strategies and studies on these systems should also be a focus.

It is evident that a multidisciplinary collaborative relationship among researchers in academia and the pharmaceutical industry will be essential to successfully develop smart nanoantibiotics, which are clearly showing potential for saving millions of lives globally from serious life-threatening infections by microorganisms.

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The authors declare that there is no con ict of interest.

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CHAPTER 6. CONCLUSIONS

6.1 General conclusions

Although antibiotics are essential for treating and managing infectious diseases, numerous disadvantages of current conventional dosage forms limit their efficacy. Alternative novel antimicrobial therapeutic strategies are being explored to address the imminent crisis with conventional antibiotics. However, as studies on novel antimicrobial systems are still in the early stages, it is necessary to identify novel materials for formulation optimisation in this field.

The research described in this thesis is intended to address the difficulties associated with current antibiotic therapy, and explores the use of novel polymeric materials to overcome the challenges related to antimicrobial resistance. The aim of the study was to: (1) explore the potential of novel antimicrobial dendrimer silver salts for enhanced antimicrobial activity and (2) explore the potential of a novel star shaped polymer for use as a stabilising agent in the preparation of silver nanoparticles. The work reported in this thesis accomplished the aim of this study and generated the following conclusions:

- Objectives 1 – 4: In addressing the first aim of this study, G1 PETIM dendron and two PETIM dendrimers containing a carboxylic acid function at the periphery were successfully designed and synthesised using reported methods. They were then used as templates to contain the silver ions, after which the PETIM dendron/dendrimers were reacted with silver nitrate to form PETIM-silver salts by means of simple techniques of mechanical stirring and solvent evaporation using a rotovap. These synthesised PETIM dendron/dendrimers were then characterised in terms of FT-IR and NMR, and the PETIM-silver salts were characterised in terms of FT-IR only, due to the hygroscopic nature of the salts. The FT-IR peaks in the range of 1459-1332 cm^{-1} from carboxylic acid terminated dendron and dendrimers disappeared, and the appearance of symmetric stretching vibrations in the range of 1288-1233 cm^{-1} was observed after transforming them into their respective silver salts. Thus, the presence of the bands due to symmetric stretching vibrations of the equivalent carbon-oxygen bonds strongly confirmed the formation of PETIM-silver salts. The cytotoxic effects of the salts were evaluated in terms of MTT assay, and the range of cell viability obtained indicated that the PETIM-silver salts

displayed a low toxicity level on all the cell lines studied. Additionally, the effects of the compounds on the cell line were not dose dependent, as no such trends were observed for any of the PETIM-silver salts at the various treatment concentrations against any of the cell lines. These PETIM-silver salts also displayed a greater percentage cell viability when compared to their respective concentrations of silver nitrate. *In vitro* antimicrobial testing revealed the enhanced antimicrobial activity of the PETIM-silver salts at low concentrations against both *S. aureus* and MRSA, and that the G1 PETIM-silver salt with the highest number of carboxylic acid functions, and ultimately the highest number of Ag^{+} ions, had the greatest antibacterial activity. The data attained for these objectives confirmed for the first time the potential of PETIM silver salts as non-toxic antimicrobial agents against sensitive and resistant bacteria.

- For Objective 5 – 7, a biocompatible and biodegradable novel G1 PETIM-m-PEG star shaped polymer was successfully designed and synthesised using a core first approach. This synthesised star polymer was then characterised in terms of IR, ^{1}H NMR, ^{13}C NMR, and XRD analysis. The formation of the G1 PETIM-m-PEG star polymer was confirmed by a disappearance of –C-Cl stretch at 653 cm^{-1} and the appearance of a strong –C-O- ether stretch at 1099 cm^{-1} in FT-IR. The characteristic peaks in ^{1}H NMR of G1 PETIM-m-PEG star polymer were of $–CH_2$-O- at 3.56 δ ppm, $-CH_2$-N- peak at 3.80 δ ppm and terminal –O-CH_3 at 3.23 δ ppm, whereas in ^{13}C NMR, those were terminal –O-CH_3 at 60.48 δ ppm, -C=O- ester at 138.25 δ ppm, and aromatic carbons at 100 and 127.84 δ ppm. The cytotoxic effects of the star polymer was assessed using the MTT assay, and the range of cell viability attained, specified that the PETIM-m-PEG star polymer displayed a very low toxicity level on all the cell lines studied. Furthermore, the effects of the compound on all the cell lines was not dose dependent, as no such trends were observed. The data attained was then used for the preparation of silver nanoparticles.
- Objectives 8 – 10, involved preparing silver nanoparticles using the G1 PETIM-m-PEG star polymer as a stabilising agent, via chemical reduction using $NaBH_4$, their formation being confirmed by UV-visible spectra and XRD analysis. The peak at a wavelength of 394.30 nm in the UV-visible spectra, due to surface plasmon resonance (SPR) of the electrons in the conduction band of silver, confirmed the formation of G1 PETIM-m-PEG star polymer silver nanoparticles. In terms of XRD, the 2θ values were in agreement with

previously reported values for silver nanoparticles. TEM and DLS studies were performed to determine the particle size, PDI and morphology of the silver nanoparticles. The DLS studies indicated a particle size of 36.44 ± 2.51, with a PDI of 0.414 ± 0.007, while the TEM studies confirmed that the nanoparticles were spherical, of uniform particle size, non-agglomerated and well dispersed in the size range of between 25-30 nm. The cytotoxic effects of the star polymer stabilised nanoparticles were also assessed, and the range of cell viability attained, which indicated similar results to that of the star polymer itself. A low toxicity level on all the cell lines studied was seen, and the effects of the compound on the cell line was not dose dependent, as no such trends were observed. The *in vitro* antimicrobial tests revealed that the star polymer stabilised silver nanoparticles are efficient enough to inhibit the growth of sensitive and resistant bacteria, and could therefore be an attractive delivery system to treat a host of infections.

In this study, novel materials and a nano-delivery system with the potential to improve antibacterial therapy were successfully prepared and characterised. The established formulation approaches and the thorough characterisation studies will benefit scientists exploring other methods of preparing novel antimicrobial systems. Further studies in this rapidly growing field will need a multidisciplinary approach to accomplish the best outcomes.

6.2 Significance of the findings

The significance of the results and outputs generated from this study are:

Optimising treatment for infectious diseases:

- With limited commercially available polymeric materials to help combat the growing difficulties that arise from bacterial resistance, identifying novel polymeric materials for antimicrobial systems as novel delivery systems is a valuable alternative to contend with the issue of AMR. This study has confirmed the formation of new antimicrobial materials and a novel delivery system, the PETIM silver salts and star polymer stabilised silver nanoparticles, which display good antimicrobial activity against both sensitive and resistant bacterial strains. These systems can improve the treatment of patients with various bacterial infections, thereby enhancing patients' quality of life and improving the economy of the country.

Identifying new pharmaceutical materials:

- This study identified PETIM silver salts and star polymer stabilised silver nanoparticles, which are new materials, with the potential for commercialisation. It expands the pool of materials for formulation scientists to develop novel antibacterial systems.

Creating new knowledge on polymers for drug delivery systems:

- New knowledge was obtained on the applicability and antimicrobial properties of PETIM dendron\dendrimers in particular, as our study confirmed the antimicrobial activity of these type of dendrimers for the first time.
- New knowledge on the application of star polymers as stabilising agents for nanoparticle formation was obtained, this being the first study to utilise a six arm star shaped PEG polymer as a stabilising agent to prepare silver nanoparticles.

Impact of this study on future research:

- This study is the building block for future research such as TEM, in depth *in vitro/in vivo* antimicrobial studies, as well as molecular mechanistic studies to optimise these materials and delivery systems for commercial use in patients. It can also be explored for application in nanomedicine and the biomedical sciences.

6.3 Recommendations for future studies

This study has set the foundation for formulating novel antimicrobial delivery systems. Further studies are essential preceding commercialisation of these new agents and can be summarised as follows:

PETIM silver salts:

- *In vitro* and *in vivo* antimicrobial studies using both sensitive and resistant strains of Gram positive and Gram negative bacteria should be performed to test whether the silver salts have a wide range of activity against different types of bacteria.
- *In vivo* antimicrobial studies using infected animals, and even human subjects, should be performed to test the silver salts antimicrobial effect. These studies may also offer details on bioavailability and related pharmacokinetics, which could provide insight into possible formulation adjustments that are essential to attain optimal bioavailability.

- The differences in activity of the three PETIM-silver salts against *S. aureus* and MRSA could be due to differences in the structure and composition of their cell walls. However, no mechanistic studies are available in the literature using novel materials and delivery systems against S. aureus and MRSA. Molecular mechanistic simulations can be done on the silver salts to establish a correlation between the in vivo and *in silico* data.
- A larger scale production technique could be designed to prepare the silver salts to enable the feasibility of the preparation method to be assessed for application in the pharmaceutical industry.
- An additional characterisation method could be employed to not only assess the purity of the silver salts, but also to better understand the structural components of them.

Silver nanoparticles:

- Numerous metal ions, such as copper, gold, etc., could be used to assess whether or not the star polymer is a good stabiliser for other metal nanoparticles.
- Further antimicrobial studies, such as the TEM analysis of bacteria, can be performed to establish the exact mechanism by which the nanoparticles affect bacteria.
- *In vivo* antimicrobial studies using infected animals, and even human subjects, should be performed to test the silver nanoparticles in terms of their antimicrobial effect. These studies could also offer details on bioavailability and related pharmacokinetics, which could provide insight into possible formulation adjustments that could be essential to attain optimal bioavailability.
- Molecular mechanistic simulations can be done on the silver nanoparticles to establish a correlation between the in vivo and *in silico* data.
- A larger scale production method could be designed to establish the feasibility of preparing the silver nanoparticles for application in the pharmaceutical industry.

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Silver salts of carboxylic acid terminated generation 1 poly (propyl ether imine) (PETIM) dendron and dendrimers as antimicrobial agents against *S. aureus* and MRSA

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Novel therapeutic strategies are essential to address the current global antimicrobial resistance crisis. Branched molecules with multiple peripheral functionalities, known as dendrimers, have gained interest as antimicrobials and have varying levels of toxicity. Silver displays activity against several micro-organisms only in its positively charged form. In this study, silver salts of generation 1 (G1) poly (propyl ether imine) (PETIM) dendron and dendrimers were synthesised and evaluated for their antimicrobial potential against sensitive and resistant bacteria. The purpose was to exploit the multiple peripheral functionalities of G1 PETIM dendron and dendrimers for the formation of silver salts containing multiple silver ions in a single molecule for enhanced antimicrobial activity at the lowest possible concentration. G1 PETIM dendron, dendrimers and their silver salts were synthesised and characterised by FT-IR, ^{1}H NMR and ^{13}C NMR. PETIM silver salts were evaluated against Hep G2, SKBR-3 and HT-29 cell lines for their cytotoxicity using the MTT assay. The G1 PETIM dendron/dendrimers, silver nitrate and silver salts of the G1 dendron (compound **13**), G1 dendrimer with an aromatic core (compound **14**) and an oxygen core (compound **15**) were evaluated for activity against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) by the broth dilution method. PETIM silver salts were found to be non-cytotoxic even up to 100 μg ml^{-1}. Minimum inhibitory concentration values of compounds **13**, **14** and **15** against *S. aureus* were 52.1, 41.7 and 20.8 μg ml^{-1} while against MRSA they were 125.0, 26.0 and 62.5 μg ml^{-1}, respectively. The calculated fractional inhibitory concentration index further indicated that compound **14** specifically displayed additive effects against *S. aureus* and synergism against MRSA. The enhanced antimicrobial activities of the PETIM dendron/dendrimer-silver salts against both sensitive and resistant bacterial strains widen the pool of available pharmaceutical materials for optimizing treatment of bacterial infections.

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Introduction

Infectious diseases, a significant portion of which are of bacterial origin, are one of the leading causes of death globally for adults and children and remain a major public health issue for developed and developing countries.[1] While antibiotics revolutionized the treatment of infections, thereby saving millions of lives, eighty years after their discovery, their effectiveness is seriously threatened by antimicrobial resistance (AMR).[2] This nullifies the use of even the most potent antibiotics, which leads to patient suffering and/or dying due to infection control failure, and results in escalated health care costs.[3]

Globally, resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA),[4] vancomycin-resistant *Enterococcus* (VRE)[5] and vancomycin-resistant *Staphylococcus aureus* (VRSA),[6] have become significant threats in community settings and hospitals for treatment of infections. Furthermore, if current escalating trends in AMR continue, several important procedures, such as cancer chemotherapy, organ transplantation and hip and other joint replacements, could no longer be performed for fear that the related compromised immune system might put the patients at severe risk of acquiring a difficult to treat and ultimately fatal infection.[7] The global AMR crisis is amplified by the decreasing development of new antibiotics by pharmaceutical companies,[8] with 20 novel classes of antibiotics being developed in between 1930–1962,[9,10] and only two of them have been marketed.[11–14] This decline in drug development is due to the high costs and lengthy delays associated with developing a new chemical entity, high attrition rates at final testing, and increasing AMR, which makes finding

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a new drug very expensive and limits the return on investment.[3,15]

It is therefore essential that alternative novel antimicrobial therapeutic strategies are explored to address the imminent crisis with conventional antibiotics. Alternative options currently being investigated are novel drug delivery systems for existing antibiotics, such as silver nanoparticles,[16–18] solid lipid nanoparticles,[19–21] liposomes[22–24] and the synthesis of new antimicrobial materials, such as dendrimers[25–27] and antimicrobial peptides.[28]

Silver is a potent antimicrobial agent, particularly in its positively charged ionic form, as it displays a strong toxicity to a wide range of micro-organisms and concurrently has a particularly low human toxicity.[29–31] Antimicrobial silver is widely used to combat organisms associated with burns and wounds.[30] In addition, silver-based medical preparations are available and frequently used in coatings of biomedical materials, such as silver impregnated catheters and dressings for wound healing.[29] Silver is also capable of disturbing key functions in a microorganism that causes AMR. It has a high affinity for negatively charged side groups on biological molecules, such as carboxyl, phosphate, sulfhydryl and others dispersed throughout microbial cells. It thereby transforms the macromolecule's molecular structure *via* this binding reaction, rendering it useless to the cell.[30] Concomitantly, silver can attack numerous sites within the cell, incapacitating critical physiological functions, such as cell wall synthesis, protein folding and function, membrane transport, nucleic acid (such as RNA and DNA) synthesis, and translation and electron transport, which are vital for cell energy production. Dispossessed of such key functions, bacterial growth can either be inhibited or, more frequently, the microorganism is killed.[30] It is highly improbable that resistance to antimicrobial silver could ever develop, as this would mean that an organism would have to undertake concurrent mutations in every critical function within just a single generation to evade the compounds multiple actions.[30] This is a crucial factor to consider when developing new antimicrobial materials to overcome resistance. However, it should be noted that silver is nontoxic to human cells only in minute concentrations.[32] This clearly limits the use of metallic silver and silver ions as an antibacterial agent only up to concentrations that are non-toxic to eukaryotic cells.

Dendrimers are repeatedly branched molecules or nanosized, radially symmetric molecules that have a well-defined, uniform and monodisperse structure that consists of branches surrounding a core.[33,34] The availability of several functional surface groups and their low polydispersity make them a rich source for finding novel and unique properties.[33,35] Due to these very distinctive properties, and the fact that they can be adapted to therapeutic needs, they are regarded as model carriers for small molecule drugs and biomolecules.[26] Dendrimers have gained further interest as likely antimicrobial agents due to the availability of numerous end groups and their compressed structure.[36,37] Therefore, if any one of the functional groups is capable of interacting with a target, other groups within close proximity of one another could make synergistic interactions for antimicrobial activity possible.[36] Specific interactions (*e.g.* quaternary ammonium based dendrimers) aim to eliminate bacterial/viral infections by inhibiting the growth of microbes, thereby killing them and nonspecific interactions (*e.g.* oligosaccharide based dendrimers), and preventing the initial attachment between bacteria/viruses and host cells.[36]

It has also been highlighted that dendrimers show promising biocompatibility in general,[26] which is essential for their application, and can themselves be used as antimicrobial agents.[38–41] Consequently, highly potent dendrimer based antibacterial agents have been synthesised.[38,40] Currently, the most extensively used dendrimers in drug delivery include poly propylene imine (PPI), polylysine, triazine[42] and polyamidoamine (PAMAM), the latter being the first and most commonly studied.[26] Unfortunately, its uses are constrained by limitations such as cytotoxicity resulting from its amine-terminated nature[43] and as a result, there are no commercially available dendrimer based formulations for systemic administration.[42] Researchers have recognized the potential of developing complexes of silver and dendrimers to enhance antimicrobial activity, with Balogh *et al.* having prepared PAMAM dendrimer based silver complexes,[44] which showed enhanced antimicrobial effect, creating a new and potent antimicrobial agent for biomedical applications.

A fairly new class of dendrimers, known as the poly (propyl ether imine) (PETIM) dendrimers, has been reported to have good biocompatibility when compared to commercial PAMAM dendrimers, and has been effectively applied for encapsulation of ketoprofen for sustained drug delivery.[42] Although it has several advantages, such as non-cytotoxicity and easy functional group modification at the periphery, its potential for antimicrobial therapy has not been exploited. This study is therefore the first combination of PETIM dendrimers and silver to identify novel antimicrobial materials effective against both sensitive and resistant bacterial strains, and will widen the pool of available pharmaceutical materials to optimize the treatment of bacterial infections.

In this study, a generation 1 (G1) PETIM dendron and two PETIM dendrimers containing a carboxylic acid function at the periphery were synthesised and reacted with silver nitrate to form dendrimer-silver salts. The PETIM dendrimers were used as templates to contain the silver ions. The rationale for using PETIM dendron and dendrimer as a template to contain silver ions were: (i) more than one silver ion can be accommodated on a single PETIM dendron or dendrimer, as it contains multiple carboxylic acid functions at the periphery; (ii) PETIM silver complexes are non-toxic to mammalian cells due to the biocompatibility of PETIM dendrimers; and (iii) PETIM on its own could display antimicrobial activity, thus the potential antimicrobial activity of PETIM silver complexes may display additive or synergistic effects. Published studies on silver complexes of organic compounds as antimicrobial agents mostly include two organic molecules complexed with one silver ion through a chemical bond formation.[45,46] In the present investigation, our goal was therefore to exploit the multiple peripheral functionalities of biocompatible PETIM dendron and dendrimers to form silver salts containing multiple silver ions in a single molecule for enhanced antibacterial activity at

the lowest possible concentration. The intention was to study the effect of the number of silver ions per molecule of these dendrimer silver salts on antimicrobial efficacy against both sensitive and resistant strains. For this reason, a G1 PETIM dendron (two carboxylic acid functions at the periphery), G1 PETIM dendrimer with oxygen core (four carboxylic acid functions at the periphery) and G1 PETIM dendrimer with aromatic core (six carboxylic acid functions at the periphery) were selected. The results of the investigations are reported in this paper.

Results and discussion

Synthesis

In this study three different compounds were employed, *viz.*, a G1 PETIM dendron **4**, a PETIM dendrimer with an aromatic core **7**, and another PETIM dendrimer with an oxygen core **12**. Synthetic steps for these compounds are depicted in Schemes 1–3 and explained hereunder.

The dendron was prepared using 3-amino-1 propanol **1** and excess *tert*-butyl acrylate **2** to afford an ester in good yield. Thereafter the resulting ester was deprotected (AcCl, H_2O) to obtain the free carboxylic acid containing G1 PETIM dendron **4** (Scheme 1).

The two dendrimers synthesised were prepared with slight modifications in a previously reported method.[47] Upon synthesis of the dendron **3**, its attachment to a selected core was carried out. Compound **3** was coupled with 1,3,5-benzenetricarbonyl trichloride **5** in the presence of DMAP to attain **6**. Thereafter the resulting ester was deprotected *via* an acetyl chloride and water system to attain the free carboxylic acid containing G1 PETIM dendrimer with an aromatic core **7** (Scheme 2).

Bis-nitrile **9** was attained from acrylonitrile **8** and aqueous NaOH (40%). Bis-nitrile was subjected to successive reactions; *i.e.* reduction of the nitrile using $LiAlH_4$ to a diamine **10**; Michael addition of *tert*-butyl acrylate to afford the tetrakis **11**; and deprotection of the ester (AcCl, H_2O) to attain the free carboxylic acid containing G1 PETIM dendrimer with an oxygen core **12** (Scheme 3).

Preparation of PETIM-silver salts (Scheme 4). PETIM silver salts (**13**, **14** and **15**) were all prepared in a similar method where silver was reacted with **4**, **7** and **12** to afford these PETIM-silver salts.

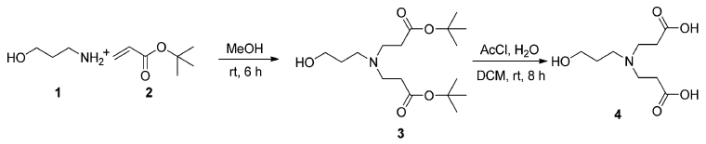
Characterisation

The synthesised dendron and dendrimers were characterised by FT-IR, ^{1}H NMR, ^{13}C NMR and HRMS and were compared with the literature values.[47] Synthesis of the silver salts were accomplished *via* reaction of silver nitrate with the corresponding dendron/dendrimer acid. Formation of silver salts was supported by observing the shifts in the positions of characteristic IR frequencies of carboxylic groups in the dendron and dendrimers.

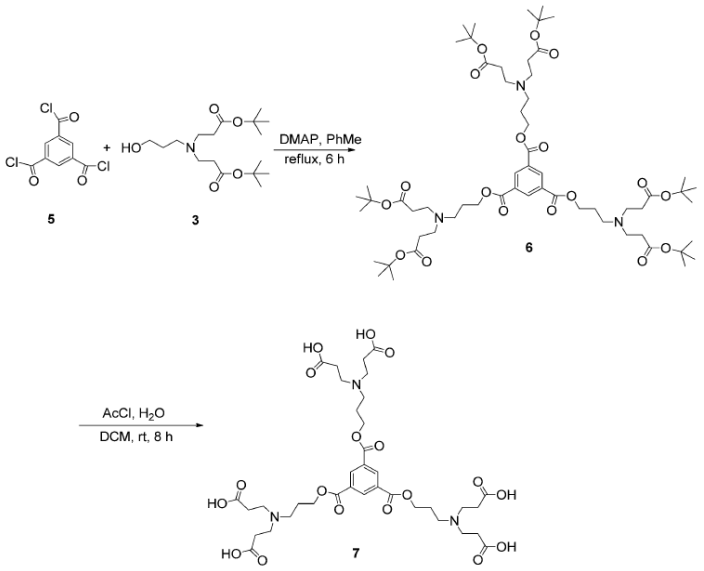
The main feature which allows one to differentiate a carboxylic acid from all other carbonyl compounds is a broad absorption band due to the strongly hydrogen bonded O–H stretching vibrations which extends from 3300–2500 cm^{-1}. The transformation of the ester function to a carboxylic acid was confirmed by the presence of this characteristic peak in FT-IR spectrum. In addition, all carboxylic acid terminated dendron/dendrimers exhibited a peak in the range of 1707–1714 cm^{-1} indicating the presence of a C=O stretching band of the –COOH group. The aliphatic C–H stretching band appeared as a jagged peak near 3000 cm^{-1}. Coupled vibrations involving C–O stretching were observed in the range of 1459–1399 cm^{-1}. Salts of carboxylic acids do not display any of the carbonyl bands rather bands owing to the asymmetric and symmetric stretching vibrations of the equivalent carbon–oxygen bonds. They are observed at 1610–1550 cm^{-1} and 1420–1300 cm^{-1} respectively, which provides evidence for the carboxylate anion.[48] In our study the peaks in the range of 1459–1332 cm^{-1} from carboxylic acid terminated dendron and dendrimers disappeared and appearance of symmetric stretching vibrations in the range of 1288–1233 cm^{-1} was observed (Fig. 1–3) after transforming them into their respective silver salts. Thus, the presence of the bands because of symmetric stretching vibrations of the equivalent carbon–oxygen bonds strongly confirms the formation of silver salts of G1 PETIM dendron and dendrimers. Further attempts to characterise silver salts using elemental analysis were not successful because of their hygroscopic nature.[47]

In vitro cytotoxicity study

An *in vitro* cell culture system was used to determine the biological efficacy of the PETIM silver salts. The MTT assay, which is based on the biochemical reduction of MTT by viable cells, was used to determine the cytotoxicities of the PETIM silver salts against Hep G2, HT-29 and SK-BR-3 cell lines.[49] Determining cell viability using cytotoxicity assays are basic steps in toxicology that explain the cellular response to a compound by providing information on cell death and their metabolic activities.[50] Cell viability of between 80% and 95% were observed for all the PETIM silver salts across all the cell lines (Fig. 2). The comparative results between the individual PETIM dendron/



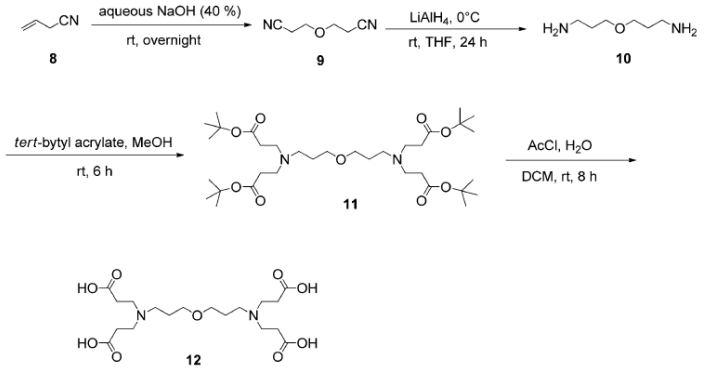
Scheme 1 Synthesis of G1 PETIM dendron.



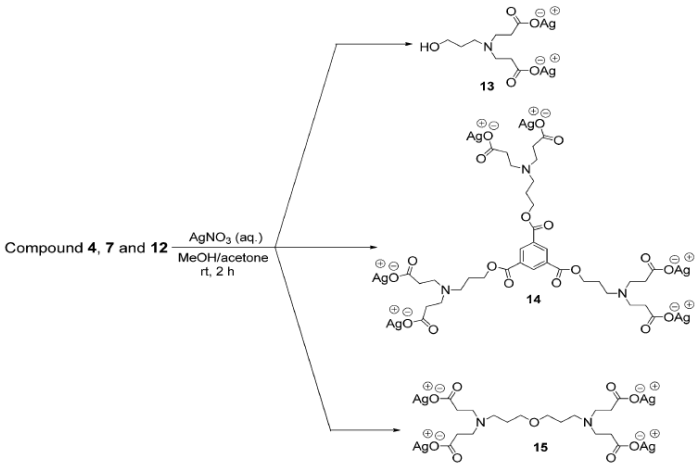
Scheme 2 Synthesis of G1 PETIM dendrimer containing an aromatic core.

dendrimers, their respective concentrations of silver nitrate, and their subsequent combinations (PETIM silver salts) were tested for cytotoxicity and are represented in Fig. 3.

The range of cell viability obtained in this study indicates that the PETIM silver salts displayed a low toxicity level on all cell lines studied.[51] The results also showed that the effects of the compounds on the cell line were not dose dependent, as no dose dependent trends were observed for any of the PETIM silver salts at the various treatment concentrations against any of the cell lines (Fig. 2). These PETIM silver salts displayed a greater percentage cell viability when compared to their respective concentrations of silver nitrate (Fig. 3). Reduced cytotoxicity of the PETIM silver salts may be due to their close-to-neutral net surface charge, which had little effect on membrane integrity.[52] These results are in line with previous findings, where acetamide-terminated G5 PAMAM dendrimers revealed to have little effect on membrane integrity, whereas positively charged G5 PAMAM dendrimers reduced the integrity of the cell membrane and prompted the release of cytoplasmic membrane proteins, lactate dehydrogenase and luciferase.[52,53] The PETIM silver salts therefore have a statistically greater cell viability than the silver nitrate ($P < 0.05$) (Fig. 3) and slightly



Scheme 3 Synthesis of G1 PETIM dendrimer containing an oxygen core.



Scheme 4 Synthesis of silver salts of G1 PETIM dendron and dendrimers.

higher cell viability when compared to the PETIM dendron/dendrimers. Therefore, they can be considered non-toxic with potential for use in the biomedical and pharmaceutical fields.

In vitro antimicrobial evaluation

The antimicrobial activities of silver nitrate, the PETIM dendron/dendrimers and the PETIM silver salts were investigated against *S. aureus* and MRSA. A summary of the results for the MIC values for *in vitro* antimicrobial activity is presented in Table 1. MIC values for the different concentrations of silver nitrate against *S. aureus* were 112.5, 87.5 and 77.5 μg ml^{-1} respectively, and against MRSA they were 93.7, 210 and 77.5 μg ml^{-1} respectively (Table 1). Ionized silver brings about structural changes in bacterial cell walls and nuclear membranes as it is highly reactive when it binds to tissue proteins. Thus it results in cell distortion and even cell death. Silver can also bind to bacterial DNA and RNA, and can therefore inhibit bacterial replication. These antimicrobial properties of silver are dependent on the quantity and the rate at which silver is released.[54,55] The MIC values for the PETIM dendron/dendrimers, *i.e.* **4**, **7** and **12** against *S. aureus* and MRSA, were all 500 μg ml^{-1} (Table 1). The MIC values obtained for the PETIM dendron/dendrimers indicate that the PETIM dendron/dendrimers alone do have some antimicrobial activity, although low, against the selected bacteria. Higher antimicrobial activity has been reported for both unmodified dendrimers and dendrimers, with additional surface modifications such as PAMAM dendrimer ammonium salts,[38] and PPI dendrimers modified with maltotriose 25% and 100%.[39] However, the unmodified dendrimers displayed higher levels of cytotoxicity when compared to the surface modified dendrimers due to the cationic nature of these dendrimers.[56] As the PETIM dendrimers in our study displayed good cell viability due to their anionic nature, this nullifies the need for surface modification procedures to minimize the toxicity. Although these MIC values are higher when compared to surface modified and unmodified dendrimers, such as PAMAM and PPI against gram positive bacteria, this does confirm for the first time the antimicrobial activity of G1 PETIM dendron and dendrimers (**4**, **7** and **12**).

The MIC values for the PETIM silver salts, *i.e.* **13**, **14** and **15** investigated in this study, were 52.1, 41.7, and 20.8 μg ml^{-1} against *S. aureus* respectively, while against MRSA they were 125.0, 26 and 62.5 μg ml^{-1} respectively (Table 1). An increase in antimicrobial activity was observed for all salts when compared to silver nitrate and PETIM dendron/dendrimers alone. This may be a result of a high local concentration of silver ions available at the periphery of the PETIM silver salts. Antimicrobial activity was reported to be less when internal complexes were applied, showing that accessibility of the silver is a vital factor, and that a high local concentration of silver needs to be accessible to have a significant effect on microorganisms.[44] The MIC values of the salts of PETIM dendron/dendrimers were markedly reduced for G1 PETIM-dendron silver salt **13** and G1 PETIM dendrimer (oxygen core)-silver salt **15** against *S. aureus*, and G1 PETIM dendrimer (aromatic core)-silver salt **14** against both organisms. Compound **13** and **15** exhibited 42% and 33% greater activity against *S. aureus* respectively when compared to MRSA. However, compound **14** displayed 62% greater activity against MRSA than *S. aureus*. The PETIM silver salts showed different degrees of antibacterial activity in relation to the bacterial species used in this study. Compound **14** displayed greater antibacterial activity against MRSA than *S. aureus*. Certain dendrimers displayed potent and broad antimicrobial activity against *S. aureus*,[37] as well as a selectivity toward this particular bacterial species.[39] Polcyn *et al.* also recently synthesised a range of modified dendrimers and interestingly, they too identified one particular dendrimer as having strong activity

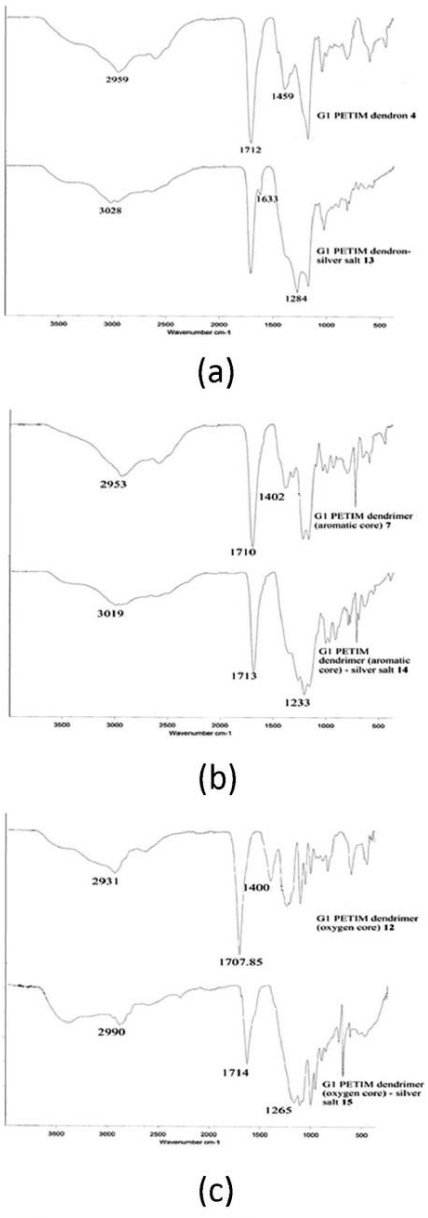


Fig. 1 (a) FT-IR spectra comparing G1 PETIM dendron **4** and G1 PETIM dendron-silver salt **13**; (b) G1 PETIM dendrimer (aromatic core) **7** and G1 PETIM dendrimer (aromatic core)-silver salt **14** and (c) G1 PETIM dendrimer (oxygen core) **12** and G1 PETIM dendrimer (oxygen core)-silver salt **15**.

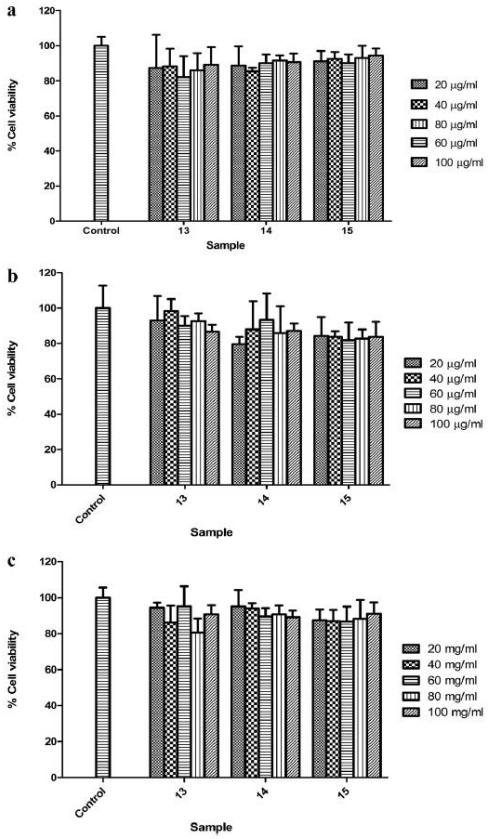


Fig. 2 (a) Cytotoxicity assay against Hep G2 cells, displaying percentage cell viability after exposure to various concentrations of PETIM silver salts [G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**] to Hep G2 cells. Results are presented as mean ± SD ($n = 6$). (b) Cytotoxicity assay against HT-29 cells, displaying percentage cell viability after exposure to various concentrations of PETIM silver salts [G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**] to HT-29 cells. Results are presented as mean ± SD ($n = 6$). (c) Cytotoxicity assay against SK-BR-3 cells, displaying percentage cell viability after exposure to various concentrations of PETIM silver salts [G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**] to SK-BR-3 cells. Results are presented as mean ± SD ($n = 6$).

against MRSA,[37] similar to the antimicrobial activity of compound **14** used in this study. Wang *et al.*, performed antimicrobial testing on norfloxacin-loaded solid lipid nanoparticles for a 144 h time period, and the results indicated antimicrobial activity for an extended time period.[19] Similarly, the antimicrobial activity of **13**, **14** and **15** were tested over a 72 h

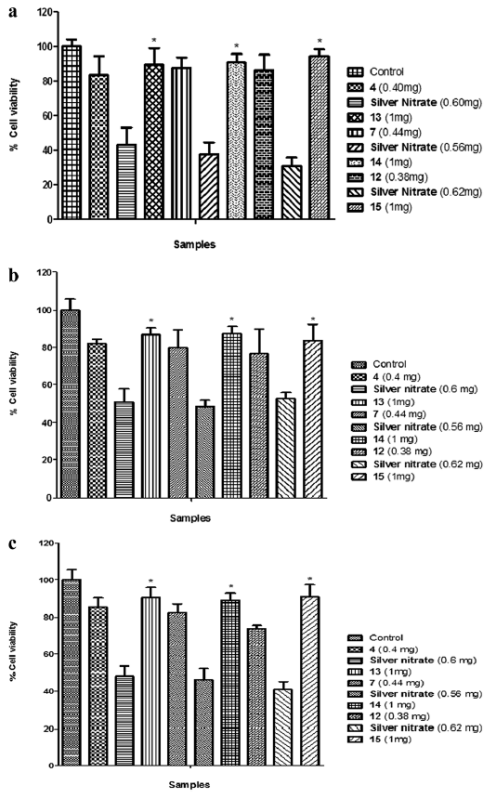


Fig. 3 (a) Cytotoxicity assay on Hep G2 cell lines, comparing percentage cell viability after exposure to PETIM silver salts against their individual parent dendron/dendrimers as well as silver nitrate concentrations. Results are presented as mean ± SD ($n = 6$). *denotes significant difference compared to the respective silver nitrate ($P < 0.05$) [G1 PETIM dendron **4**; G1 PETIM dendrimer with aromatic core **7**; G1 PETIM dendrimer with oxygen core **12**; G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**]. (b) Cytotoxicity assay on HT-29 cell lines, comparing percentage cell viability after exposure to PETIM silver salts against their individual parent dendron/dendrimers as well as silver nitrate concentrations. Results are presented as mean ± SD ($n = 6$). *denotes significant difference compared to the respective silver nitrate ($P < 0.05$) [G1 PETIM dendron **4**; G1 PETIM dendrimer with aromatic core **7**; G1 PETIM dendrimer with oxygen core **12**; G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**]. (c) Cytotoxicity assay on SK-BR-3 cell lines, comparing percentage cell viability after exposure to PETIM silver salts against their individual parent dendron/dendrimers as well as silver nitrate concentrations. Results are presented as mean ± SD ($n = 6$). *denotes significant difference compared to the respective silver nitrate ($P < 0.05$) [G1 PETIM dendron **4**; G1 PETIM dendrimer with aromatic core **7**; G1 PETIM dendrimer with oxygen core **12**; G1 PETIM dendron-silver salt **13**; G1 PETIM dendrimer (aromatic core)-silver salt **14**; G1 PETIM dendrimer (oxygen core)-silver salt **15**].

period, with the results being consistent throughout this time span, indicating that they have the potential for sustained antimicrobial activity.

MIC values alone did not contribute toward a clear indication of the combined effects of the PETIM dendron/dendrimers and silver nitrate. Hence, the effects of the combination of G1 PETIM dendron/dendrimers and silver nitrate were also investigated, and these effects were evaluated using ΣFIC. A summary of the results for the ΣFIC values for *in vitro* antimicrobial activity experiments is presented in Table 2. All of the combinations displayed different degrees of effectiveness against the bacteria tested, and no antagonistic relations were observed. Compound **13** presented a ΣFIC value of 1.58 against MRSA (Table 2), which represents indifference (Table 3). Compound **13** and **14** presented a ΣFIC value of 0.57 and 0.56 against *S. aureus* (Table 2), and **15** presented a ΣFIC value of 0.93 against MRSA (Table 2), which are all indicative of additive effects (Table 3). Compound **14** presented a ΣFIC value of 0.18 against MRSA (Table 2) and **15** presented a ΣFIC value of 0.31 against *S. aureus* (Table 2), which signify synergistic effects (Table 3). Of the three PETIM silver salts tested, **13** was observed to be the least active salt, whereas **14** was most active. This pattern of antibacterial activity of G1 PETIM-silver salts against both *S. aureus* and MRSA can be correlated to the structures of the compounds. The order of antibacterial potency of dendron/dendrimer-silver salts was G1 PETIM dendrimer (aromatic core)-silver salt **14** (six Ag^+ ions in the structure) > G1 PETIM dendrimer (oxygen core)-silver salt **15** (four Ag^+ ions in the structure) > G1 PETIM dendron-silver salt **13** (two Ag^+ ions in the structure). The G1 PETIM-silver salt with the highest number of carboxylic acid functions, and ultimately the highest number of Ag^+ ions, had the greatest antibacterial activity. As the G1 PETIM-silver salts contain positively charged Ag^+ ions and the bacterial cell wall has an overall negative charge, which has more affinity towards positively charged compounds, it may be possible that **14** had the best activity because of the highest number of Ag^+ ions present. The synergistic effect of **14** could therefore be a result of the combination of different mechanisms of actions of both silver and the dendron/dendrimers. Silver is known for its growth inhibitory capacity against microorganisms,[57] and by using dendrimers as a template to incorporate silver, dendrimers themselves can become potent antimicrobials.[58] This activity can then be further enhanced if the functional groups of the dendrimers are within close proximity to one another.[36]

The interesting differences in activity of the three compounds against *S. aureus* and MRSA as well as specifically the significant synergistic activity against MRSA as compared to *S. aureus* in **14** may be due to differences in the structure and composition of their cell walls. For example one of the most widely reported mechanisms of resistance in *S. aureus* is the development of a modified penicillin binding protein (PBP) known as PBP 2a found in MRSA.[59,60] Biosynthesis of peptidoglycan, which comprises the outermost layer of Gram-positive bacteria, is achieved by the membrane-bound enzymes PBP.[59] With MRSA the modified PBP known as PBP 2a, is intrinsically resistant to inhibition by β-lactams and stays active even in the

Appendix A

Table 1 MIC results for *in vitro* antimicrobial activity of PETIM dendron/dendrimers, PETIM silver salts and their corresponding individual silver nitrate concentrations against *S. aureus* and MRSA

	MIC (μg ml^{-1})	
	Organism	
Sample	*S. aureus*	MRSA
G1 PETIM dendron **4**	500[a]	500[a]
Silver nitrate	112.5[a]	93.7[a]
G1 PETIM dendron-silver salt **13**	52.1 ± 18.04	125[a]
G1 PETIM dendrimer with an aromatic core **7**	500[a]	500[a]
Silver nitrate	87.5[a]	210[a]
G1 PETIM dendrimer (aromatic core)-silver salt **14**	41.7 ± 18.04	26 ± 9.04
PETIM dendrimer with oxygen core **12**	500[a]	500[a]
Silver nitrate	77.5[a]	77.5[a]
G1 PETIM dendrimer (oxygen core)-silver salt **15**	20.8 ± 11.07	62.5[a]

[a] Denotes SD = 0.

presence of antibiotics that typically inhibit most endogenous PBP enzymes, thereby replacing their functions in cell wall synthesis and permitting growth in the presence of β-lactam inhibitors such as Methicillin.[59]

The significant increase in activity of **14** against MRSA may be attributed to its higher valency compared to **15**. The higher valency of **14** might have resulted in better binding affinity to PBP 2a of MRSA than PBP of *S. aureus*. This plausible mechanism of action could be supported by the recent findings where multivalent vancomycin-conjugated G5 PAMAM dendrimers exhibited enhancement in avidity in the cell wall models of *S. aureus* and VRSA as compared to free vancomycin. In this particular study authors have observed that the vancomycin-conjugated PAMAM dendrimers had binding avidity of 2–3 and 5 orders of magnitude with D-Ala–D-Ala, a cell wall precursor of *S. aureus* and D-Ala–D-Lac, a cell wall precursor of VRSA respectively.[61] The absence of PBP 2a in *S. aureus* could have been the reason behind low activity of **14** against *S. aureus* as compared to **15**. In the case of *S. aureus* **15** may have greater binding affinity to PBP resulting in its higher antibacterial activity against *S. aureus* than **14**.

Whilst the paper by Choi *et al.* attempts to provide a mechanistic understanding of the vancomycin-conjugated G5 PAMAM dendrimers against *S. aureus* and VRSA, there are no such mechanistic studies available in the literature using novel materials and delivery systems against *S. aureus* and MRSA. There is also the possibility of multiple simultaneous mechanisms of actions of **14** and **15** against both *S. aureus* and MRSA, therefore, the mechanism of action postulated to explain the differences in antibacterial activity of **14** and **15** against MRSA and *S. aureus* respectively is a hypothesis based on previous literature and needs to be confirmed by future in depth experimental mechanistic studies.

Table 3 FIC index[65]

Index	Synergy	Additive	Indifference	Antagonism
FIC	≤0.5	>0.5–1	>1 to <2	≥2

Experimental

Materials and methods

Acrylonitrile, *tert*-butyl acrylate and 3-amino-1-propanol were purchased from Alfa Aesar (Germany). 4-(Dimethylamino) pyridine (DMAP), lithium aluminum hydride ($LiAlH_4$), acetyl chloride (AcCl), 1,3,5-benzenetricarbonyl trichloride, silver nitrate and silica gel were purchased from Sigma-Aldrich (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Merck Chemicals (Germany). All other chemicals and solvents used were of analytical grade, used without further purification and purchased from Merck Chemicals (Germany). Purified water used during the study was produced in the laboratory with a Milli-Q purification system (Millipore corp., USA). Nutrient Broth, Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA) were obtained from

Table 2 ΣFIC results for *in vitro* antimicrobial activity of the PETIM silver salts

	ΣFIC		Results	
Sample	*S. aureus*	MRSA	*S. aureus*	MRSA
G1 PETIM dendron-silver salt **13**	0.57	1.58	Additive	Indifference
G1 PETIM dendrimer (aromatic core)-silver salt **14**	0.56	0.18	Additive	Synergy
G1 PETIM dendrimer (oxygen core)-silver salt **15**	0.31	0.93	Synergy	Additive

Biolab (South Africa). The bacterial cultures used were *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *Staphylococcus aureus* (MRSA) (*Staphylococcus aureus* Rosenbach ATCC BAA 1683). Optical density (OD) was measured using a Mindray MR-96A microplate spectrophotometer (China). FT-IR spectra of all the compounds were recorded on a Bruker Alpha-p spectrometer with diamond ATR (Germany) as per standard protocols. ^{1}H NMR and ^{13}C NMR measurements were performed on a Bruker 400/600 Ultrashield™ (United Kingdom) NMR spectrometer. HRMS was performed on a Waters Micromass LCT Premier TOF-MS (United Kingdom).

Synthesis of dendron 4 (Scheme 1)

The G1 PETIM dendron with a carboxylic acid function at the periphery was synthesised by hydrolysis of the dendron as reported in the literature.[47] In summary, a mixture of 3-amino-1-propanol **1** (5 g; 67 mmol) in methanol (20 ml) was added drop wise to a solution of *tert*-butyl acrylate **2** (51.2 g; 399 mmol) in methanol (100 ml), and was stirred for 6 h at room temperature. Surplus *tert*-butyl acrylate and solvent were removed *in vacuo*, with the crude product obtained being diluted with dichloromethane and washed with brine (3 × 25 ml). The organic layer was dried over anhydrous sodium sulphate and concentrated to yield **3** as a clear colourless liquid (21 g; 96%). Acetyl chloride (0.95 g; 12 mmol) and water (0.22 ml; 12 mmol) were added to a solution of **3** (0.5 g; 1.5 mmol) in dichloromethane (30 ml), and the solution was stirred at room temperature for 8 h. Solvents were removed under vacuum to afford **4** as a viscous material (0.3 g; 91%). FT-IR (neat) ν: 2959, 1712, 1399, 1179, 929 cm^{-1}. ^{1}H NMR (400 MHz, CD_3SOCD_3) δ: 1.82 (q, 2H), 2.50 (t, 4H), 2.82 (t, 2H), 3.47 (t, 4H), 3.76 (t, 2H). ^{13}C NMR (100 MHz, CD_3SOCD_3) δ: 28.3, 37.0, 52.2, 58.9, 59.0, 174.4. HRMS (ES-TOF): $[M]^+$ calcd for $C_9H_{17}NO_5$ 220.1185; found 220.1181.

Synthesis of G1 PETIM dendrimer with an aromatic core 7 (Scheme 2)

A mixture of **3** (3 g; 9 mmol) and DMAP (3.3 g; 27 mmol) in PhMe (60 ml) was refluxed for 3 h and cooled to room temperature. 1,3,5-benzenetricarbonyl trichloride **5** (0.6 g; 2.3 mmol) was then added to the mixture and the reaction was refluxed for 6 h. PhMe was removed *in vacuo* and the crude product was purified *via* column chromatography (silica, mesh size 60–100) (hexane/EtOAc, 4 : 6) to obtain **6** as a colourless oil (1.5 g; 60%). Acetyl chloride (3.63 g; 46 mmol) and water (0.73 ml; 41 mmol) were added to a solution of **6** (1.06 g; 0.92 mmol) in dichloromethane (40 ml), and the resulting solution was stirred vigorously at room temperature for 8 h. Solvents were then removed *in vacuo* and the subsequent residue was triturated with dichloromethane and hexane to obtain **7** (0.7 g; 93%) as a white foamy solid. FT-IR (neat) ν: 2601, 1710, 1232, 1402, 944, cm^{-1}. ^{1}H NMR (400 MHz, CD_3SOCD_3) δ: 2.23 (b, 6H), 2.86 (t, 12H), 3.29 (t, 6H), 3.36 (t, 12H), 4.44 (t, 6H), 8.70 (s, 3H). ^{13}C NMR (100 MHz, CD_3SOCD_3) δ: 22.8, 28.3, 50.6, 52.1, 62.9, 131.28, 134.3, 167.5, 174.1.

Synthesis of G1 PETIM dendrimer with an oxygen core 12 (Scheme 3)

Acrylonitrile **8** (11.66 g; 0.22 mmol) was added drop wise to aqueous sodium hydroxide (40%) (2 ml), while maintaining the temperature below 30 °C. The reaction mixture was stirred overnight at room temperature and then neutralized with hydrochloric acid (32%) (w/w). The product was extracted with chloroform (3 × 50 ml) and washed with 5% sodium hydroxide (100 ml) followed by brine (50 ml). The organic layer was dried over anhydrous sodium sulphate and concentrated under vacuum to yield **9** (7.44 g; 55%). To a solution of $LiAlH_4$ (1.38 g; 48 mmol) in dry THF (40 ml) at 0 °C, **9** (3 g; 24 mmol) was added drop wise, a solution of **9** in THF (10 ml). The reaction was allowed to come to room temperature and was then stirred for 1 h, after which cold water (2.2 ml; 122 mmol) was added drop wise to the reaction mixture. The reaction mixture was stirred overnight at room temperature to afford **10**, a diamine (2.63 g; 80%) after filtration of the reaction mixture and evaporating the solvent. A solution of **10** (2.63 g; 20 mmol) in methanol (60 ml) was added drop wise to *tert*-butyl acrylate (14.02 g; 0.11 mmol) in methanol (50 ml), and the reaction was stirred for 6 h at room temperature. After column chromatographic purification (silica, mesh size 60–100) (hexane/EtOAc, 7 : 3) and removal of the solvents, **11** was obtained as a colourless liquid (3.45 g; 27%). Finally, acetyl chloride (1.33 ml; 15 mmol) and water (0.28 ml; 16 mmol) were added to a solution of **11** (0.5 g; 0.77 mmol) in dichloromethane (10 ml), and the solution was stirred vigorously at room temperature for 8 h to afford **12** (0.3 g; 94%) after removing the solvent *in vacuo* and trituration of residue with hexane and dichloromethane several times. FT-IR (neat) ν: 2931, 1707, 1240, 1400, 930 cm^{-1}. ^{1}H NMR (400 MHz, D_2O) δ: 2.0 (b, 4H), 2.88 (t, 8H), 2.93 (b, 4H), 3.31 (b, 4H), 3.47 (t, 8H). ^{13}C NMR (100 MHz, D_2O) δ: 22.50, 2.28, 52.08, 58.9, 62.7, 176.5.

Silver salt of G1 PETIM dendron 13

To a solution of **4** (0.1 g; 0.46 mmol) in methanol (10 ml), an aqueous solution of silver nitrate (0.154 g; 0.9 mmol) in H_2O (5 ml) was slowly added and stirred vigorously for 2 h. The solvents were removed *in vacuo* to obtain **13** (0.19 g; 96%). FT-IR (neat) ν: 3028, 1722, 1633, 1284 cm^{-1}.

Silver salt of G1 PETIM dendrimer with an aromatic core 14

An aqueous solution of silver nitrate (0.25 g; 0.31 mmol) in H_2O (30 ml) was slowly added to a solution of compound **7** (0.2 g; 1.18 mmol) in methanol and stirred vigorously for 2 h. The solvents were removed *in vacuo* to obtain **14** (0.33 g; 92%). FT-IR (neat) ν: 3019, 1713, 1287, 1233, 1181 cm^{-1}.

Silver salt of G1 PETIM dendrimer with an oxygen core 15

Compound **12** (0.25 g; 0.59 mmol) was dissolved in acetone (50 ml), to which an aqueous solution of silver nitrate (0.405 g; 2.38 mmol) in H_2O (30 ml) was slowly added and stirred vigorously for 2 h. The solvents were removed *in vacuo* to afford **15** (0.5 g; 99%). FT-IR (neat) ν: 2932, 1714, 1265, 1210 cm^{-1}.

In vitro cytotoxicity study

Cell culture against hepatocellular carcinoma (Hep G2), colorectal adenocarcinoma (HT-29) and breast adenocarcinoma (SK-BR-3) cell lines were cultured with complete medium (minimum essential medium, supplemented with 10% bovine calf serum, 100 units per ml of penicillin, and 100 mg ml^{-1} of streptomycin). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Solutions: The compounds were dissolved in DMSO and distilled water as a stock solution,[62] and diluted in the culture medium at concentrations of 20, 40, 60, 80 and 100 μg ml^{-1} as working-solutions.[63]

MTT assay: The cell lines were harvested from the exponential phase, were seeded equivalently into a 96-well plate (2.2×10^3) and incubated for 24 h to allow for adherence. Thereafter, the culture medium was removed and replaced with fresh medium (100 μl per well), with the samples being added to the wells to achieve final concentrations. The control wells were prepared by adding the culture medium only. Wells containing the culture medium without cells were used as blanks. All experiments were performed with six replicates. Upon completion of the incubation for 48 h, the culture medium and compounds were removed and replaced with fresh medium (100 μl) and 100 μl of MTT solution (5 mg ml^{-1} in PBS) in each well. After 4 h incubation, the media and MTT solution was removed and 100 μl of DMSO was added to each well to solubilize the MTT formazan. The OD of each well was measured on a microplate spectrophotometer at a wavelength of 540 nm.[64] The percentage cell viability was calculated as follows:

$$\%\text{cell survival} = [\text{A}540\text{ nm treated cells}]/[\text{A}540\text{ nm untreated cells}] \times 100 \quad (1)$$

A540: absorbance at a wavelength of 540 nm.

Antimicrobial evaluation

Determination of minimum inhibitory concentrations (MICs): the MICs of the PETIM dendron/dendrimers, silver nitrate and dendrimer-silver salts were determined in triplicate using the broth dilution method. Stock solutions of **4** (0.4 mg ml^{-1}), **7** (0.44 mg ml^{-1}) and **12** (0.38 mg ml^{-1}), as well as silver nitrate in three different concentrations (0.60 mg ml^{-1}, 0.56 mg ml^{-1}, 0.62 mg ml^{-1}), were prepared in dimethyl sulfoxide (DMSO). The quantities were equivalent to the amount of individual components present in 1 mg ml^{-1} solutions of the respective dendrimer-silver salt. Stock solutions of the various dendrimer-silver salts (1 mg ml^{-1}) were prepared in distilled water **13** and DMSO **14** and **15**. The compounds were tested against *S. aureus* and MRSA, which were grown overnight in Nutrient Broth at 37 °C and adjusted to 0.5 McFarlands standard with distilled water. Serial dilutions of the dendron/dendrimers, silver nitrate and dendrimer-silver salts were prepared in MHB from the stock solutions. The test bacteria were added to each dilution and incubated overnight at 37 °C. Thereafter, each dilution was spotted on MHA plates and incubated overnight at 37 °C. After incubation, the MHA plates were examined for growth and the MIC's was determined, with DMSO being used as a control.

Determination of fractional inhibitory concentration (FIC): the effects of the combination of G1 PETIM dendron/dendrimers and silver nitrate were investigated by determining the Σ FIC. The European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)[65] described the method for quantifying MIC results in terms of the FIC index, defined as the sum of FIC values of two drugs in combination. An example of the method used to calculate the ΣFIC is as follows:

For two antibacterials A and B alone and in combination (**4** and silver nitrate).

$$\text{FIC}_{(\text{A})} = \frac{\text{MIC}_{(\text{A in presence of B})}}{\text{MIC}_{(\text{A alone})}} = \frac{52.08}{500} = 0.10416 \quad (2)$$

$$\text{FIC}_{(\text{B})} = \frac{\text{MIC}_{(\text{B in presence of A})}}{\text{MIC}_{(\text{B alone})}} = \frac{52.08}{112.5} = 0.46293 \quad (3)$$

$$\Sigma\text{FIC} = \text{FIC}_{(\text{A})} + \text{FIC}_{(\text{B})} = 0.10416 + 0.46293 = 0.56709 \quad (4)$$

The FIC index is shown in Table 3. Indifference is when the effect of a combination of antimicrobials is equal to the effects of the most active compound. The additive effect refers to the effect of a combination of antimicrobials, where the effect of the combination is equal to that of the sum of the effects of the individual components. Synergistic action of a combination of two antimicrobials is present if the effect of the combination exceeds the additive effects of an individual compound.[65]

Statistical analysis

The results are expressed as mean ± standard deviation (SD) and were analysed using one-way analysis of variance (ANOVA), followed by the Mann–Whitney test using GraphPad Prism® (Graph Pad Software Inc. Version 5, San Diego, CA). A p value of less than 0.05 was considered to be statistically significant.

Conclusion

The results obtained in the present study confirm the enhanced antimicrobial activity of the PETIM-silver salts at low concentrations against both *S. aureus* and MRSA. These results also demonstrate that the PETIM-silver salt with the highest number of Ag^+ ions, had the greatest antibacterial activity. At the same time these salts display low cytotoxicity, which paves the way to synthesise silver salts of higher generation PETIM dendrimers, and to evaluate them as effective antimicrobials against a range of sensitive and resistant micro-organisms. A combination of such antimicrobial agents increases the spectrum of organisms that can be targeted and circumvent the emergence of resistance in microorganisms. The synthesised G1 PETIM-silver salts in this study show potential for applicability in pharmaceutical as well as biomedical fields.

Appendix A

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RSC Advances

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A poly (ethylene glycol) six-arm star-shaped polymer as an efficient stabiliser for the synthesis of antibacterial and non-cytotoxic silver nanoparticles

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UNIVERSITY OF KWAZULU-NATAL ™
INYUVESI YAKWAZULU-NATALI

SILVER SALTS OF CARBOXYLIC ACID TERMINATED GENERATION 1 POLY (PROPYL ETHER IMINE) (PETIM) DENDRON AND DENDRIMERS AS NOVEL ANTIMICROBIAL AGENTS AGAINST *S. AUREUS* AND MRSA

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INTRODUCTION

Alternative novel antimicrobial therapeutic strategies are essential to address the current global antimicrobial resistance crisis. Silver displays activity against several micro-organisms only in its positively charged form (1). Branched molecules with multiple peripheral functionalities, known as dendrimers, have gained interest as likely antimicrobial agents (2). Although PAMAM silver salts are reported to have antimicrobial activity, PETIM-silver salts displayed not only good antimicrobial activity but low toxicity as well.

AIM The purpose was to exploit the multiple peripheral functionalities of G1 PETIM dendron and dendrimers for the formation of silver salts containing multiple silver ions in a single molecule for enhanced antimicrobial activity at the lowest possible concentration.

METHODS

SYNTHESIS OF DENDRON AND DENDRIMERS
The ester terminated generation 1 (G1) PETIM dendron, dendrimer with an aromatic core and the dendrimer with an oxygen core were synthesized following a previously reported method (3).

SYNTHESIS OF G1 PETIM-SILVER SALTS
Silver nitrate was added to the dendron/dendrimers in acetone/methanol and stirred for 2 h. The solvents were then removed under vacuum to obtain the silver salts (Figure 1).

STRUCTURAL EVALUATION
FT-IR spectra of all the compounds were recorded on a Bruker Alpha-p spectrometer with diamond ATR (Germany) as per standard protocols. ^{1}H NMR and ^{13}C NMR measurements were performed on a Bruker 400/600 Ultrashield™ (United Kingdom) NMR spectrometer at 400 and 100 MHz respectively.

IN VITRO CYTOTOXICITY STUDY
G1 PETIM-silver salts were evaluated for their cytotoxicity by MTT assay using Hep G2 cells.

ANTIMICROBIAL EVALUATION
The minimum inhibitory concentrations (MICs) of the PETIM dendron/dendrimers, silver nitrate and dendrimer-silver salts were determined in triplicate using the broth dilution method against *S. aureus* and MRSA and incubated overnight at 37°C. The effects of the combination of G1 PETIM dendron/dendrimers and silver nitrate were investigated by determining the fractional inhibitory concentration (FIC).

DATA ANALYSIS
The results expressed as mean ± standard deviation (SD), were analyzed using one-way analysis of variance (ANOVA) followed by the Mann-Whitney test using GraphPad Prism® (Graph Pad Software Inc. Version 5, San Diego, CA). A p value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

***IN VITRO* CYTOTOXICITY STUDIES:**

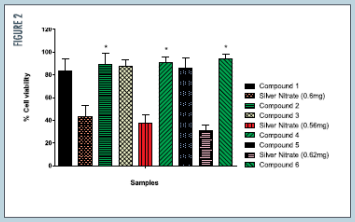

Figure 2. Cytotoxicity assay on Hep G2 cell lines, comparing percentage cell viability after exposure to PETIM silver salts against their individual silver nitrate concentrations. Results are presented as mean ± S.D. (n = 6). *denotes significant difference as compared to the respective silver nitrate ($P < 0.05$) [compound 1: G1 PETIM dendron; compound 3: G1 PETIM dendrimer with aromatic core; compound 5: G1 PETIM dendrimer with oxygen core; compound 2: G1 PETIM dendron-silver salt; compound 4: G1 PETIM dendrimer (aromatic core)-silver salt; compound 6: G1 PETIM dendrimer (oxygen core)-silver salt]

The range of cell viability obtained in this study indicates that the PETIM silver salts displayed a low toxicity level. These PETIM-silver salts displayed a greater percentage cell viability when compared to their respective concentrations of silver nitrate and PETIM dendron/dendrimers (Figure 2). Reduced cytotoxicity of the PETIM-silver salts may be due to their close-to-neutral net surface charge, which had little effect on membrane integrity (4). These results indicate that the novel PETIM-silver salts synthesized showed enhanced cell viability as compared to their individual constituents and can therefore be considered non-toxic.

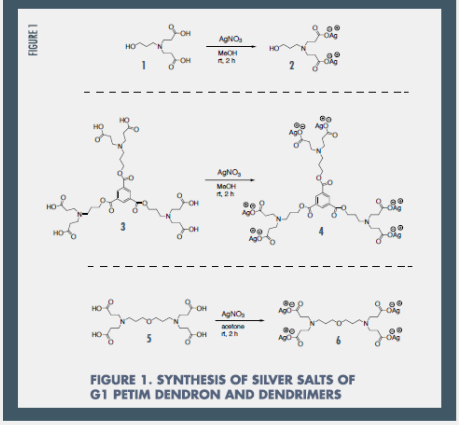

FIGURE 1. SYNTHESIS OF SILVER SALTS OF G1 PETIM DENDRON AND DENDRIMERS

ANTIMICROBIAL EVALUATION:

TABLE 1. MIC results for *in vitro* antimicrobial activity of PETIM dendron/dendrimers, PETIM-silver salts and their individual silver nitrate concentrations against *S. aureus* and MRSA.

SAMPLE	MIC (µg/ml) ORGANISM	
	S. aureus	MRSA
Compound 1	500	500
0.6 mg Silver Nitrate	112.5	93.7
Compound 2	52.08	125
Compound 3	500	500
0.56 mg Silver Nitrate	87.5	210
Compound 4	41.67	26.03
Compound 5	500	500
0.62 mg Silver Nitrate	77.5	77.5
Compound 6	20.82	82.5

An increase in antimicrobial activity was observed for all salts when compared to silver nitrate and PETIM dendron/dendrimers alone. This may be a result of a high local concentration of silver particles available at the periphery of the PETIM silver salts (5).

TABLE 2. ΣFIC results for *in vitro* antimicrobial activity of the PETIM silver salts.

SAMPLE	ΣFIC		RESULTS	
	S. aureus	MRSA	*S. aureus*	MRSA
Compound 2	0.57	1.58	Additive	Indifference
Compound 4	0.56	0.18	Additive	Synergy
Compound 6	0.31	0.93	Synergy	Additive

The effects of the combination of G1 PETIM dendron/dendrimers and silver nitrate were also investigated using ΣFIC. Of the three PETIM-silver salts tested, compound 2 was observed to be the least active salt whereas compound 4 was most active. This pattern of antibacterial activity can be correlated to the structures of the compounds. The G1 PETIM-silver salt with the highest number of carboxylic acid functions and ultimately the highest number of Ag+ ions had the greatest antibacterial activity. The synergistic effect of compound 4 could therefore be a result of the combination of different mechanisms of actions of both silver and the dendron/dendrimers. Additionally, compound 4 displayed greater affinity towards MRSA.

CONCLUSION

Enhanced antimicrobial activity of the PETIM-silver salts were observed at lower concentrations. This paves the way to synthesize silver salts of higher generation PETIM dendrimers and to evaluate them as effective antimicrobials against a range of sensitive and resistant micro-organisms. These antimicrobial agents increases the spectrum of organisms that can be targeted and can prevent the emergence of resistance in microorganisms. The identification of these novel, non-toxic antimicrobials will widen the pool of available pharmaceutical materials for optimizing the treatment of bacterial infections. The synthesized novel G1 PETIM-silver salts in this study show potential for applicability in pharmaceutical as well as biomedical fields.

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Novel Poly(ethylene glycol)- Star-shaped Polymer coated Silver Nanoparticles: Synthesis, In vitro Cytotoxicity and antibacterial activity

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Abstract:
The identification of polymeric materials for stabilisation of silver nanoparticles (Ag NP) is essential for their optimal formulation and activity. Star polymers (SPs), wherein at least three linear polymer chains with essentially identical lengths are attached to only one branching point (Inoue, 2000) , have attracted considerable attention due to their unique topological structure and attractive physical and chemical properties which are different from their linear counterparts (Wu *et al*., 2015; Jia *et al*., 2014). These SPs could be a novel family of stabilising agents for the preparation of colloidal silver nanoparticles (Ag NPs). Despite their numerous advantages there is a lack of literature on the design, synthesis and application of SPs to prepare stable Ag NPs for biomedical and pharmaceutical applications.
In this paper we report, the synthesis of a novel generation 1 poly propyl ether imine (G1-PETIM) dendrimer derived 6-arm polyethylene glycol (PEG) SP (G1-PETIM-mPEG SP) and its application as a stabiliser for Ag NPs. The G1-PETIM-mPEG SP was characterised using Fourier-transform infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C) and X-ray diffraction (XRD) analysis. Silver nanoparticles (G1-PETIM-m-PEG SP@Ag NPs) were prepared via chemical reduction using the G1-PETIM-m-PEG SP as a stabiliser and their formation was verified using FT-IR, UV-vis spectroscopy, dynamic light scattering, transmission electron microscopy and XRD analysis. The G1-PETIM-m-PEG SP and G1-PETIM-m-PEG SP@Ag NPs were evaluated for their cytotoxicity against MCF-7, HeLa and Hep G2 cell lines using MTT assay. G1-PETIM-m-PEG SP@Ag NPs, silver nitrate, G1-PETIM-m-PEG SP and a physical mixture of the latter two were evaluated for antibacterial activity against *S. aureus*, MRSA, *E. coli* and *P. aeruginosa*. The synthesised G1-PETIM-m-PEG SP@Ag NPs were non-agglomerated, spherical and monodisperse as compared to Ag NPs prepared with m-PEG and without any stabilizer (Fig. 1a-c). The average particle size of G1-PETIM-m-PEG SP@Ag NPs was 36.44 ± 2.51nm, and were found to be non-cytotoxic even up to 100 µg/ml. The minimum inhibitory concentration values against *S. aureus* and MRSA (Gram-positive bacteria) were 18.5 and 74 µg/ml respectively, and against *E.coli* and *P. aeruginosa* (Gram-negative bacteria), the values were 9.25 and 74 µg/ml respectively. These low MIC values proved that nanoparticles retained their antibacterial potential upon stabilisation by the G1-PETIM-m-PEG SP. The results obtained in this study suggest that the synthesised G1-PETIM-m-PEG SP is an attractive biocompatible star polymer for the stabilisation of Ag NPs.

Keywords: dendrimer, polyethylene glycol, silver nanoparticles, cytotoxicity, antibacterial, *S. aureus*, MRSA.

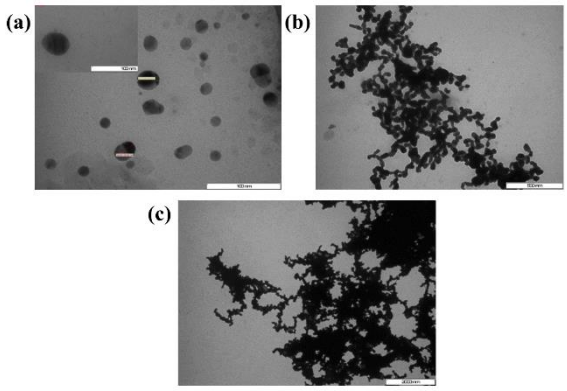

Figure 1: TEM images of: (a) G1-PETIM-m-PEG SP@Ag NPs; inset shows a single SP Ag NP, (b) m-PEG@Ag NPs and (c) plain Ag NPs.

References:

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Appendix D

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From: setcor@srv.setcor.org [mailto:setcor@srv.setcor.org] **On Behalf Of** Setcor Conferences and Events
Sent: 20 October 2015 06:50 PM
To: Rahul Kalhapure
Subject: Conference Abstract Confirmation

Dear Rahul Kalhapure,

We reviewed your research work abstract you submitted for the conference Nanotech France 2016 and we would like to confirm to you that your abstract is **Accepted for a poster**.

Sincerely yours