

Synthesis of new potent “composite” biocides with both *N*-chloramine and quaternary ammonium moieties and strategies of converting them into selective biocides

By

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ABSTRACT

Antibiotic resistance is one of many life-threatening problems for humans in the 21st century as mutations in microorganisms become increasingly difficult to treat. Scientists from all over the globe have been trying hard to find a solution for this issue and save people's lives. This study combines approaches from both organic chemistry and materials science in order to combat these issues. First part of the research includes finding a solution to the quench of active biocides in high protein media (HPM), in which we synthesized "composite" biocides by covalently bonding a long alkyl chain quaternary ammonium salt (QAC) with an amine-based *N*-chloramine. It was hypothesized that using a secondary amine-based *N*-chloramine can improve biocides stability in HPM; therefore, 2,2,6,6-tetramethyl-piperidinol was linked to dodecyltrimethylammonium chloride or tetradecyltrimethyl-ammonium chloride via a triazole bridge (5a or 5b). The monofunctional QACs 5a and 5b were then converted to corresponding "composite" biocides 6a and 6b using tertbutyl hypochlorite. The antibacterial challenge was performed against various Gram-negative and Gram-positive bacteria 5a, 5b, 6a, 6b, hydantoin-based composite biocides previously synthesized in our lab [3-(3-chloro-4,4-dimethyl-2,5-dioxo-imidazolidin-1-yl)-propyl]-dimethyl-tetradecyl-ammonium chloride, C18, and its unchlorinated precursor, C17). In this antibacterial assay, the compound with shorter alkyl chain (N-(2-(4-((1-chloro-2,2,6,6-tetramethylpiperidin-4-yloxy)methyl)-1H-1,2,3-triazol-1-yl)ethyl)-N,N-dimethyldodecan-1-ammonium chloride, 6a) showed superior antibacterial activity in both phosphate-buffered saline (PBS) and HPM through higher efficiency in inactivating Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*), and wild-type *P. aeruginosa* (PA01) than the commercially available biocide benzyldimethyltetradecylammonium chloride (BC).

In the second part of this research, we intended to develop a strategy to convert highly potent yet non-selective cytotoxic biocides into selective non-cytotoxic antibacterial agents. We achieved it by encapsulating non-selective biocides in biocompatible solid lipid nanoparticles (SLNs) conjugated with a MRSA-specific antibody (termed as "Ab"). A model biocide (C17) has been utilized for the proof-of-concept study. The C17-loaded Ab-conjugated (C17-SLN-Ab) SLNs demonstrated superior antimicrobial activity over the antibody-free (C17-SLN) and nonspecific antibody conjugated (C17-SLN-IgG) counterparts. To see the effect of the antibody on selective toxicity of SLNs, we developed an assay in which toxicity of SLNs towards fibroblast cells was determined in the presence of MRSA. In this assay, C17-SLN-Ab revealed more selective toxicity towards MRSA than fibroblast cells. C17-SLN-Ab also exhibited double selectivity with higher toxicity to MRSA than *P. aeruginosa*. The interaction between different SLNs was evaluated using TEM imaging in which SLN-Ab showed more tendency to bind to MRSA than *P. aeruginosa*. To confirm the versatility of this method, anti *E. coli* antibody and BC were used, and similar results were achieved.

Overall, we successfully designed a potent biocide with the capacity for use in HPM and PBS, which mitigates the issue of active biocides being quenched in high protein fluids. Also, a nanotechnology method was used to turn potent broad-spectrum non-selective biocides (C17 and BC) into selective ones.

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1 Chapter 1: Introduction

1.1 Antibiotic resistance concerns and global effects

Antibiotic resistance has been recognized as the most challenging healthcare issue in the 21st century [1]. Every year, around 2 million people in the United States (U.S.) are severely infected by antibiotic-resistant bacteria, and at least 23,000 people die as a direct result of antibiotic resistance [2]. This is even higher than the annual number of deaths by HIV/AIDS (6721 deaths in 2015) in the U.S. [3]. According to the Canadian Ministry of Health, in 2014 [4] there were over 18,000 reported infection cases in Canadian hospitals due to resistance to antibiotics [5]. Antibiotic resistance is not only a problem in Canada and the U.S. but all over the globe. Every year, over 700,000 people worldwide die because of drug-resistant bacteria [6]. In addition to death, antibiotic-resistant pathogens place a huge economic burden on healthcare systems, responsible for \$20 billion in annual healthcare costs in the U.S and €1.5 billion in the European Union in 2010 [2] and the use of primary clinical antibiotics has been restricted by the ever-growing antibiotic resistance [7]. World Health Assembly Member States agreed that it is crucial to have a global action plan against antibiotic resistance, which resulted in the formation of a comprehensive action plan in 2014 [8].

1.1.1 Definition of antibiotic resistance

Antibiotic resistance is the ability for bacteria to increase their tolerance to antibiotics and overcome the effect of antibacterial medicines [8]. Antibiotic resistance can be intrinsic (a biological characteristic of bacteria) or acquired.

Intrinsic resistance is the inherent ability of bacteria to resist against antibiotics. This natural insusceptibility can be caused by different factors such as outer membrane permeability and multi drug resistance (MDR) efflux pumps. The fluidity of cytoplasmic membranes affects

permeability. The more flexible a membrane is, the more permeable it is. Reductions in the fluidity of the cytoplasmic membrane and outer membrane cause changes in the structure of many membrane proteins and therefore impacts their performance. Also, some bacterial membranes have additional external structures which can prevent toxic materials by making a finer molecular sieve, letting only ions and nutrients enter the cell, and preventing antibiotics from penetrating the bacterial cell. For example, in the case of *Bacillus anthracis*, a poly- γ -D-glutamic acid (PGA) capsule helps bacterial self-defense against the actions of proteins in sera or phagocytes [9]. Also, in the case of Gram-positive bacteria, a thick peptidoglycan layer coupled with teichoic acid polymers and covalently-bound proteins adds additional improvements to self-preservation of the permeability of the bacterial membrane. However, the permeability threshold of Gram-positive bacteria is very broad (30-57 kDa) which makes them more susceptible to antibiotics than Gram-negative bacteria, which has an outer membrane with an unusual structure of polysaccharide units bonded with lipid A [10].

The efflux pump is the second innate pathway bacteria have for developing resistance to antimicrobial agents [11]. Almost all bacteria have efflux pumps and the encoding genes exist on chromosomes or plasmids. There are five different types of efflux pumps: the resistance-nodulation-division (RND) family, the major facilitator superfamily (MFS), the ATP (adenosine triphosphate)-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family [12]. These proteins can extrude antimicrobial agents from bacterial cells before the drugs can reach their targets [11] [12].

Acquired resistance is a random mutation in bacterial DNA which can be achieved from the accession of the gene by plasmids (conjugation or transformation), transposons (conjugation), integrons and bacteriophages, and bacterial cell gene mutation. These gene acquisitions and

mutations cause modifications in the bacteria which can lead to different kinds of antibiotic resistance mechanisms such as antibiotic inactivation (modification ability), target modification capability, and reduction of antibiotic accumulation within the cells (by increasing of efflux pumps expression and decreasing the outer membrane permeability), as well as bypassing antibiotic inhibition [7].

Antibiotic inactivation (or modification) is usually caused by enzymes such as β -lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases. These enzymes inactivate or modify antibiotics using different mechanisms such as hydrolysis, group transfer, and the redox process, all of which are chemical reactions which change the molecular structures of antibiotics in such a way that the antibiotics lose their antimicrobial effects. Antibiotic-target interaction and binding can be affected by small changes in the molecular structures of the targets of the antibiotics [13]. For example, in drug-resistant *S. aureus*, the main resistance pathway is Erm, a methyltransferase enzyme which decreases the affinity of erythromycin for RNA by changing the peptidyl transferase loop of the 23S RNA component of the ribosome.

Vancomycin-resistant *Enterococci* (VRE) has used a target reprogramming strategy to develop resistance against vancomycin. In this process, new enzymes (which are encoded by the *vanHAX* genes) reduce pyruvate to D-lactate (*vanH*), add D-alanine and D-lactate together to produce D-Ala-D-Lac (*vanA*), and then hydrolyze the normal metabolite D-Ala-D-Ala while sparing D-Ala-D-Lac (*vanX*), which reduces the binding efficacy of vancomycin by 1,000-fold [7].

Although bacterial resistance to antibiotics is one of the biggest threats to the public health, antibiotics are not the only source of resistance; it has been shown that biocides in the environment can lead to bacterial cross-resistance to antibiotics.

1.1.2 Definition of cross-resistance

Cross-resistance is the toleration of toxic material by microorganisms as a result of subjection to another similar substance [14], [15].

Various biocides have been studied for induced cross-resistance to antibiotics, in particular: triclosan, chlorhexidine, aminoglycosides, ethidium bromide and benzalkonium chloride [16], [17]. Unlike antibiotics, which attack specific targets on bacteria, biocides attack one or several non-specific sites which exist in the majority of bacterial cells resulting in bacterial death. There are several similar mechanisms that bacteria use to resist antibiotics and biocides. These mechanisms mostly decrease the amount of drug accumulated within the cells, change the cell wall or membrane permeability, modify the target, and pump the attacking drug out from the cell using efflux pumps. Among all the mentioned mechanisms, the efflux mechanism is the common cause of cross-resistance to antibiotics [18]. Braoudaki et al [16], examined the cross-resistance of bacteria to a wide range of antibiotics, such as amoxicillin, amoxicillin-clavulanic acid, chloramphenicol, erythromycin, ciprofloxacin, clindamycin, colistin sulfate, gentamicin, imipenem, rifampin, tetracycline, trimethoprim, fusidic acid and vancomycin, caused by different biocides such as benzalkonium chloride, chlorhexidine, and triclosan in *Salmonella serovar Enteritidis*, *Salmonella serovar Typhimurium*, *Salmonella serovar Virchow*, and *E. coli* O157. Braoudaki et al demonstrated that cross-resistance took place in the majority of cases (except for *Salmonella serovar Enteritidis* where cross-resistance happened only between erythromycin and chloramphenicol). Cross-resistance between biocides and antibiotics in *P. aeruginosa* has been reported by different authors [14], [19], [20].

1.2 Strategies to combat antibiotic resistance and cross-resistance

There are different strategies suggested by the WHO (the World Health Organization) to control and reduce the pace of antibiotic resistance, including the cautious use of antibiotics in animal care or breeding, household applications, as well as the more well-informed use of antibiotics in healthcare. These strategies are recommended to different groups of society (individuals, policy makers, health professionals, the healthcare industry and the agriculture sector) based upon their roles and interactions with antibiotics. For example, individuals have been asked not to take non-prescription antibiotics and follow their health workers' advice [21].

1.2.1 Antibiotics: discovery, classification, and potentiation

While positive changes have been created by the cautious use of existing antibiotics in society, material scientists and chemists are working hard to develop new antibiotics, find new methods to potentiate current antibiotics, find different alternative to antibiotics, as well as synthesize new and potent biocides to disinfect different environments, surfaces, and topical wounds.

1.2.1.1 History of discovery and resistance development of the antibiotics

Bacterial infection was one of the deadliest diseases of the early 20th century [22]. Having a simple injury or a small cut could cause death, and bacteria constantly endangered human life. 1928 marked the end of the reign of bacteria and the beginning of the modern era of antibiotics. While Penicillin was discovered by Fleming at Saint Mary's Hospital London accidentally, the prescription of antibiotics for treatment of serious infections didn't start until the 1940s. Since then, antibiotics have saved many people's lives [23].

The glorious era of antibiotics was the 1940-1960s when many potent novel antibiotics were utilized in the war against bacteria [24]. Despite the synthesis of different antibiotics following the discovery of penicillin, bacteria began to show resistance to new antibiotics after their introduction into the market (**Figure 1-1**) [25]. Because bacteria and other pathogens possess the capability of rapid evolution, within only a couple of decades, microbes once again gained the upper hand with antibiotic resistance, which was exacerbated by a dramatic reduction in the number of newly discovered antibiotics [26]–[29]. Subsequently, a push for new solutions led to numerous studies on the discovery of new antibiotics as well as the potentiation of existing antibiotics. Because of the high rate at which bacteria now develops resistance to new antibiotics, investigations into new antibiotics is no longer been profitable, which has resulted in a much slower pace of drug development. In addition, improving existing antibiotics does not prevent existing genetic antibiotic resistance pathways. Therefore, the treatment of infections continues to prove difficult and continues to have a high cost [22], [30]. On the other hand, the number of isolates of antibiotic-resistant pathogens is increasing; for instance, based on the report issued in 2004 [31], the increase in the rate of antibiotic-resistance isolated in ICU between 1998-2002 was 12%, 11%, 47%, 15% and 20% for vancomycin-resistant *Enterococci*, MRSA, Cephalosporin (3rd generation) – resistant *K. pneumonia*, imipenem-resistant *P. aeruginosa* and cephalosporin (3rd generation) – resistant *P. aeruginosa*, respectively.

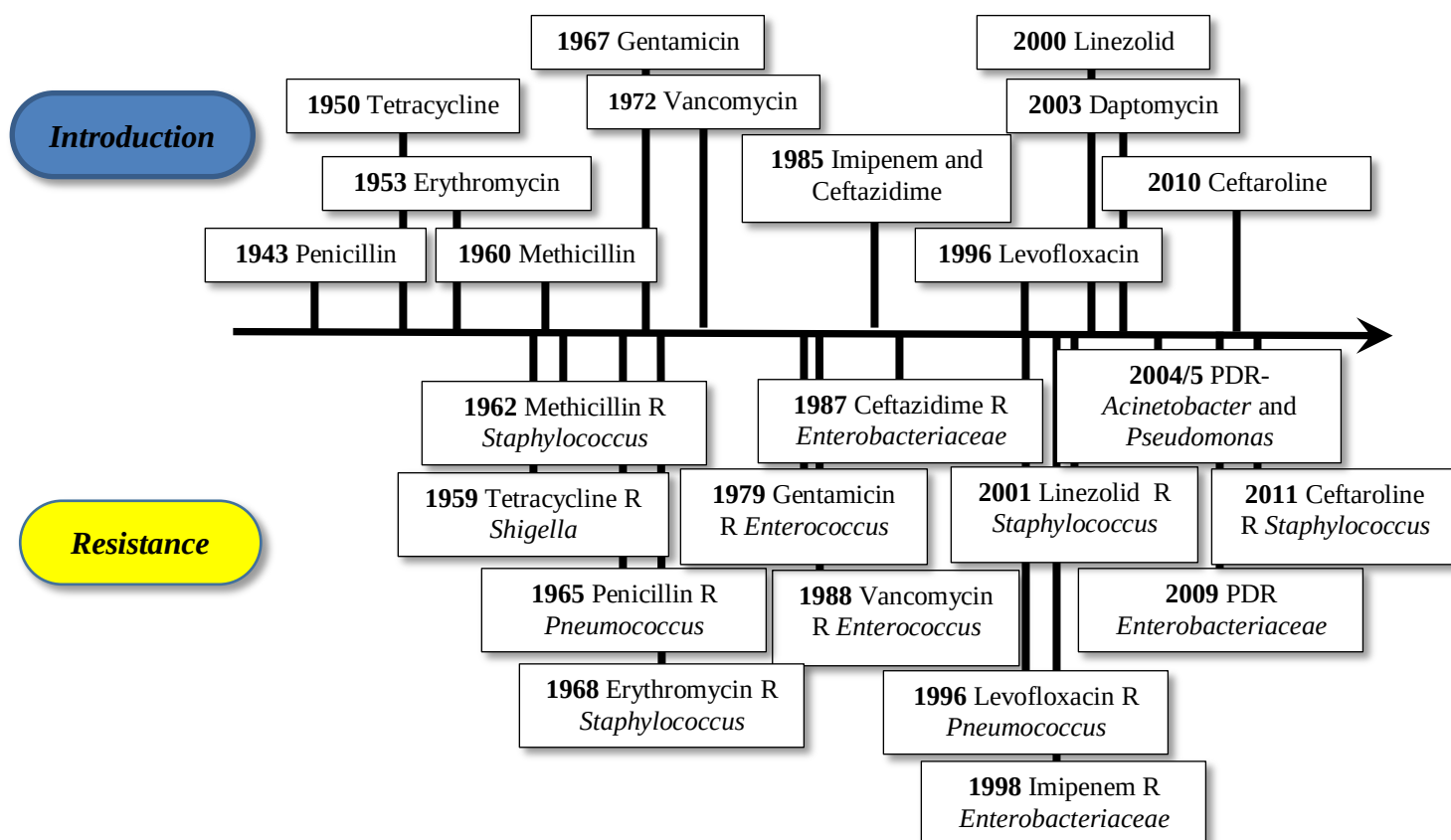


Figure 1-1. Antibiotics discovery and resistance. R: Resistant, PDR: Pan-drug resistant

There is a pressing need to develop new antibiotics as well as potentiate the existing antibiotics against resistant bacterial strains. Before touching upon these two strategies against antibiotic resistance, it is vital to discuss the modes of action of existing antibiotics and their corresponding mechanisms of resistance.

1.2.1.2 Different classes of antibiotics (via their modes of action and resistance mechanisms)

Bacterial mechanisms of resistance to antibiotics are controlled by the molecular structure and modes of action of the specific antibiotic. There are six main classes of antibiotics namely, β -Lactams, Macrolides, Fluoroquinolones, Tetracyclines, Sulfonamides, and Aminoglycosides. These different classes of antibiotics combat bacteria through different mechanisms [32].

β -Lactam antibiotics (including penicillin) were the first to be discovered in the 1930s and are used extensively as antibacterial drugs, contributing to 65% of the total world market of antibiotics (\$15 billion USD in 2003) [32]. The main characteristic of these drugs is the presence of the β -lactam ring in their chemical structures, which results in a variety of β -lactam antibiotics depending on substitutions of the β -lactam core [33]. This class of antibiotics inhibits the biosynthesis of the peptidoglycan layer of bacterial cell walls by binding to the DD-transpeptidases which are vital to that biosynthesis, thus compromising the integrity of the cell wall. β -Lactam antibiotics are especially effective in Gram-positive bacteria [33].

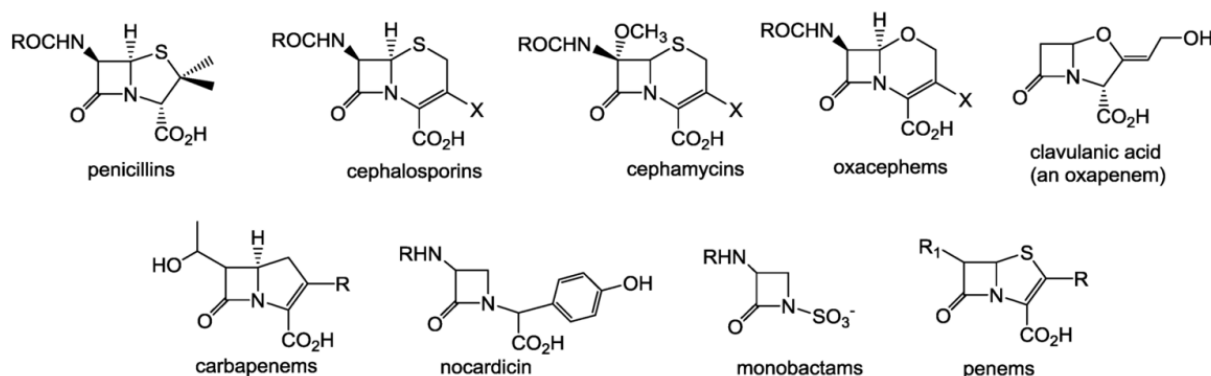


Figure 1-2. Various β -lactam ring substitutions

The main resistance mechanism to β -lactam antibiotics is β -lactamase production, which is common in Gram-negative bacteria. These hydrolytic enzymes destroy the amide bond in the lactam ring interfering with the antimicrobial activity of these antibiotics [34].

Macrolides are natural antibiotics (polyketides) possessing a large macrocyclic lactone ring with one or two deoxy sugars. They not only are antibiotics but also have antifungal properties as well. This class of protein synthesis inhibitors prevents peptidyl

transferase from adding the tRNA-attached growing peptide to the next amino acid during ribosomal translation, and it also inhibits early stage dissociation of the peptidyl-tRNA from the ribosome [35]. Resistance to this type of antibiotic takes place through different pathways, including enzymatic detoxification (of the antibiotic), target modification, and removal through efflux pumps [36].

Fluoroquinolones are a subcategory of quinolones, which are broad-spectrum antibiotics. These natural antibiotics use selective inhibition of the topoisomerase II ligase domain to prevent bacterial DNA from unwinding and duplicating, thereby hindering bacteria reproduction because the two nuclease domains remain intact [35]. The most common pathway to develop resistance against fluoroquinolones is the mutation of primary and secondary targets, type II topoisomerases, which in turn causes alterations in the structure of the target proteins. This change affects the affinity of the drug to its target. There are also other resistance mechanisms to fluoroquinolones such as changing the bacterial cell membrane permeability and effluxing the antibiotics out of the cells [37].

Tetracyclines have played a crucial role in decreasing the death rate from cholera and are commonly utilized to treat acne and rosacea. Similar to macrolides, these broad-spectrum antibiotics are a subclass of polyketides and are produced by the *Actinobacterial* genus *Streptomyces*. Tetracyclines inhibit protein synthesis by interfering with the attachment of charged aminoacyl-tRNA to the A site of the ribosome. Tetracyclines bind to the ribosomal 30S subunit and inhibit the addition of new amino acids to the nascent peptide chain [38]. There are three different strategies used by bacteria to resist the effects of tetracyclines: (i) preventing the tetracyclines from reaching their targets by changing the cell membrane permeability and effluxing them out, (ii) protection of the ribosome by associating a cytoplasmic protein (a 68-kDa protein on sodium dodecyl sulfate polyacrylamide gel

electrophoresis) with the ribosome to make it insensitive to tetracyclines, and (iii) tetracycline inactivation via a 44-kDa cytoplasmic protein which reacts with tetracycline [39].

Aminoglycosides show antimicrobial activity against most gram-negative aerobic and facultative anaerobic *Bacilli*. They are commonly used to treat *Pseudomonas aeruginosa* infections. These amino-modified glycosides inhibit protein synthesis by disturbing peptide addition at the 30S subunit of the microorganism's ribosome in the cytosol [11], [40]. Resistance to aminoglycosides usually happens through two different pathways: (i) Enzymatic modification of aminoglycosides (which causes a covalent change in amino or hydroxyl groups and therefore changes the binding affinity of the drug to its targets) or (ii) drug uptake reduction (by changing the membrane permeability and active effluxing) [41].

1.2.1.3 Synthesis of new antibiotics

One of the most direct and important approaches toward antibiotic resistance is to synthesize new antibiotics against which bacterial resistance is less likely to develop.

1.2.1.3.1 Progress and challenges of antibiotic discovery

Synthesis of new antibiotics is one of the effective strategies for defeating antibiotic resistance. The different approaches to new antibiotic discovery are: (i) screening natural products and find new lead compounds from which to synthesize new antibiotics, (ii) utilizing non-natural chemical routes like combinatorial chemistry, (iii) Genomics, which can be used to select potential antibacterial targets, (iv) extracting natural products from nonculturable bacteria, and (v) targeting non-multiplying bacteria [42].

Penicillin is one of the antibiotics which is a natural compound and has provided lead structures, such as 6-amino penicillanic acid, for the discovery of new drugs like amoxicillin.

Zhanel et al [43], showed successful attempts for using this strategy to develop new antibiotics. Non-natural compounds also can be used to develop new antibiotics such as Prontosil (the precursor to sulpha drugs), metronidazole, isoniazid, and oxazolidinones [42].

Another approach for pharmaceutical chemists is to review old antibiotics which, at the time of their discovery, could not find their way onto the market because they either did not meet the required potency or were not broad spectrum enough [44].

Finding potential targets in bacteria for antibiotic discovery is another approach which researchers have been working on for many years. This approach has resulted in the identification of a large number of leads resulting in the discovery of highly potent enzyme inhibitors. However, a lot of these enzyme inhibitors have not shown an effective antimicrobial activity [44].

Antibiotics can be produced by bacteria to inhibit or kill their bacterial opponents in the environment. There are several commercially available antibiotics such as streptomycin which were extracted from artificially cultured bacteria. However, most bacteria found in the environment are non-culturable in the laboratory. Such a huge population of (non-culturable) bacteria can provide a large pool of antibiotics which have not yet been discovered. There are some researchers who have shown that it is now possible to clone non-culturable bacteria genomes and then express them by recombinant DNA technology [42].

On the other hand, it has been proven that 60% of bacteria contributing to infections are non-multiplying bacteria which are hard to kill. It has been observed that if the level of antibiotic in a person's blood is below the effective concentration, these non-multiplying bacteria will start multiplying to cause infection. Therefore, an additional dose of antibiotics is required to remove the multiplying bacteria emerging from the non-multiplying bacteria. Consequently,

it is necessary to find antibiotics which are effective against non-multiplying bacteria. One advantage of this approach is that the treatment time could be reduced [42].

There are different drawbacks for new antibiotics discovery which have led to the failure of this approach. One of the most important reasons is that in the past few years the level of new antibiotics discovered is lower than the rate of antibiotic resistance developed by pathogens [45]. Also, FDA approval for newly discovered antibiotics has become costly for pharmaceutical companies, which makes them reluctant to invest in it [46]. Therefore, more innovative, less expensive and simpler methods, such as potentiation of existing antibiotics and adoption of antibiotic alternatives, are now being considered.

1.2.1.4 Ways to potentiate existing antibiotics

Potentiation of existing antibiotics has been used by medicinal chemists to tackle antibiotic resistance. Several methods such as combination therapy and encapsulation have been utilized to potentiate antibiotics.

1.2.1.4.1 Combination Therapy

Combination therapy is a strategy used by clinicians to treat difficult-to-treat infections. The very basic method is a combination of different antibiotics to treat MDR pathogens.

However, there is other compounds which can be utilized as adjuvants responsible for: (i) signal integration and processing, (ii) regulation of virulence and resistance gene expression, (iii) activation of effector molecules [47],[48].

Microorganisms gather information and signals from their surrounding environment using systems which include two main components: a sensor kinase and a response regulator.

Signals microbes respond to can include pH and temperature changes as well as the existence

of nutrition or antibiotics in the environment. After signal integration, microbes respond to these signals in different ways. The combination of antibiotics and the inhibitors of two-component signal integration systems has been used in different studies as an effective method for combination therapy. For example, the inhibitor of WalR, an essential component in microbial signal integration systems that are directly involved in metabolism, was used by Gotoh et al against MRSA [49]. Other non-essential components have been used by different scientists such as QseC/QseB [50], PhoQ/PhoP [51], and GacS/GacA [52].

It has been observed that hydroxy benzimidazoles inhibit some proteins which are responsible for the control of virulence and resistance gene expression, such as XylS/AraC in Gram-negative pathogens [53]. There are other recent studies which focused on compounds that inhibit the activity of EthR, a transcriptional repressor in *Mycobacterium tuberculosis* (Mtb) [54].

The combination of inhibitors for effector molecules, such as effector enzymes and efflux pumps, is another strategy used extensively in combination therapy. The β -lactamases inhibitor is one of the effector inhibitors which have been used to potentiate β -lactam antibiotics [55].

There are various ways to inhibit efflux pumps such as (i) interrupting the efflux gene expression, (ii) changing the molecular structure of the drug by adding functional groups to diminish the recognition, (iii) channeling protein assembly interference, (iv) utilizing compounds which can compete with the drug for efflux, (v) blockage of the efflux channel, or (vi) interfering with the efflux energy transfer [56]. The combination of an efflux inhibitor with an antibiotic has been used in different studies [57]. A dipeptide amide compound, MC-207,110, (phenylalanine-arginyl b-naphthylamide), was one of the first efflux inhibitors to inhibit MexAB/OprM-mediated efflux of quinolones in *P. aeruginosa* [58]. MC-207,110

effectively decreased the MIC value of levofloxacin against *P. aeruginosa* by 64 fold and also diminished *P. aeruginosa* resistance to levofloxacin.

Although there are a lot of studies indicating the benefits of antibiotic combination therapy, the therapy has its own disadvantages and side effects. Irrational use of combination therapy can diminish its effect and worsen the antibiotic resistance situation, which can, in turn, result in superinfection [59]. Antagonism, side effects, drug-drug interaction, and cost are other disadvantages of combination therapy [60].

1.2.1.4.2 Encapsulation

Encapsulation of antibiotics into nano-carriers (or nano-antibiotics for short) represents a promising formulation approach to overcome the mucus barrier and prolonging the antibiotic retention time in the body. It has been shown that antibiotic encapsulation can improve the antibacterial activity of certain antibiotics up to 1000 times [61]. However, the loading efficacy of existing nano-carriers is low, and a large portion of antibiotics end up being wasted. This problem has not stopped researchers from using nano-antibiotics, and they have tried to solve the loading efficacy problem by using more efficient methods such as the core-shell method in which nano-sized antibiotics make the core while the shell consists of a biodegradable polymer[62]. Moreover, other nano-carriers such as liposomes, SLNs, and polymeric nanoparticles are still under investigation [63].

Liposomes are one of the nano-carriers utilized extensively to generate nano-antibiotics [63]. Liposomal antibiotics are smaller than 500 nm. This small size helps them to overcome the mucus barrier and facilitates their penetration through mucous pores [61], [64]. Fattal and his coworkers [65] encapsulated the ampicillin in liposomes and observed an improvement in antimicrobial activity of encapsulated ampicillin in comparison to the free antibiotic. They

observed a 20-fold increase in activity and the lifespan of mice treated with a nano-antibiotic rose from 10 to 20 days in comparison to the mice treated with the free antibiotic. Desiderio et al. [66] used liposome encapsulated cephalothin to act against *S. typhimurium* in mice and observed that the bacterial viability dropped from 10^6 to 10^2 CFU/mL in 7 days, which showed that the antimicrobial activity of liposome entrapped cephalothin is higher than free antibiotics. Ampicillin encapsulated liposomes were utilized to treat mice infected with *L. monocytogenes* and a 90 fold increase in antibiotic efficiency was observed [67]. Desiderio et al claimed that the improved antimicrobial activity was due to better delivery to the infection sites which were liver and spleen.

Liposomal polymyxin B is one of the successful examples of liposome-encapsulated antibiotics. These nano-antibiotics were used to treat the *P. aeruginosa* infections. Polymyxin B is cytotoxic, and its use is restricted due to its toxic effect. The nephrotoxicity and ototoxicity of polymyxin B have been dramatically decreased when it is encapsulated in liposomes. This liposomal antibiotic acts against the *P. aeruginosa* by membrane infusion and delivers the drug into the bacterial cells [68]. There are many other liposomal antibiotic drugs to inactivate different bacteria [69]–[72]. For example, liposomal benzyl penicillin was utilized against the penicillin-sensitive strain of *Staphylococcus aureus* and showed higher efficiency as well as less time of exposure in comparison to the free drug [72]. Also, ciprofloxacin-loaded liposomes showed a very good activity and inhibition of *Salmonella Dublin* in mouse spleens [71].

Biodegradable polymers have been used in large scale drug delivery applications. The worldwide market of polymer-encapsulated drugs is over USD 60 billion [73]. These nanocarriers were primarily used to encapsulate drugs with poor water solubility. Due to the hydrophobic nature of the core of polymeric nanoparticles, they are also currently utilized in

antibiotic drug encapsulation to enhance the antimicrobial properties of drugs [63]. Espuelas et al. [74] encapsulated amphotericin B in poly(ϵ -caprolactone) nanospheres with a non-ionic surfactant coating and observed greater antimicrobial activity towards *Leishmania Donovan*. Ampicillin was encapsulated in poly(iso hexyl cyanoacrylate) nanoparticles and used against *Listeria monocytogenesis* mouse peritoneal macrophages [75]. There are other examples of antibiotic-loaded polymeric nanoparticles which all showed an enhancement of antimicrobial activity in comparison to free drugs [76]–[80].

Solid lipid nanoparticles (SLNs) are another class of nanocarriers used for the encapsulation of antimicrobial drugs. SLNs can be utilized as a topical cream applied to the skin or administered orally in the form of tablets, pellets, and capsules [81]. SLNs can help to bypass efflux pumps and increase the efficacy of antibiotics. For example, tobramycin-loaded SLNs enhanced the antimicrobial activity of tobramycin by diminishing the effect of the ATP-dependant efflux pump responsible for the poor absorption rate of the free drug [82]. SLNs also can facilitate the delivery of drugs to the lung and lymphatic systems through pulmonary delivery [83], [84]. There are several other cases of using SLNs to improve antimicrobial activity of certain drugs [85], [86]. Although the potentiation of existing antibiotics is a proven method of increasing the efficacy of existing antibiotics and slowing down resistance development, it does not intrinsically bypass all the genetic resistances. Bacterial resistance can still develop through overexpression of target enzymes, alteration of binding sites, and enzymatic degradation of encapsulated antibiotics.

1.2.2 Alternative to antibiotics

Alternatives to antibiotics are innovative methods in which natural compounds and pathways are used to tackle antibiotics resistance.

1.2.2.1 Antibacterial vaccines

Vaccines are one of the innovative approaches to defeat antibiotic resistance. They have a high efficacy and 90% of target population coverage [87]. Vaccine development against bacteria seems to be a promising strategy to defeat antibiotic resistance and there are no reports of vaccine-resistant bacteria [88].

The oldest vaccines, which were discovered in the last century, are directed to toxins such as the vaccines used for tetanus and diphtheria infections. Vaccines which currently can be found in the market have pre-existing antibodies in the serum. These vaccines do not prevent infections but instead prevent the diseases caused by bacteria. Disease prevention usually happens under different mechanisms, for example, toxin neutralization in the case of tetanus and diphtheria, and phagocytes activation and bacterial destruction in case of *Pneumococci*, *Meningococci* and *Hib* [87]. In general, vaccines can provide immunity to bacteria or toxins by imitating the infection.

Vaccines do not kill bacteria directly but facilitate the production of T-lymphocytes and antibodies. Therefore, it is less likely for bacteria to develop resistance against vaccines. However, vaccines can cause allergic responses in patients and they are not effective when the patient is already infected with bacteria. In addition, their spectrum is narrow and limited to the specific pathogens for which they were designed [88].

1.2.2.2 Phage therapy

Bacteriophages or phages are viruses that kill bacteria by occupying the bacterial cells and interrupting the bacterial metabolism. Phages were first discovered in 1896 and studies on phage therapy started in 1919 [89].

Phages have shown higher antibacterial activity than antibiotics in human and animal infections [90], [91]. These phages can get into the target bacterial cell and destroy the cell by replicating and lysing. Also, it has been observed that different phages show different duplication cycles. Bacteriophage therapy has been used for many years in Eastern Europe and administrated to humans in different ways, such as orally, rectally, locally and intravenously [89].

There are different advantages and disadvantages for bacteriophage therapy. Phages can be antibacterial, their population increases during the process, they are nontoxic, the chance of resistance and antibiotic cross-resistance is low, and the discovery of them is fast. Also, there have not been any reported side effect caused by phage therapy [89]. On the other hand, all phages are not therapeutic and phages have both a specific host and a narrow spectrum [92].

1.2.2.3 Immunostimulants

Immunostimulants are materials (drugs or nutrients) which improve the immune system in a non-specific manner and result in the enhancement of the host's resistance to diseases [93]. Immunostimulants are usually classified as biological response modifiers. These compounds are generally cytokines or soluble chemicals which cause reinforcement in the natural resistances of the body. Immunostimulants help patients to cope with different viral or bacterial infections by allowing communication between different populations of cells [94] or the stimulation of different factors of the immune system such as the phagocytosis, properdin and complement systems, protective secretory IgA antibodies, α - and γ - interferon release, T- and B- lymphocytes, cytokines, and pulmonary surfactants [95].

Nonspecific immunotherapy and immunoprophylaxis are promising approaches to modulate immune responses in a beneficial way since the discovery of new specific vaccines or treatments for various infections are a time-consuming process [95].

1.2.2.4 Anti-virulence therapies

Anti-virulence therapies are techniques that disarm the bacteria, preventing it from causing any damage to the host organisms. These therapies disable the bacteria without killing it [96], [97]. There are different virulence factors used by bacteria to cause diseases such as adhesins, toxins, and specialized secretion systems to deliver effectors. Anti-virulence therapy is generally based on the inhibition of these virulence factors [96].

The major advantage of this method is the lower survival evolutionary pressure on the bacteria to develop resistance because most virulence factors are not crucial for bacterial survival, for example, toxins and adhesins, and removing them does not kill the bacteria [26]. Also, these virulence factors are specific to pathogens and the anti-virulence therapies do not cause any toxicity to mammalian cells. However, inhibitors can interfere with the similar pathways in normal microbiota. Because of the lack of large-scale clinical trials, it is hard to evaluate the potential side effects of this therapy.

There are two approaches for antivirulence therapies, namely, broad-spectrum and narrow-spectrum therapies [27]. Some of the virulence factors seem to be common in different pathogens, therefore, anti-virulence drugs that target these elements show broader spectrum activity than others. However, a large number of these drugs could be specific to one pathogen and its unique virulence factors [96].

1.2.2.5 Probiotics

Probiotics are unharmed and non-pathogenic microorganisms which provide health benefits for the host. Probiotic therapy was first used to treat diarrhea and rotavirus shedding in toddlers in 1917 [98].

Probiotics show activity against pathogens by generating antimicrobial agents such as bacteriocins and organic acids. There are some reported side effects such as animal poisoning, allergies, and diarrhea. However, probiotics are usually safe microorganisms.

There are different challenges to making probiotics practical such as the limited number of safe bacteria, the possibility of inactivation of probiotics in different preparation steps, the vulnerability of probiotics under acidic pH, and the difficulty of maintaining a sufficient amount of viable microorganism in the intestine [93].

1.2.2.6 Therapeutic antibodies

Serum therapy began to be used to treat the infectious diseases in the beginning of the 20 century and showed great success at treating infections caused by *Pneumococcal pneumonia*, *Meningococcal meningitis*, *Erysipelas*, and *Anthrax* [99].

Antibodies can act through various mechanisms such as blocking the antigen receptor in the effector cells [100], inducing bacterial agglutination [101] and neutralizing bacterial toxins [102]. Antibodies are highly selective to a specific pathogen and to a limited number of patients. They also do not kill the bacteria directly. It is, therefore, less likely for bacteria to develop resistance against them [103].

There are different advantages to the use of therapeutic antibodies such as ease of use, low toxicity, and no damage to the microflora. On the other hand, these antibodies have some

drawbacks: high cost, the need for a specific diagnosis, and difficulties with industrial production [99].

1.2.3 Synthesis of potent biocides

Antibiotics and their alternatives are not the only tools in the combat against resistant bacteria; biocides are another promising weapon in this war.

1.2.3.1 Definition and history of biocides

Biocides, including disinfectants, antiseptics, and preservatives, are chemical substances that destroy, inhibit, or control the harmful effect of a wide range of microorganisms [104]. The first use of antibacterial agents in water treatment was the preservation of boiled water in silver and copper containers in Persian Empire in 450 BC. The first medical application for a biocide was in the Middle Ages when Arab physicians used mercuric chloride in wound dressings. Use of bleaching powder and iodine as a disinfectant, chlorinated water for hand washing in hospitals and phenol as wound disinfectant started in the 1830s, 1840s, and 1860s, respectively. The evaluation of the antibacterial efficacy of some biocides happened after the mid-nineteenth century. The minimal inhibitory concentration (MIC) of phenol, creosote, salicylic acid, and benzoic acid were determined by Bucholtz in 1875. In 1893, Traugott introduced hydrogen peroxide as a disinfectant. Chlorine-releasing compounds were utilized as biocides for the first time in 1915 by Dakin [105].

Nowadays, there are different applications for biocides such as water treatment, hospital and household disinfection, food preservation, and crop protection. These applications can be used to categorize various biocides. Biocides can also be grouped based on their mode of action and their target region of microorganisms such as membrane active biocides, cell wall inhibitors, cytoplasm active biocides (which attack targets in the cytoplasm and interfere with

their metabolism), and genotoxic biocides (which react with DNA and affect the biosynthesis of DNA). Chemical reactivity of biocides, such as oxidizing, non-oxidizing, and electrophilicity, is another biocide categorization strategy [105].

One of the most important problems with biocides is that all biocides are toxic and many of them are corrosive. It is, therefore, necessary to consider international regulatory procedures for biocides including biocide toxicity and ecotoxicity data [105].

1.2.3.2 Resistance to biocides

In a similar method to the way they resist antibiotics, bacteria can intrinsically resist against biocides and the level of this intrinsic insusceptibility differs from one bacterial strain to another. Bacterial mechanisms for intrinsic resistance to biocides are the same as bacterial methods for intrinsic resistance to antibiotics, in particular, the permeability of the cell wall and the membrane, and the efflux pump. However, intrinsic resistance to biocides is not the only bacterial resistance mechanism and bacteria can also acquire resistance genes through mutation, natural selection, or from other bacteria in the division state [106]. These resistance acquisitions happen in different ways such as inactivating or modifying biocides, decreasing the concentration of biocides within bacterial cells using efflux systems, generating of new specific targets, and modifying targets using enzymatic pathways [105].

1.2.3.3 Various broad-spectrum biocides

As biocides are one of our best options to fight bacteria in the post-antibiotic era, the bacterial resistance mechanisms to biocides must be fully understood to guarantee that the misuse of these compounds will not happen. The bacterial mechanisms for developing resistance to different biocides vary depending upon the chemical structure of the biocides and their modes of action [107].

1.2.3.3.1 *N*-Chloramine

N-chloramines are the most promising biocidal agents. There is no documented bacterial resistance against them [25]. *N*-chloramines are compounds containing one or more chlorine atoms attached to nitrogen covalently. The antimicrobial properties of *N*-chloramines are caused by the oxidation state $+δ$ of chlorine (Cl^+) atoms. The existence of $α$ -hydrogen governs the stability of *N*-chloramines, where the $α$ -hydrogen can facilitate dehydrogenation due to heat or UV exposure [108]. In addition, the stability of chlorine on nitrogen in *N*-chloramines is different in aqueous solutions and biological fluids based on their dissociation constants because the chlorine can get reduced or transfer to other amines in high protein media and loses its activity. The stability of *N*-chloramines has the following order: amine>amide>imide based *N*-chloramine (**Figure 1-3**). On the other hand, the unstable chlorine makes *N*-chloramines better antimicrobial agents. Therefore the antimicrobial activity of different *N*-chloramines has an inverse trend of imide > amide >> amine [109].

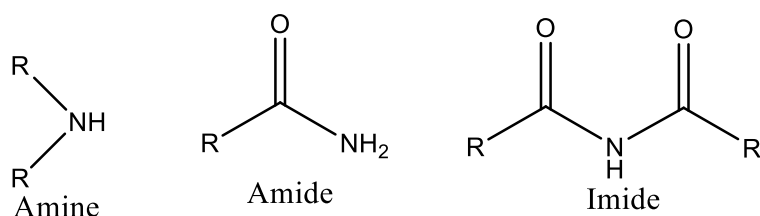


Figure 1-3. General molecular structure of amine, amide, and imide

The antimicrobial behavior of *N*-chloramines occurs through different pathways: (i) formation of chlorine cover and denaturation of bacterial cell wall proteins, (ii) penetration into a bacterial cell and (iii) destruction of the intracellular vital component. *N*-chloramines attack various targets in living cells, particularly sulfur-containing amino acids such as cysteine and methionine, interfere with hydrogen bonding in proteins and affect protein functions governed by their structures [110], [111].

N-chloramines are very strong antimicrobial agents which are toxic to both bacteria and human cells. These compounds cause tissue irritation, pain and prolong the wound healing process [112]. In addition to the antimicrobial effect of *N*-chloramines, they show some non-microbicidal effects such as destruction of toxins, anticoagulant function, and anti-inflammatory activity [113].

Selk et al. [114] evaluated the antibacterial effect of different mild *N*-chloramines as well as their skin irritation. The mild *N*-chloramines reached the sterilization stage (total kill) after 2.5 min with the concentration of 10 ppm. It was further demonstrated that introducing carboxylic groups to the *N*-chloramine molecules increased their antibacterial efficacy by 5 to 50 fold in comparison with chlorhexidine, while skin irritation decreased. Selk et al. showed that these new compounds were more effective in preventing the expansion of skin microflora under occlusion than chlorhexidine by 10 to 100 fold. This research showed that *N*-chloramines are toxic to cells which make it hard to use *in vivo* applications. However, if this toxicity can somehow be reduced, *N*-chloramines would be a good alternative to antibiotics for wound disinfection.

Taurine chloramine is one of the most famous natural *N*-chloramines existing in the human immune system and has been used against photogenes for many years [113]. Stimulated human granulocytes and monocytes produce *N*-chlorotaurine which has shown antibacterial, antifungal, and antiviral activities [115].

Although *N*-chloramines were introduced about a hundred years ago as a disinfectant, there is, as yet, no documented resistance against them because of their non-specific interaction with biological targets [110].

1.2.3.3.2 Quaternary ammonium compounds

The general molecular structure of QACs consists of a quaternary amine attached to four different organic groups, which can be hydrogen, a plain alkyl group or other functional groups substituted for the alkyl group. Since the nitrogen is positively charged, there is an anion attached to the positively charged nitrogen in this compound [116]. Scientists found the antimicrobial effect of long chain QACs, with at least one substituting alkyl chain consisting of 8-18 carbons, in early 1900, when it was used as a hand sanitizer in surgeries. [117]

The use of QACs as an antimicrobial agent has dramatically increased in last few decades [118]. There are many different applications for QACs, including surface disinfection, water treatment, antifungal treatment, hygiene products and wound disinfection. [116] Various QACs are available on the market now such as Benzalkonium chloride, Cetrimonium Chloride/Bromide (cetrimide), cetylpyridinium chloride, alkyl amino alkyl glycines and didecyl dimethyl ammonium chloride (DDAC).

Benzalkonium chloride is one of the most common QACs. In reality, a mixture of alkyl-benzyl-dimethyl ammonium chloride with different alkyl chain length, 8-18 carbons, is typical. However, as a pharmaceutical product, benzalkonium chloride is formulated using chain length in the range of 12 to 14 carbons. [117]

The antimicrobial efficacy of QACs is governed by the lipophilicity of the molecule which is, in turn, determined by the N-alkyl chain length. When the chain length is 12-14 carbons, the QAC is more effective against Gram-positive bacteria and yeast. Increasing the length of at least one of the substituting alkyl chains to 14-16 makes it a good antimicrobial agent for Gram-negative bacteria. QACs are completely inactive when the chain length is below 4 or above 18. [118]

The attraction between the positively charged nitrogen on QACs and negatively charged head groups of membrane phospholipids initiates the antimicrobial action of QACs. As soon as this ionic bond forms, the long alkyl chain penetrates and integrates into the membrane core [116]. When the concentration of QAC is high, bacterial membrane desolation accrues due to the formation and aggregation of micelles [118]. QACs can also disrupt and denature the structure of proteins and enzymes. [116]

Different QAC resistance mechanisms include resistance by reduced expression of porins, mobile genetic elements, and overexpression of efflux pumps [119]. Tabata et al. [120] showed a correlation between the resistance to QACs and the expression of outer membrane protein (porin) and OprR in *P. aeruginosa*. It has also been observed that the existence of benzalkonium chloride in subinhibitory concentrations reduces the level of outer membrane proteins [121], [122]. In addition, bacterial strains with an acquired genetic unit, such as a plasmid, transposon or integrin, demonstrated higher minimal inhibitory concentration for QACs [116].

QACs are broad spectrum antibacterial agents with MIC range between 10-500 ppm against different bacterial strains from Gram-positive to Gram-negative bacteria [116]. *In vivo* application of QACs is restricted due to the high cytotoxicity of this biocide to mammalian cells (<0.1 ppm). However, different researchers have demonstrated that turning QACs into a less cytotoxic antimicrobial agent using polymerization techniques make them a good alternative to antibiotics [123].

1.2.3.3.3 Iodine

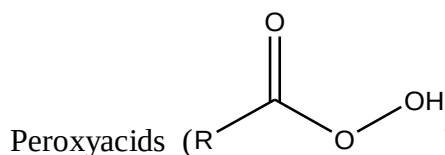
Iodine-based products are one of the oldest disinfectants and have been in use for over 170 years in aqueous or alcoholic solution forms, mostly for the disinfection of wounds [124].

Despite the reactivity of iodine being less than that of chlorine, strong antibacterial, antiviral, sporicidal and fungicidal activities have been reported in the literature [125]. Aqueous iodine solutions, which consist of at least seven different species, are unstable and generally associated with staining and irritation. The most important member of this family (which is primarily responsible for the antibacterial activity of iodine solutions) is molecular iodine (I_2) [126]. The MIC of iodine compounds against different bacterial strains has been found to be 70 to 150 ppm of available iodine [127]. The instability of iodine solutions can be improved by the development of “iodine carriers” or “iodine-releasing agents” called iodophors, which are a mixture of iodine and a solubilizing agent or a carrier [125].

The exact mode of action of iodine remains unknown; however, the antibacterial activity of iodine, similar to that of chlorine, is fast even at lower concentrations. Iodine attacks key functional groups of proteins (such as sulfur containing amino acids, cysteine and methionine) on the bacterial membrane, or it can penetrate into bacterial cells quickly and attack proteins as well as nucleotides, and fatty acids resulting in bacterial death [125].

Although there is no documented resistance to iodine and its compounds, due to multiple potential targets, iodine is toxic to human tissue and can cause irritation and staining. It cannot be utilized as an alternative to antibiotics unless the cytotoxicity of iodine is decreased. There are strategies for decreasing the cytotoxicity of iodine. For example, iodophors, “iodine carriers” or “iodine-releasing agents”, are less toxic [128].

1.2.3.3.4 Peroxyacids



are bactericidal, sporicidal, virucidal and fungicidal agents and are active at lower concentrations, as well as being more potent, than hydrogen peroxide. By-products of the decomposition reaction of peroxyacid are carboxylic compounds and oxygen which makes it safe to be used in different applications such as disinfecting medical devices, hemodialyzers and environmental surfaces [125].

Unlike peroxidases do with hydrogen peroxide, peroxidases do not decompose peroxyacid. Peroxyacid attacks protein and enzymes by denaturing them. It also affects the bacterial cell wall by damaging the wall's sulfhydryl (-SH) and disulfide (S-S) bonds [125].

Peroxyacid is considered to be a regenerable antibacterial agent and as such, it has been used as a powerful hospital disinfectant. The carboxylic acid byproducts of the decomposition of peroxyacid can react with oxidant chlorine bleach to regenerate peroxyacid groups. There has been a great deal of research regarding how compounds with carboxylic acid groups were grafted on a surface to make an antibacterial substrate by converting the carboxylic groups to peroxyacid groups using oxidant bleach [129].

Bridier et al. [130] tested different disinfectants including peracetic acid (PAA), which is the most famous member of peroxyacid containing compounds, and observed a higher tolerance to peracetic acid and some other oxidising agents in a strain of bacteria isolated from an endoscope washer disinfectant which was in contact with a high amount of oxidising biocides. Although PAA has been used to disinfect drinking water instead of sodium hypochlorite, it is cytotoxic ($\text{TC}_{50} > 1 \text{ mM}$) [131], [132]. Bridier et al. observed that PAA is effective against different bacterial strains and its antimicrobial activity is even higher than benzalkonium

chloride. The effective concentration of PAA to reach 3 log reduction against different bacteria was found to be 5 –10 ppm where this concentration for benzalkonium chloride was 5-30 ppm. Viola et al. [133] showed that a solution of 1% PAA is more cytotoxic to mouse fibroblast cells than a solution of 2.5% of NaOCl.

1.2.3.3.5 Glutaraldehyde

Glutaraldehyde is a dialdehyde which has been used as a disinfectant for many years. Generally, glutaraldehyde is used for disinfecting and sterilizing surgical equipment and endoscopes at low-temperatures. It is also used to fix the bacterial membrane in electron microscopy [125]. Glutaraldehyde has shown activity against different pathogens such as bacteria, spores, fungi, and viruses. The MIC of glutaraldehyde has been reported to be 1375 to 3250 ppm against different bacterial species, which makes it a broad spectrum antimicrobial agent [134].

The first reports on the antibacterial activity of glutaraldehyde were published in the 1960s [135]. Different studies showed different effects of glutaraldehyde on microorganisms such as (i) outer layer strong attachment, (ii) dehydrogenase and periplasmic enzymes inhibition, (iii) lysostaphin-induced lysis inhibition in *S. aureus*, and (iv) RNA, DNA, and protein synthesis inhibition. Consequently, the modes of action of glutaraldehyde are (i) association with an outer layer of bacterial cells and crosslinking proteins and lipids, (ii) inhibition of vital enzymes leading to the production of spheroplasts or protoplasts, and (iii) prevention of lysis [125], [136]. Although glutaraldehyde is a good antibacterial agent, it is cytotoxic for human cells [137]. The CT 50 for glutaraldehyde was measured to be 85.14 ppm (0.85 mM) against WI-38 (human embryonic lung) fibroblasts cells [138].

There are different bacterial resistances reported against glutaraldehyde. Griffith et al [139] have reported glutaraldehyde-resistant *Mycobacterium chelonae* in endoscope washers in hospitals. Tschudin-Sutter et al [140] found glutaraldehyde-resistant *Pseudomonas aeruginosa* strain in endoscopes which were washed with glutaraldehyde for disinfection. It has been reported that resistance to glutaraldehyde is due to a genetic factor which can be acquired by contact glutaraldehyde. Outer layer proteins contribute to a bacteria's resistance to glutaraldehyde by increasing the adhesion of the membrane to glutaraldehyde which results in a reduction in the penetration of the glutaraldehyde into the bacterial cells [141]. The efflux pump is another mechanism of resistance to glutaraldehyde reported by Vikram et al. [136] which also causes cross-resistance to antibiotics.

1.2.3.3.6 Antimicrobial polymers

Antimicrobial polymers have been used for decades, and there are a lot of reviews about them. Conventional antimicrobial agents have some issues, such as residual toxicity which can cause serious problems to the environment, and antimicrobial polymers can improve this residual toxicity [142]. Therefore, the use of antimicrobial polymers is increasing, and this is happening in both research and industrial applications. Biocidal polymers demonstrated other advantages such as chemical stability, low permeation rate through the skin and non-volatility.

There are three main classes of antimicrobial polymers: (i) polymeric biocides, (ii) biocidal polymers, and (iii) biocide-releasing polymers which all have different mechanisms of action based upon their type [143]. Polymeric biocides are prepared by the polymerization of bioactive repeating units. These repeating units act similarly to biocidal monomers (**Figure 1-4**). The main problem with this group of antimicrobial polymers is the reduced water solubility of the biocide after polymerization. In this case, each repeating unit acts as a

biocide which attacks different targets in the bacterial cell. Biocidal polymers are macromolecules in which the whole molecule acts as one biocidal agent (**Figure 1-4**). Therefore, in this case, the repeating unit is not necessarily biocidal. Due to the similarity of their structure, polymeric biocides were initially considered as polymer biocides with immobilized quaternary ammonium salts. The attraction between the negatively charged bacterial membranes and polycations is the main mode of antimicrobial action of polymeric biocides and the macromolecule act as a single antimicrobial agent. This electrostatic interaction causes membrane disruption resulting in bacterial death. There are different examples of such polymers with quaternary ammonium and phosphonium, tertiary sulfonium and guanidinium functions [143]. The polymer backbone can act as a matrix to hold different biocides. The nature of polymers gives them the ability to use one biocide or a combination of various biocides. The polymer properties such as molecular weight and hydrophilicity can also significantly affect the antimicrobial activity [123].

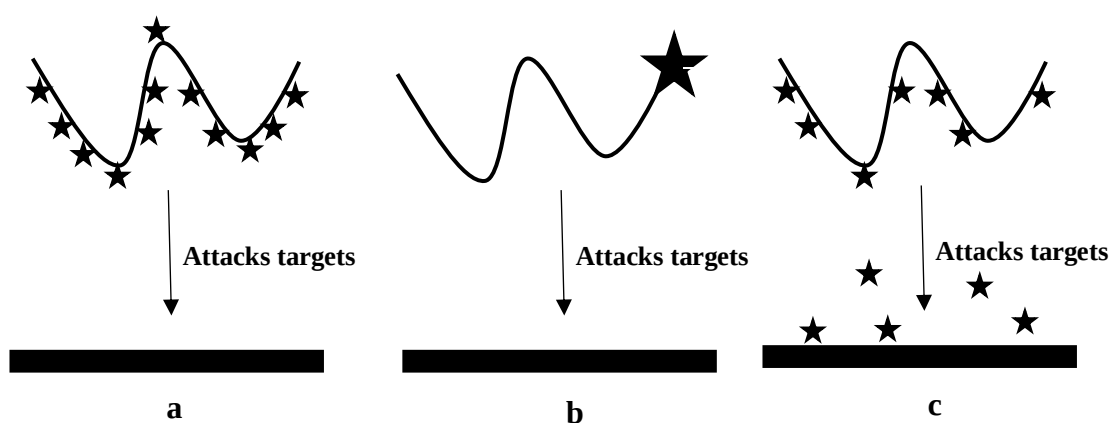


Figure 1-4.General working principles of antimicrobial polymers: (a) polymeric biocides; (b) biocidal polymers; (c) biocide-releasing polymers [143].

The first biocide-releasing polymer prepared by polymerization of salicylic acid did not show antimicrobial activity. However, it helped scientists to understand that degradation of ester can release salicylic acid in a controlled manner which led to the discovery of tributyltin esters of polyacrylates [143], [144]. Nitric oxide (NO) releasing polymers are another member of biocide-releasing polymers. NO shows antimicrobial behavior in two ways, when the concentration is low, NO act as signaling molecules which promotes the activity and growth of immune cells. In high concentrations, NO covalently binds DNA, proteins, and lipids resulting in inhibiting or killing the microorganism [145].

Although there is almost no documented resistance against antimicrobial polymers and there is a low risk of developing resistance to these polymeric antimicrobials, there is a chance of resistance to them when antibiotic releasing polymers are involved or biocides with existing resistance against them are utilized as building blocks [146].

1.2.3.3.7 Antimicrobial peptides

Antimicrobial peptides are broad spectrum antimicrobial agents which are effective against viruses, bacteria, and other parasites. These peptides are oligopeptides in which the number of amino acids varies from five to a hundred. There are different classes of antimicrobial peptides based on their molecular structure such as cationic/anionic peptides and β -sheet/ α -helical/extended/ loop peptides [147].

Antimicrobial peptides were discovered in 1939 by Dubos during his effort to extract an antimicrobial agent from a soil bacillus strain. This fractioned peptide was identified and named gramicidin and was then used to treat *Pneumococci* infection on mice. Only a couple of years later, another antimicrobial peptide, tyrocidine, was discovered. Tyrocidine is

effective against both Gram-negative and Gram-positive bacteria. However, this peptide is toxic to human blood cells [148].

Today there are more than 5000 antimicrobial peptides extracted from nature or synthesized in the lab. Most of the antimicrobial peptides in animals are found in organs which are subject to airborne pathogens. For instance, frog skin makes over 300 different antimicrobial peptides to protect the frog as its first line of immune defense [149].

The mechanism of action of antimicrobial peptides is well-known and is not an enzymatic mechanism. The majority of antimicrobial peptides are cationic. They can interact with the negatively charged bacterial membrane and disrupt the lipid bilayer. These antimicrobial peptides are also conjugated and have both a hydrophobic and a hydrophilic part. Lipids can attract the hydrophobic part, and the hydrophilic component can bind to phospholipid groups [147]. Some researchers have shown that antimicrobial peptides could kill bacteria without disintegration of the membrane at low concentrations through the inhibition of some critical reactions within the cell such as protein production [150].

Antimicrobial peptides are used against different pathogens such as viruses, bacteria, and fungi. Also, these peptides are utilized in biofilm and persisted control [150].

There are different mechanisms for resistance to antimicrobial peptides such as constitutive (passive) resistance, inducible (adaptive) resistance, and the combination of both passive and adaptive resistance [151].

1.2.4 Nanotechnology use in antimicrobial discovery

Nanoparticles (NPs) can be used to combat antibiotic resistance by either demonstrating intrinsic antimicrobial activity, such as active therapeutic agents or improving the activity and

safety of other antimicrobial agents, including antibiotics and biocides, as delivery vehicles to transport and deliver drugs [152].

In section 1-4-3-2 we mentioned the utilization of NPs to encapsulate antibiotics to improve their MICs and bypass bacterial resistance mechanisms. The same logic can be used to justify the use of NPs as nano- carriers for biocides. Most of the potent biocides (such as *N*-chloramine and QACs) are toxic to human cells. This toxicity limits the *in vivo* application of these effective biocides. In addition, biocides are mostly unstable in biological fluids. These drawbacks can all be reduced by encapsulation in NPs. In order to make these potent broad-spectrum biocides applicable for *in vivo* applications, they need to be isolated from other cells and released directly inside the bacteria. One of the solutions developed to protect unstable drugs is the use of nanocarriers [61], [63], [152], [153]. Various nano-carriers are employed for antimicrobial drug delivery, namely liposome and polymer-based NPs [154].

1.2.4.1 Delivery vehicles to transport drugs to pathogens

An improvement in antimicrobial activity using nano-carriers can be achieved by bypassing common antibiotic resistance pathways such as regulation of cell membrane permeability, the efflux pump, antibiotic destruction, and target binding interference by overexpression or changing the molecular structure. Also, NPs can provide different ways to destroy biofilm, infections and reduce antibiotic resistance development by providing several different antibacterial mechanisms [155].

1.2.4.1.1 Liposomes

Liposomes are made of a phospholipid bilayer with an aqueous core and have a size of nanometer to micrometer. Liposomes have been extensively studied as an acceptable vehicle to deliver different materials such as proteins, enzymes, and drugs [156]. The aqueous core

can encapsulate hydrophilic drugs while the hydrophobic drugs can be carried in the lipid bilayer without any further modification so they can then be delivered to a specified target. The very first drug encapsulated in liposome (in 1995) was doxorubicin (with the commercial name of Doxil) which is approved by the FDA. Liposomes have been utilized extensively for delivery of antimicrobial agents due to their bilayer structure. Because this bilayer structure is similar to a bacterial membrane, it can cause the fusion of the liposome and bacterial membranes resulting in the delivery of the drug directly into the bacterial intracellular [156].

Different factors affect liposome activity and efficacy such as physicochemical properties of lipid, the nature of the loaded drug, the size and size distribution, the surface charge, and the stability of liposome in storage.

One of the problems scientists have faced during the administration of liposomal drugs was fast liposome disappearance due to the mononuclear phagocytic system (MPS). To solve this issue, certain glycolipids were utilized to increase the circulation time of the liposomes and reduce the uptake by MPS. Another strategy for dealing with fast liposome disappearance was the conjugation of molecules like PEG to the surface of the liposome to improve *in vivo* applications. Having the PEG on the surface can facilitate the conjugation of targeting ligands such as antibodies and aptamers to the surface of the liposomes.

Like other encapsulation techniques, liposomes can be used to reduce the toxicity and side effects of a drug, for example, polymyxin B which is active against *P. aeruginosa*. Encapsulating of gentamicin and ceftazidime could prolong the circulation of the drug in the blood. Also, liposomal antimicrobial agents are more stable and have a low elimination rate by MPS [152].

1.2.4.1.2 Polymeric NPs

Biocompatible polymers have been used as carriers for controlled drug release since the 1980s, and there are many studies examining how to enhance intracellular release and increase the retained time of the drug in the body by the reticuloendothelial system (RES) [157]. There are different advantages of using polymer NPs as a carrier for antimicrobial drug release: (i) they provide more structural stability during processing and within the biological fluid; (ii) their physical characteristics, such as size, ζ -potential, drug uptake, and release profile, can be regulated by changing the molecular weight of the polymer, the solvent, the concentration of the drug and the surfactant [158]; (iii) They provide some functional group as a joint point for drugs and targeting molecules such as peptides, antibodies, and aptamers [159].

Polymeric nanoparticles which have been investigated for drug carriers are divided into two main classes: linear polymers and amphiphilic block copolymers. Linear polymers have been mostly used in two different forms for drug delivery: nano capsules (in which the polymer acts as a permeable membrane around drug NPs and control the release) and nanospheres (in which the drugs are loaded homogeneously into the polymeric matrix) [160], [161].

Block copolymers form micelles during a self-assembly process while the hydrophobic drug is encapsulated in the core, and hydrophilic segments form a shield around it. The drug can be released from the core by the opening or degrading of the conjugated bond between the hydrophilic and hydrophobic segments [162]. Targeting ligands have recently been employed by conjugating them to the end of block polymer chains for targeted release of the drug. An example of this is the encapsulation of amphotericin B in poly(ϵ -caprolactone) nano-spheres [74], [163], [164].

1.2.4.1.3 SLNs

Solid lipid nanoparticles (SLNs) have become the center of attention for many drug delivery studies since the early 1990s. SLNs are particles between 50 and 1000 nm in size. The difference between emulsions and SLNs is the replacement of a liquid lipid with a solid lipid. Lecithin is also used in the emulsion as a stabilizer since stabilization of SLNs can be done using other surfactants or polymers. The advantage of SLNs over polymeric nanoparticles is that the conventional oil in water (O/W) emulsion technique, which has been established and scaled up for over 70 years in the pharmaceutical industry, is utilized as a production method. The production lines for emulsion pharmaceutical products with temperature control facilities already exist. These production lines can be used to synthesize a large quantity of SLNs.

SLNs provide the advantages of both polymeric nanoparticles and liposomes. However, they do not have the disadvantages of either component [165]. Liposomes, as described above, have a low circulation time and expensive pharmaceutical ingredients, whereas polymeric nanoparticle ingredients are cytotoxic and their production scale-up, in this case, is hard. Researchers have been looking for an alternative for polymeric nanoparticles and SLNs have been found to meet those needs [165]. SLNs improved the bioavailability and target release of both of the conventional methods of encapsulation (liposomes and polymeric nanoparticles) for antimicrobial drugs. Antimicrobial agents encapsulated in SLNs can be taken orally in the form of tablets, capsules, and pellets. Similar to other nano-carriers, SLNs provide many benefits, including increasing the circulation time of drug, combating resistance mechanisms (such as blocking the efflux pump), decreasing the amount of drug trapped in the kidneys, increasing the concentration of the drug in the desired organs and enhancing the bioavailability of the drug. [152]

Antimicrobial loaded SLNs can be uptaken undesirably by the MPS (the mononuclear phagocytic system). Therefore it is necessary to improve targeting, release, and drug delivery within SLNs. Inhalable SLNs are more stable than polymeric nanoparticles and liposomes and the risk of organic solvent remaining in the SLNs is low. [152]

There are two different production methods for SLNs namely the hot homogenization technique and the cold homogenization technique. Both approaches include dissolving the drug in a lipid melt at 5-10 °C above the lipid melting point. In the case of hot homogenization, after the drug is dissolved in the lipid melt, the solution is dispersed in a hot aqueous surfactant solution while being stirred. The primary emulsion achieved by this process is homogenized using a piston-gap homogenizer. After being cooled down, the temperature lipid is recrystallized and makes solid lipid nanoparticles. This method is even suitable for temperature sensitive drugs due to the short time of heat exposure. In the cold homogenization technique, the solution of lipid melt is cooled down, and the solid lipid microparticles are dispersed in a surfactant solution which is cold. The cavitation forces break the micro particles into nano size particles. Cold techniques avoid melting the solid lipid in order to minimize the loss of hydrophilic drugs. [165]

1.2.4.2 Engineered NPs as active therapeutic agents

In addition to encapsulating antibacterial materials by nano-carriers, engineered nano-sized antimicrobial metals and materials are another class of nano antimicrobial agents. In general, decreasing the size increases the surface area to volume ratio, which results in unique chemical-physical properties and improves the antimicrobial's activity against pathogens [152].

1.2.4.2.1 Silver

Silver has been used for several centuries as an antimicrobial agent for burn and chronic wound infection control. In 1000 B.C. silver was used to sanitize drinking water and since 1700 silver has been used pharmaceutically. Silver nitrate was used to remove granulated tissues and facilitate the formation of wound crusts in the 19th century. Discovery of penicillin decreased the antimicrobial use of silver dramatically. However, demands for silver increased in the 1960s when Moyer used a 0.5% silver nitrate solution to improve the treatment of burn wounds. This treatment does not disrupt the proliferation of epidermal cells and shows antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* [166]. The first antimicrobial cream of silver (silver sulfadiazine) was a combination of silver and sulfonamide. Nowadays, due to the limitation of antibiotics, use of silver wound dressings has once more increased [167].

Silver NPs have a big impact on the antimicrobial activity of silver by providing a huge surface area which can increase the effective contact with bacteria and therefore increase antimicrobial efficacy [168]. It has also been observed that decreasing the particle size of materials can improve their biocompatibility [169].

The exact mechanism of silver nanoparticles antimicrobial activity is not yet clear. However, various mechanisms have been proposed. Membrane disruption is one of the possible mechanisms, in which silver NPs bind to the bacterial membrane and penetrate into the cell. This penetration causes a change in cell membrane structure resulting in bacterial death. There are some studies using an electron spin resonance spectroscopy technique which demonstrated the formation of free radicals by Ag NPs. These free radicals attack bacterial cell membranes and make them porous. Silver ions are another described mechanism for the antibacterial activity of Ag NPs [170]. These ions interact with the thiol groups on many

essential cell components, such as proteins and enzymes, and inactivate them. Also, silver is considered a soft acid which can react with cellular components (like DNA, which have the soft basic elements sulfur and phosphorus) and destroy them, leading to the death of bacteria [168].

The biggest issue with silver in high concentrations is its nano-toxicity. These researchers suggested that the nano-toxicity of silver can cause health and environmental problem and they noted that it is difficult to remove deposited silver from the body completely [168].

Nano-silver antibacterial, antiviral, and antifungal activity have been studied by many researchers [167]. The first case of silver resistance was found in a burn wound, which was treated with silver nitrate, in the 1960s. There have been many reported silver-resistant bacteria isolated from burn wounds, hospitals, and clinical environments, since then [171].

1.2.4.2.2 Titanium dioxide (TiO₂)

Titanium dioxide (TiO₂) has been extensively studied since 1972. It is one of the most powerful semiconductive photocatalytic materials and has high oxidative activity, long stability and strong antimicrobial properties [172].

UV irradiation (both near-UV and UV-A) activate TiO₂ NPs' antibacterial properties by generating reactive oxygen species (ROS) such as free radicals and peroxides. Free hydroxyl radicals are very potent oxidant species which primarily attack the surfaces of bacterial cells and disrupt their membranes [173]. This membrane disruption leads to an interruption in some important biological functions such as impermeability, respiration, and oxidative phosphorylation reactions [152]. The effective antibacterial concentration of TiO₂ NPs, depend upon their size as well as the intensity of the UV light used and varies from 10 to 1000 ppm. Scientists have reported that the complexity, density, and structure of bacterial

membranes or walls govern the antimicrobial efficacy of TiO₂ NPs. In the study[173], bacteria were shown to be vulnerable to TiO₂ in the following order: *E. coli* > *P. aeruginosa* > *S. aureus* > *E. faecium* > *C. albicans*. The density and complicity of cell wall increase in an opposite order, i.e. *E. coli* and *P. aeruginosa* (Gram-negative) have a thin cell wall, *S. aureus* and *E. faecium* (Gram-positive) have a thick cell wall and *C. albicans* has the thickest eukaryotic cell wall among all the studied species. The photocatalytic antimicrobial effect of TiO₂ NPs is more effective against viruses than against bacteria and bacterial spores [173]. It was also reported that TiO₂ NPs inhibit the growth of *Enterobacter cloacae* more than that of *E. coli* and *P. aeruginosa* [174].

It has been observed that metal doping of TiO₂ NPs improved their light absorbance and increased their antimicrobial activity. Ag/TiO₂ NPs also showed interesting light-independent antimicrobial properties against both *E. coli* and *B. subtilis* spores [175].

There have been different reported resistances to TiO₂ NPs in *E. faecalis*, *S. aureus* and *E. coli* using enzymatic mechanisms [176]. In this resistance pathway, antioxidant enzymes, particularly catalase, decrease the susceptibility of bacteria against oxidants.

1.2.4.2.3 Zinc oxide (ZnO)

Research on the antimicrobial activity of zinc oxide (ZnO) began in the early 1950s. In 1995 Sawai and his colleagues determined the antimicrobial activity of different metal oxides, including MgO, CaO and ZnO powders [177].

The antibacterial behavior of ZnO particles has been investigated in both microscale and nano scale formulations. Nano-sized zinc oxide (ZnO NP) has exhibited significant antimicrobial activity against a broad spectrum of bacterial strains. ZnO NPs were found to be non-toxic to human cells with a good biocompatibility [178]. ZnO NPs are effective

against food-borne pathogens and have been utilized to protect agricultural products. ZnO NPs showed different advantages over silver NPs including low preparation cost, and anti-UV properties [152].

While there are several theories explaining the antimicrobial activity of ZnO against both Gram-positive and Gram-negative bacteria, the real mode of action of ZnO is still unclear [177]. The primary mode of action for ZnO NPs is the destruction of proteins and lipids located on the bacterial cell membranes. This demolishing act causes leakage of the intracellular contents of bacteria cells and results in the death of the bacteria. ZnO NPs also produce reactive oxygen species (ROS) and zinc ions in different media [179]. It has been found that the concentration of free hydrated zinc ion (Zn^{2+}) or labile zinc complexes determined the antimicrobial efficiency of ZnO NPs and the chemical properties of the media affected the antimicrobial activity of ZnO NPs [180].

There are various reports of zinc and zinc oxide resistance and tolerance by different microorganisms [181].

1.2.4.2.4 Gold

Gold nanoparticles (Au NPs) have been utilized in biosensors, DNA labeling, and vapor sensors for many years. The antibacterial activity of Au NPs has become very important because of their remarkable physical properties [182], [183].

Au NPs can absorb near-infrared light and turn it into heat to treat bacterial infections. To activate the antimicrobial effect of Au NPs a laser light with a proper wavelength is usually used [184]. Strong laser light can induce hyperthermia effects with the bubble formation around the Au NPs which can destroy bacteria.

Au NPs are effective against both Gram-negative and Gram-positive bacteria including the drug resistance ones.

Conjugation of Au NPs to different antibodies and peptides improves selective binding to the target bacteria [184]. For instance, conjugation of anti-protein A antibodies to Au NPs have been used to provide a selective antimicrobial activity of Au NPs against *S. aureus* [184]. It has been observed that the combination of or coating of Au NPs with antibacterial drugs such as gentamicin, streptomycin, and neomycin can improve the antimicrobial activity of both Au NPs and antibiotics as well as improve their application in serious infection treatments. For example, coupling ampicillin with chitosan-capped Au NPs increased the antimicrobial activity of ampicillin by two-fold [185].

There is no reported resistance against Au NPs and studies on MRSA have indicated that resistance to Au NPs did not happen even after 20 generations [186].

1.3 Potential ligands to enhance targeted delivery of antimicrobials to bacteria

Although nano-carriers facilitate drug circulation and distribution among cells, they do not deliver the drug in a selective manner. There are different methods to provide targeted release of a drug such as using targeting ligands, utilizing pH, and heat sensitive groups.

Recently, the first form of targeting (using a ligand) has made substantial progress in the ability of nanoparticles to target certain cell populations with high selectivity for both oncology applications and other therapeutic areas. These active, selective targeting strategies rely on highly specific interactions of antibodies, aptamers, peptides, and oligonucleotides with cell surface receptors known to be expressed only on the target cells. These active targeting strategies are discussed in more detail below.

1.3.1 Antibody

The conjugation of antibody (Ab, also known as an immunoglobulin (Ig)), to nanoparticles, gives the nanoparticles selective recognition and the ability for specific binding to the target while the properties of the nanoparticles remain unchanged [100]. This conjugation can also facilitate cellular uptake of nanoparticles as well as the stability of nanoparticles within the cells.

Antibodies are a subcategory of glycoproteins which play a significant role in disease defense mechanisms in vertebrates. All of the antibodies have a Y-shape structure with two antigen recognition domains which are identically the same and two similar effector functions domains. These antigen-binding domains are specific to individual antigens. There are billions of antibodies which either already exist or can be generated within the human body. These antibodies have different antigen-binding domains which will bind to a specific antigen.

Antibodies have two heavy (55– 70kD) and two light (24-25kD) chains which are connected by disulfide bonds. There are five isotypes of antibodies within vertebrate animals (IgG, IgE, IgD, IgA, and IgM) which are categorized based on their heavy domains. Disulphide bridges not only connect the chains, but the stability of the antibody is dependent upon them because they provide intracavitary bridges.

There are six different functions for which antibodies are responsible:

1. Neutralization: Antibodies neutralize and block the pathogens and toxins.
2. Complement activation: IgM and IgG antibodies cause openings in the pores of cell membranes via a proteolytic cascade activation.

3. Phagocytosis and opsonization: IgG can bind to receptors on both macrophages and dendritic cells as well as facilitate the phagocytosis of pathogens.
4. Cytolysis facilitation: Antibodies can facilitate cytolysis by directing antibody-dependent cellular cytotoxicity (ADCC), Natural Killer (NK) cells, and macrophages, which lyse cells, to the target cells.
5. Participation in parasite removal and allergic responses: IgE gets involved in allergic responses by activating mastocytes. This antibody also helps eosinophils eliminate parasites.
6. Improving mucosal protection: IgA can be found in secretions and improves the mucosal protection in the respiratory and digestive systems .[100]

Antibody conjugated nanoparticles can be developed for two different purposes: treatment and recognition. Diagnosis applications can be further divided into two distinct categories: *in vivo* and *in vitro*. There has been a lot of research done on using antibody conjugated NPs for therapeutic applications. A significant portion of this research has focused on target release and selective delivery.[100]

Monoclonal antibodies (mAb), which are the largest group of antibodies on the market, have also been utilized to treat different cancers and infectious diseases. They can act through various mechanisms: blocking the antigen receptors in the effector cells [100], inducing bacterial agglutination [101] and neutralizing the bacterial toxin[102]. Monoclonal antibodies are highly selective to a particular pathogen, so it is unlikely for bacteria to develop resistance against them.

1.3.2 Aptamers

Aptamers are short DNA or RNA molecules with the ability of specific binding to target molecules with high efficiency. SELEX (systematic evolution of ligands by exponential enrichment) is the method used to find effective aptamers [187]. There are different aptamers which act against various small organic molecules or macromolecule targets.[187] There are various advantages of aptamers over antibodies such as low preparation cost, better directional amplification by Polymerase chain reaction (PCR) and simpler labeling methods for molecular recognition.[188] Aptamers also show a high degree of selectivity and affinity in comparison to monoclonal antibodies, and their smaller size facilitates tissue uptake. These advantages make aptamers the best targeting ligand for drug delivery and nanotechnology applications.

1.3.3 Peptides

Peptides are short chains of amino acid monomers linked by peptide (amide) bonds. Peptides can be effectively used for cellular targeting and precise delivery. They are stable and can be easily synthesized and conjugated to any drug or nanoparticles [189]. The first targeting peptide was glycine-aspartic acid (RGD) peptide, which can bind to proteins on the cell membrane, discovered by Pierschbacher and Ruoslahti [190]. Short peptides can bind to antigens on the surface of immunoglobulin receptors. These targeting peptides can be cytotoxic too, which is favorable in the case of anticancer nano materials [191].

Cell penetrating peptides (CPP) can be used as cargo vectors. It has been shown that the conjugation of a penetrating peptide to an antibiotic can boost the antimicrobial activity of that antibiotic [192]. Being a delivery vector means CPPs can efficiently facilitate the delivery of different molecules to their targets. The efficacy of the delivery is dependent on

the type of peptide, but the cell penetration happens by endocytosis. First, the peptide and cargo make a complex, then they interact with the cell plasma membrane. Endosomal uptake and escape are the next steps following by cytoplasmic or nuclear localization. The peptide-cargo complex can be covalent, for example, a disulfide bond and amide bond, or noncovalent, for example, with electrostatic and hydrophobic interactions between negatively charged cargo molecules. Penetrating peptides can be conjugated to nanoparticles and help them to penetrate through the membrane and enter into the cells. [193]

1.4 Hypotheses and Objectives

Previous work by our lab [194] has demonstrated that covalently joining hydantoin based *N*-chloramine with long-chained quaternary ammonium (QA) compounds results new “composite” biocides with enhanced biocidal activity, outperforming the formulation of two separate mono-functional biocides (hydantoin-based *N*-chloramine and long-chained QA). We hypothesized that covalently joining an amine based *N*-chloramine with a long-chained QA unit can also give a biocide with enhanced antimicrobial activity. Also, secondary and primary amine-based *N*-chloramines might be less likely to be quenched by organic load than amide based *N*-chloramines which makes them better suited for applications with high protein loading such as wound infection control. Therefore, a secondary amine based *N*-chloramine was attached to QAC moieties with different chain length to explore the possible synergistic antimicrobial effect, stability in biological fluid and cytotoxicity to skin cells. Based on the results, *in vivo* application of these biocides might be hampered because of their non-selective toxic nature. Therefore, we intended to develop a strategy to convert such potent non-selective biocides into selective ones, enabling their *in vivo* applications for wound infection control.

It was hypothesized that highly potent but non-selective, cytotoxic disinfectants can be converted into selective, noncytotoxic antimicrobial agents using nanoparticles (NPs). The proposed idea was to first encapsulate a model potent biocide, 3-(4,4-Dimethyl-2,5-dioxo-imidazolidine-1-yl)-propyl]-dimethyl-tetradecyl-ammonium chloride, (C17), into solid lipid nanoparticles (SLNs) , followed by the conjugation of an antibody specific to a target bacterium to enable selective delivery of the toxic cargo to the bacterium.

Goal, Objectives, and Methods: The main goal of this study is to synthesize highly potent “composite” biocides with both amine-based *N*-chloramine and QA moieties and develop a strategy to convert this kind of highly potent but non-selective antimicrobial agents into selective non-cytotoxic antimicrobial agents using nanotechnology. The specific objectives are to:

- 1- Synthesize and characterize a series of “composite” biocides comprising amine-based *N*-chloramines and a QA unit.
- 2- Carry out a series of quantitative *in vitro* antimicrobial assays to evaluate the antimicrobial activity of the synthesized compounds and commercial ones at the same time.
- 3- Prepare and characterize SLNs loaded with C17 using the emulsion solvent diffusion method and conjugate an antibody (NYR MRSA 16 antibody and rabbit anti-*E. coli* IgG type antibody) to make them selective.

Establish and perform various assays to evaluate bioactivity and selectivity of the fabricated C17 loaded nanoparticles.

2 Chapter 2. New Generation of *N*-Chloramine-QAC Composite

Disinfectants: A Superior Antimicrobial Agent to Target Gram-negative Bacteria in Biological Fluids

We previously reported [194] that the combination of *N*-chloramine with Quaternary Ammonium Compound (QAC) can boost the antibacterial efficacy without inducing bacterial resistance. However, similar to other *N*-chloramines and QACs, the antimicrobial activity of this high-performance composite biocide deteriorated in high protein fluids. In this study, we replaced the *N*-chloramine moiety, amide-based, with a secondary amine-based one to improve the efficacy of biocide in biological fluids. N-(2-(4-((1-chloro-2,2,6,6-tetramethylpiperidine-4-yloxy)methyl)-1H-1,2,3-triazole-1-yl)ethyl)-N,N-dimethyl dodecane-1-aminium chloride (6a) with 12 carbon on QAC alkyl chain showed superior antimicrobial activity in high protein media (HPM) in comparison to other composite biocides and biocides benzyldimethyltetradecyl-ammonium chloride (BC). The bactericidal activity of this high-performance biocide was found to be higher in case of Gram-negative bacteria, which are hard to kill.

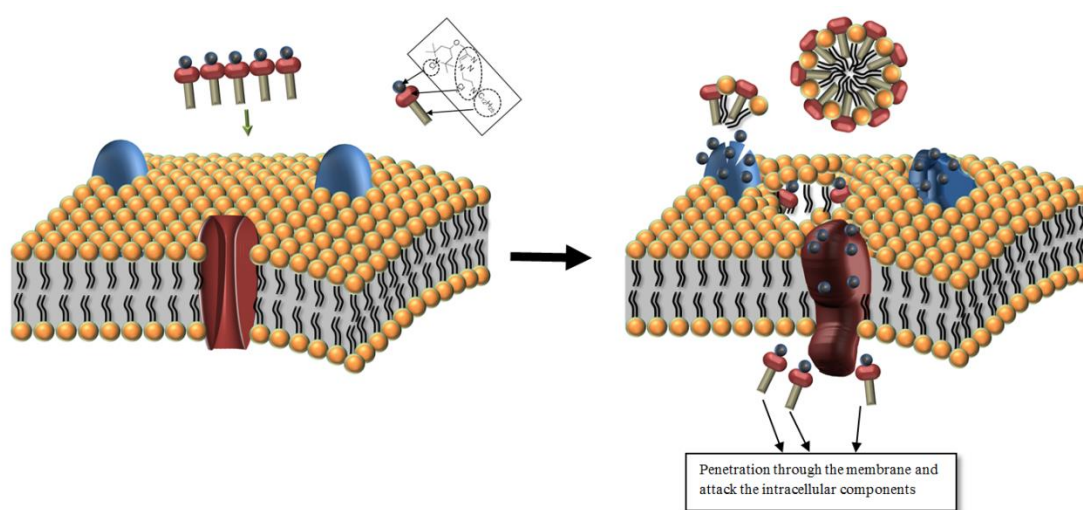


Figure 2-1. Schematic of composite biocide's mode of action

2.1 Introduction

Disinfectants and biocides are widely used in food production industry to kill bacteria and provide food safety [195]. However, the application of disinfectants in food is limited as a result of antimicrobial resistance, lack of high-performance antibacterial agents which can stay effective in biological fluids, and food contamination with disinfectants [196],[197]. In addition, disinfectants overuse can causes cross-resistance to antibiotics and worsen the antimicrobial resistance issue which challenges our ability to cure infectious diseases in the 21st century [14], [15].

There are a vast variety of antimicrobial agents used to disinfect different substrates and environments, especially in the food industry, such as quaternary ammonium salts (QASs), *N*-chloramines, and amphoteric [198].

QACs are one of the extensively used disinfectants to sanitize the environment in food industries. The lipophilicity of the QACs affects the antimicrobial efficacy. This lipophilicity is determined by the *N*-alkyl chain length and QACs with 12-16 alkyls are more effective [118]. In addition, Jono et al. [199] observed that in a high protein media (HPM) with 10% bovine serum albumin (BSA) the binding of biocides benzalkonium chloride to BSA rose with the increase of alkyls chain length on QAC and the most binding happened in the case of benzalkonium chloride with 14 and 16 carbons. Therefore, the lower alkyls attached to QACs molecules, the lower amount of deactivation due to binding to proteins. On the other hand, our study showed that QACs with 14 carbons were more effective against both Gram-negative and Gram-positive bacteria [194]. It has been also demonstrated that covalently joining hydantoin (amide) based *N*-chloramine with long-chained QACs made new “composite” biocides with enhanced biocidal activity, outperforming the formulation of two separate mono-functional biocides [200]. This combination not only demonstrated a superior

antimicrobial activity in aqueous solutions but also provides a biocide without any genetically acquired resistance [201]. This activity enhancement gives the benefit of having the same or better activity with the shorter alkyl chain on QACs. However, like QACs, different *N*-chloramines have different stability in biological fluids based on their dissociation constant because the chlorine can transfer to other amines in high protein media or get reduced by sulfur-containing amino acids such as cysteine and methionine and lose its activity. The stability of *N*-chloramines has the following order: amine>amide>>imide [109].

In this study, a composite biocide was designed to have a maximum antimicrobial activity in high protein media. To this end, a secondary amine-based *N*-chloramines linked to a QAC with the 12 and 14 alkyl groups via a triazole ring linkage and the antibacterial activity of these compounds evaluated against Gram-negative and Gram-positive bacteria. This strategy led us to make a superior antimicrobial agent which is better suited for applications with high protein loading such as food industries.

To this goal, 5,5-dimethyl hydantoin was replaced with 2,2,6,6-tetramethylpiperidinol linked to the QACs with 14 and 12 carbons using the click chemistry. Afterwards, these newly synthesized biocides, hydantoin-based biocides and a commercially available one, BC, were challenged with different bacterial strains such as Methicillin-resistant *Staphylococcus aureus* (MRSA) (Gram-positive species), *E.coli*, Multi-drug resistant *Pseudomonas aeruginosa* (MDR *P. aeruginosa*), and wild-type *P. aeruginosa* (Gram-negative species). N-(2-(4-((1-chloro-2,2,6,6-tetramethylpiperidine-4-yloxy)methyl)-1H-1,2,3-triazole-1-yl)ethyl)-N,N-dimethyldodecan-1-aminium chloride (6a) demonstrated a superior antimicrobial activity over all other biocides against Gram-negative and Gram-positive bacteria in both phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and high protein media (5% Fetal bovine serum (FBS)

in PBS). The redox titration has been utilized to track the chlorine loss in the presence of high protein media.

2.2 Experimental

2.2.1 Materials

All the reagents and solvents, which were analytical grades and no further purification been used, were purchased from suppliers namely, Sigma, Fisher, and VWR. NMR spectra were performed using Bruker Avance 300 MHz NMR spectrometer at room temperature and in 5 mm NMR tubes. High-resolution mass measurements were recorded by AB SCIEX Triple TOF 5600+ (ESI-MS), Concord, ON with Direct infusion method. Multi-drug resistant (MDR) *P. aeruginosa* #73104 and Community-associated (CA)-MRSA #70065, and wild-type *P. aeruginosa* (PA01) which were obtained have been achieved from the CANWARD (Canadian Ward Surveillance) study assessing antimicrobial resistance in Canadian hospitals, <http://www.canr.ca>. *Escherichia coli* #25922 was purchased from ATCC.

2.2.2 Synthesis and analysis

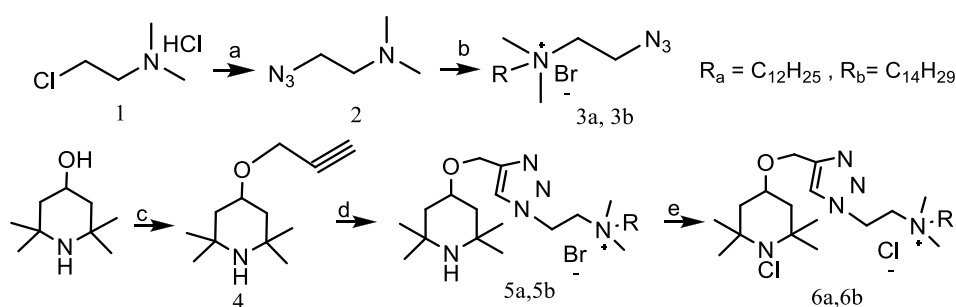


Figure 2-2. Chemical reactions pathways a) NaN_3 , KOH , H_2O , reflux, overnight, b) R-Br , acetonitrile, reflux, overnight, c) Propargyl bromide, NaH , THF , N_2 , 60°C , 5 hrs d) CuSO_4 , Cu , $\text{MeOH}/\text{H}_2\text{O}$, rt, overnight, e) anion-exchange resin (Amberlite R IRA-900, Cl^-) tert butyl

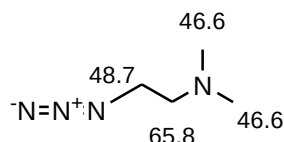
hypochlorite, acetone/H₂O, 0°C, 1 hr

(2-Azido-ethyl)-dimethyl-amine (2) (Figure 2-2):

20 g (139 mmol) of 2-Chloro-N,N-dimethylethylamine hydrochloride (**1**) was dissolved in 50 mL water and then 45.13 (0.69 mol, 5 equiv) of sodium azide was added to the solution and reaction continued overnight at reflux. Then, 7g of potassium hydroxide added to the mixture followed by extraction with dichloromethane (3×100 mL). After solvent evaporation 10.3 g (95 mmol, 65% yield) of a yellow liquid was achieved.

¹H-NMR (CDCl₃, 300 Hz) δ [ppm]: 3.35 (t, *J*= 6.1 Hz, 2H: N₃CH₂CH₂N(CH₃)₂), 2.5 (t, *J*= 6.2 Hz, 2H: N₃CH₂CH₂N(CH₃)₂), 2.27 (s, 6 H: N₃CH₂CH₂N(CH₃)₂)

¹³C-NMR (CDCl₃, 75 Hz) δ [ppm]: 46.6, 46.6, 48.7, 65.8; HRMS (ESI-TOF) *m/z*: [M+H]⁺ calculated for C₄H₁₀N₄⁺, 114.0905; found: 114.0911

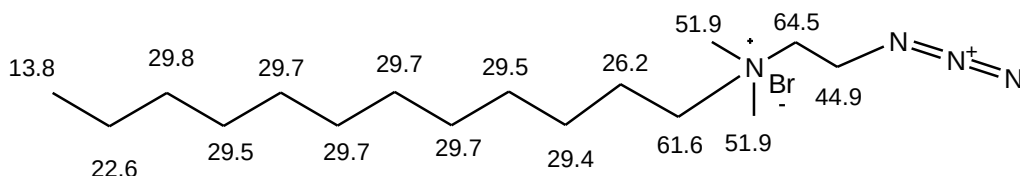


3a, 3b (Figure 2-2):

5.0g (44 mmol) of 2-azido-N,N-dimethylethylamine (**2**) was dissolved in 50 mL of acetonitrile and then 48.4 mmol of 1-bromoalkane, 1-bromotetradecane (**3a**) or 1-bromotetradecane (**3b**), was added to the solutions and the reactions continued overnight at reflux. After solvent evaporation, compounds were dissolved in 5 mL of methanol followed by participation in 150 mL of 50/50 ethyl acetate/ hexane **3a** (11.20 g, 35.11 mmol, 79% yield) and **3b** (11.6 g, 33.4 mmol, 76% yield) of white solids were achieved.

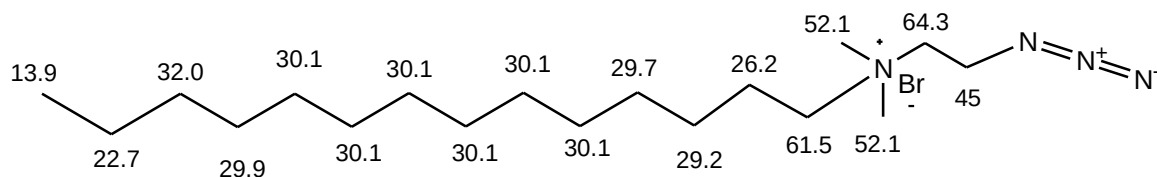
3a: $^1\text{H-NMR}$ (D_2O , 300 Hz) δ [ppm]: 4.04 (t, $J=6.2$ Hz, 2H: $\text{N}_3\text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_2$), 3.62 (t, $J=6.2$ Hz, 2H: $\text{N}_3\text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_2$), 3.43 (t, $J=7.8$ Hz, 2H: $(\text{CH}_3)_2\text{N}^+\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$), 3.22 (s, 6 H: $(\text{CH}_3)_2\text{N}^+\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$), 1.73-1.9 (m, 2H: $(\text{CH}_3)_2\text{N}^+\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$), 1.23-1.48 (m, 18H: $(\text{CH}_3)_2\text{N}^+\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$), 0.91 (t, $J=6.3$ Hz, 3H: $(\text{CH}_3)_2\text{N}^+\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$)

$^{13}\text{C-NMR}$ (D_2O , 75 Hz) δ [ppm]: 13.8, 22.6, 26.2, 29.4, 29.5, 29.7, 29.8, 31.9, 44.9, 51.9, 61.6, 64.5; HRMS (ESI-TOF) m/z : $[\text{M-Br}]^+$ calculated for $\text{C}_{16}\text{H}_{35}\text{N}_4^+$, 283.2856; found: 283.2864.



3b: $^1\text{H-NMR}$ (D_2O , 300 Hz) δ [ppm]: 4.04 (t, $J=6.2$ Hz, 2H: $\text{N}_3\text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_2$), 3.62 (t, $J=6.2$ Hz, 2H: $\text{N}_3\text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_2$), 3.43 (t, $J=7.5$ Hz, 2H: $(\text{CH}_3)_2\text{N}^+\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$), 3.22 (s, 6 H: $(\text{CH}_3)_2\text{N}^+\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 1.73-1.9 (m, 2H: $(\text{CH}_3)_2\text{N}^+\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 1.23-1.48 (m, 22H: $(\text{CH}_3)_2\text{N}^+\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 0.91 (t, 3H: $(\text{CH}_3)_2\text{N}^+\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$)

$^{13}\text{C-NMR}$ (D_2O , 75 Hz) δ [ppm]: 13.9, 22.7, 26.2, 29.2, 29.7, 29.9, 30.1, 32.0, 45.0, 52.1, 61.5, 64.3; HRMS (ESI-TOF) m/z : $[\text{M-Br}]^+$ calculated for $\text{C}_{18}\text{H}_{39}\text{N}_4^+$, 311.3169; found: 311.3174.

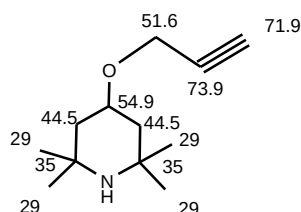


2,2,6,6-tetramethyl-4-(prop-2-ynyloxy)piperidine (4) (Figure 2-2):

9.0 g (57 mmol) of 2,2,6,6-tetramethylpiperidin-4-ol was added to 100 mL anhydrous THF and kept under a nitrogen atmosphere for 30 min, stirring at room temperature, followed by addition of 2.26 g (57.0 mmol) of NaH (60%). After 30 min of mixing, the flask sealed with a rubber stopper and transferred into a 60 °C oil bath where 6.356 mL (57.00 mmol) of propargyl bromide (80%) was added to the mixture and the reaction continued overnight at 60 °C. Afterward, solid settlements were removed by filtration and THF evaporated using rotary evaporator. After solvent removal, the compound dissolved in 50 mL of 1N HCl solution and was washed with 3×50mL DCM. Then sodium hydroxide was used to increase the pH to 10 (in an ice bath). Then the aqueous solution was washed with DCM (3×50 mL). The organic layer was dried on sodium sulfate, and the solvent was evaporated. 5.10g (26.2 mmol, 46% yield) of yellow solid was achieved.

¹H-NMR (CDCl₃, 300 Hz) δ [ppm]: 4.17 (s, 2H: OCH₂CCH), 3.83-3.96 (m, 1H: (CHCH)₂CHO), 2.39 (s, 1H: OCH₂CCH), 1.97-1.96 (d, 1 H), 1.93-1.92 (d, *J*=4 Hz, 2 H: (CHCH)₂CHO), 1.17 (s, 6H: NH(C(CH₃CH₃))₂), 1.12 (s, 6H: NH(C(CH₃CH₃))₂), 0.99 (t, *J*=11.8 Hz, 2H: (CHCH)₂CHO))

¹³C-NMR (CDCl₃, 75 Hz) δ [ppm]: 29.0, 35.0, 44.5, 51.6, 54.9, 71.9, 73.9; HRMS (ESI - TOF) *m/z*: [M+H]⁺ calculated for C₁₂H₂₂NO⁺, 196.1696; found: 196.1716



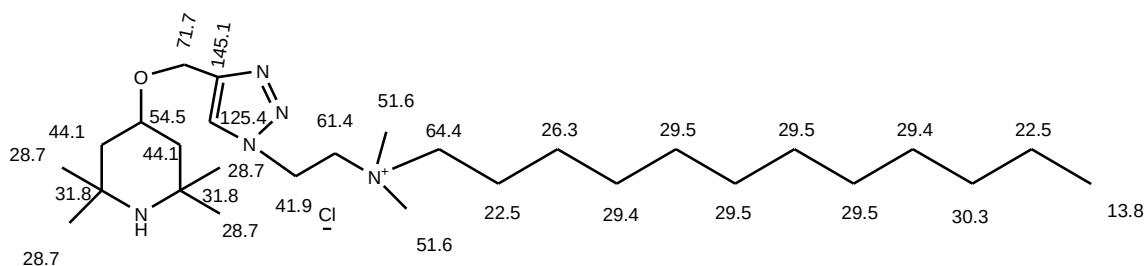
Click reaction, 5a, 5b (Figure 2-2):

2.91 g (15.0 mmol) of **4** was dissolved in 15 mL of MeOH. Then 12.5 mmol of azide **3a** (4.62 g) or **3b** (4.92 g) was added to the solution. Afterward, 0.312 g (1.95 mmol, 10%) CuSO₄ was dissolved in 2 mL water and added to the solutions. Then 2.38 g (37.4 mmol) Cu was added to the solutions and reactions continued overnight at room temperature. After filtration to remove the copper particles, the solution passed through Sorbtech CR20-01 to remove Cu (I).

5a: ¹H-NMR (D₂O, 300 Hz) δ [ppm]: 8.22 (s, 1H: OCH₂(CCHN-N=N)CH₂), 5.057 (s, 2H: OCH₂(CCHN-N=N)CH₂), 4.72 (t, *J*=6.1 Hz, 2H: OCH₂(CCHN-N=N)CH₂), 4.01 (t, *J*=6 Hz, 2H: (CCHN-N=N)CH₂CH₂), 4.09-4.24 (m, 1 H: (CHCH)₂CHOCH₂(CCHN-N=N)), 3.31 (t, *J*=7.4 Hz, 2H: N⁺(CH₃)₂CH₂CH₂(CH₂)₉CH₃), 3.18 (s, 6H: N⁺(CH₃)₂CH₂CH₂(CH₂)₉CH₃), 2.30 (d, *J*=3.5 Hz, 1H: (CHCH)₂CHO), 2.25 (d, *J*=3.5 Hz, 1H: (CHCH)₂CHO), 1.61 (m, 2H: N⁺(CH₃)₂CH₂CH₂(CH₂)₉CH₃), 1.50 (s, 6H: NH(C(CH₃)₂))₂, 1.48 (s, 6H: NH(C(CH₃)₂))₂, 1.23-1.38 (m, 18H: N⁺(CH₃)₂CH₂CH₂(CH₂)₉CH₃), 1.18 (t, *J*=11.1 Hz, 2H, (CH(CHCH)₂CHO)₂CHO), 0.88 (t, *J*=5.5, 3H: N⁺(CH₃)₂CH₂CH₂(CH₂)₉CH₃)

¹³C-NMR (D₂O, 75 Hz) δ [ppm]: 13.8, 22.5, 26.3, 28.7, 29.4, 29.5, 30.3, 31.8, 41.9, 44.1, 51.6, 54.5, 61.4, 64.4, 71.7, 125.4, 145.1; HRMS (ESI-TOF) *m/z*: [M-Cl]⁺ calculated for C₂₈H₅₆N₅O⁺, 478.4479; found: 478.4482.

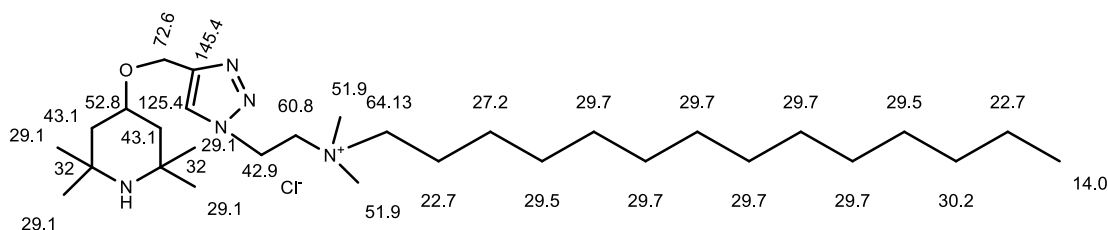
Purity QNMR > 99%



5b: $^1\text{H-NMR}$ (D_2O , 300 Hz) δ [ppm]: 8.22 (s, 1H: $\text{OCH}_2(\text{CCHN-N=N})\text{CH}_2$), 5.057 (s, 2H: $\text{OCH}_2(\text{CCHN-N=N})\text{CH}_2$), 4.72 (t, $J=6.1$ Hz, 2H: $\text{OCH}_2(\text{CCHN-N=N})\text{CH}_2$), 4.01 (t, $J=6$ Hz, 2H: $(\text{CCHN-N=N})\text{CH}_2\text{CH}_2$), 4.09-4.24 (m, 1 H: $(\text{CHCH})_2\text{CHOCH}_2(\text{CCHN-N=N})$), 3.31 (t, $J=7.4$ Hz, 2H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 3.18 (s, 6H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 2.30 (d, $J=3.5$ Hz, 1H: $(\text{CHCH})_2\text{CHO}$), 2.25 (d, $J=3.5$ Hz, 1H: $(\text{CHCH})_2\text{CHO}$), 1.61 (m, 2H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 1.50 (s, 6H: $\text{NH}(\text{C}(\text{CH}_3)_2)_2$), 1.48 (s, 6H: $\text{NH}(\text{C}(\text{CH}_3)_2)_2$), 1.23-1.38 (m, 22H, $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 1.18 (t, 2H, $J=11.1$ Hz, 2H, $(\text{CH}(\text{CHCH})_2\text{CHO})_2\text{CHO}$), 0.88 (t, $J=5.5$, 3H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$)

$^{13}\text{C-NMR}$ (D_2O , 75 Hz) δ [ppm]: 14.0, 22.7, 27.2, 29.1, 29.5, 29.7, 30.2, 32.0, 42.9, 43.1, 51.9, 52.8, 60.8, 64.13, 72.6, 125.4, 145.4; HRMS (ESI-TOF) m/z : $[\text{M-Cl}]^+$ calculated for $\text{C}_{30}\text{H}_{60}\text{N}_5\text{O}^+$, 506.4792; found: 506.4788.

Purity QNMR > 97%



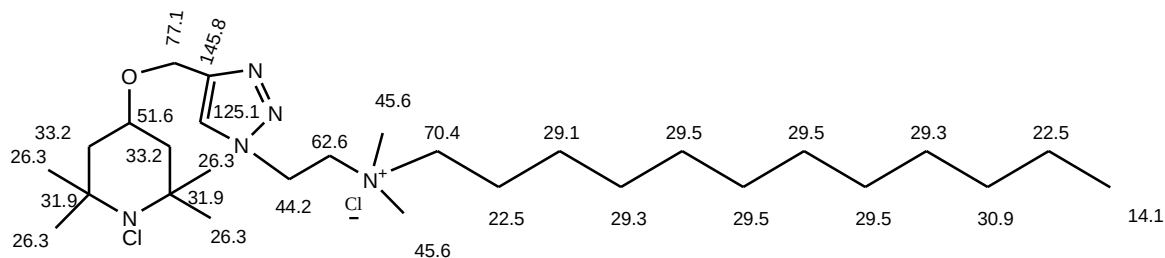
Chlorination of compounds 5a and 5b (Figure 2-2):

500 mg of **5a** or **5b** was dissolved in 10 mL of water /acetone (2/8 by volume), then 3 equiv of tert-butyl hypochlorite (450 μl) was added to vials which completely wrapped with aluminum foil at 0°C . Reactions continued for 60 min. Afterward, air flow was employed to remove acetone and unreacted tert-butyl hypochlorite and water were removed using vacuum.

6a: ^1H -NMR (CDCl_3 , 300 Hz) δ [ppm]: 8.53 (s, 1H: $\text{OCH}_2(\text{CCHN-N=N})\text{CH}_2$), 5.28 (s, 2H: $\text{OCH}_2(\text{CCHN-N=N})\text{CH}_2$), 4.55 (t, $J=6.3$ Hz, 2H: $\text{OCH}_2(\text{CCHN-N=N})\text{CH}_2$), 4.36 (t, $J=6.4$ Hz, 2H: $(\text{CCHN-N=N})\text{CH}_2\text{CH}_2$), 3.68-3.81(m, 1 H: $(\text{CHCH})_2\text{CHOCH}_2(\text{CCHN-N=N})$), 3.4 (t, $J=7.1$ Hz, 2H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$), 3.34 (s, 6H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$), 2.1 (d, $J=3.1$ Hz, 1H: $(\text{CHCH})_2\text{CHO}$), 2.05 (d, $J=3.1$ Hz, 1H: $(\text{CHCH})_2\text{CHO}$), 1.52 (m, 2H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$), 1.23-1.38 (m, 32H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$, $\text{NCl}(\text{C}(\text{CH}_3)_2)_2$ and $(\text{CH}(\text{CHCH})_2\text{CHO})_2\text{CHO}$), 0.88 (t, $J=6.5$, 3H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$)

^{13}C -NMR (CDCl_3 , 75 Hz) δ [ppm]: 14.1, 22.5, 26.3, 29.1, 29.3, 29.5, 30.9, 31.9, 33.2, 44.2, 45.6, 51.5, 62.6, 70.4, 77.1, 125.1, 145.8; HRMS (ESI-TOF) m/z : $[\text{M}-\text{Cl}]^+$ calculated for $\text{C}_{28}\text{H}_{55}\text{ClN}_5\text{O}^+$, 512.4090; found: 512.4080

Purity QNMR > 98%



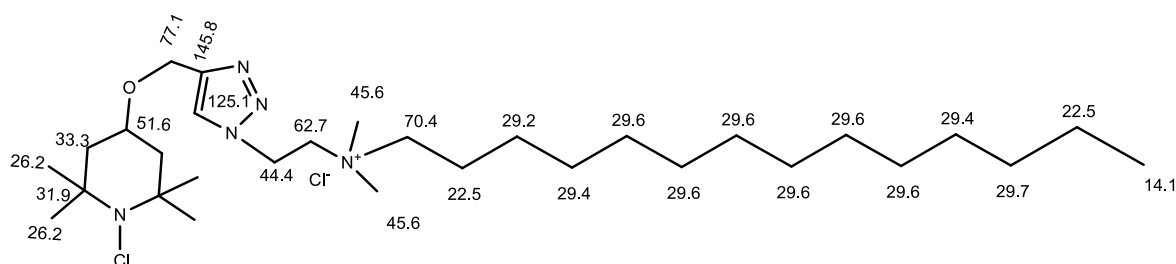
6b: ^1H -NMR (CDCl_3 , 300 Hz) δ [ppm]: 8.5 (s, 1H: $\text{OCH}_2(\text{CCHN-N=N})\text{CH}_2$), 5.24 (s, 2H: $\text{OCH}_2(\text{CCHN-N=N})\text{CH}_2$), 4.58 (t, $J=6.4$ Hz, 2H: $\text{OCH}_2(\text{CCHN-N=N})\text{CH}_2$), 4.32 (t, $J=6.4$ Hz, 2H: $(\text{CCHN-N=N})\text{CH}_2\text{CH}_2$), 3.7-3.83 (m, 1 H: $(\text{CHCH})_2\text{CHOCH}_2(\text{CCHN-N=N})$), 3.4 (t, $J=7.6$ Hz, 2H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 3.33 (s, 6H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 2.10 (d, $J=3.3$ Hz, 1H: $(\text{CHCH})_2\text{CHO}$), 2.05 (d, $J=3.3$ Hz, 1H: $(\text{CHCH})_2\text{CHO}$), 1.54 (m, 2H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 1.11-1.38 (m, 36H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$,

$\text{NCl}(\text{C}(\text{CH}_3)_2)_2$ and $(\text{CH}(\text{CHCH})_2\text{CHO})_2\text{CHO}$, 0.88 (t, $J=6.5$, 3H:

$\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$)

^{13}C -NMR (CDCl_3 , 75 Hz) δ [ppm]: 14.1, 22.5, 26.2, 29.2, 29.4, 29.6, 29.7, 31.9, 33.3, 44.4, 45.6, 51.6, 62.7, 70.4, 77.1, 125.1, 145.8; HRMS (ESI-TOF) m/z : $[\text{M}-\text{Cl}]^+$ calculated for $\text{C}_{30}\text{H}_{59}\text{ClN}_5\text{O}^+$, 540.4403; found: 540.4386.

Purity QNMR > 95%



2.2.3 Copper analysis:

Antibacterial effect of copper has been reported in many studies [202]. Copper has been used as the catalyst for click reaction and this copper can interfere the antibacterial activity of compounds. Therefore, copper removal to the non-effective concentration was crucial. To this end, Sorbtech CR20-01 beads have been used. Copper concentration in compounds was measured using ICP Optical Emission Spectrometer, Varian 725-ES.

2.2.4 Quantitative NMR (q-NMR):

Q-NMR is a technique to determine the purity of the compounds introduced in 1963 [203]. There are different advantages for Q-NMR such as structure determination at the molecular level, intensity calibration free, short measurement times, non-destructive character, and ability to the determination of different analytes simultaneously and no isolation need for the

analytes in the mixture. Q-NMR is based on the fact that signal response (integration) I_x is the related directly to number nuclei N_x generating the corresponding resonance which can be shown as $I_x = K_s N_x$ where the K_s is spectrometer constant. Therefore, determination of the relative area ratio for two elements is: $\frac{I_x}{I_y} = \frac{N_x}{N_y}$

And the molecular ratio of two compounds is n_x/n_y , and it is equal to: $\frac{n_x}{n_y} = \frac{I_x N_y}{I_y N_x}$,

Therefore, the fraction ratio of compound X in the mixture is [204]: $\frac{n_x}{\sum_{i=1}^m n_i} = \frac{I_x/N_y}{\sum_{i=1}^m I_i/N_i}$

To determine the purity of a compound, an internal standard should be used. The internal standard is a molecule with high purity (>99%) added to the mixture. Afterward, the mixture is dissolved in an NMR solvent and placed in the machine for NMR determination. The weight of each sample must be determined accurately (10^{-2} mg, in our case we used the PerkinElmer autobalance AD6 10^{-3} mg). The purity of the compound can be calculated as following:

$$P[\%] = \frac{n_{IC} \cdot Int_t \cdot Mw_t \cdot m_{IC}}{n_t \cdot Int_{IC} \cdot Mw_{IC} \cdot m_s} \cdot P_{IC}$$

Where:

m_{IC} = weight (mass) of the internal standard (IC)

m_s = weight mass) of the sample

Int_{IC} = area (integral) of the IC resonance signal being used for quantification

Int_t = area (integral) of the target analyte (t) resonance signal being used for quantification

n_{IC} = number of protons that give rise to Int_{IC}

n_t = number of protons of the target analyte that give rise to Int_t

$M_{w_{IC}}$ = molecular weight of the internal standard

M_{w_t} = molecular weight of the target analyte

P_{IC} = purity of the internal standard, as percent value

2.2.5 Redox titration:

Redox titration is a titration technique in which a redox reaction takes place between the analyte and titrant [205]. A redox indicator and conductometer might be used during the titration. In this study, redox titration was carried on to track the chlorine lost in high protein loaded fluid (5% FBS) for 6a, 6b, and C18.

To this end, 0.25 mmol of each compound dissolved in 10 mL of Milli-Q water to have an aqueous solution of each compound. Afterward, 50 μL of fetal bovine serum (FBS) was added to 950 μL of the prepared solution and mix them on an orbital shaker for different time frames (1, 5, 20, 60, 90 and 120 min). 100 μL of the mixture was added to a mixture of 30 mL of Milli-Q water, 2 mL of 5% acetic acid buffer solution, 1 g (6 mmol) potassium iodide and five drops of freshly prepared 1% starch solution to get a solution with a dark purple color. 0.001 N sodium thiosulfate standard solution was used to titrate (dropwise addition) the mixture using burette. The titration stopped when the solution color turned clear, and the chlorine content was calculated as below: $Cl\% = \frac{M_w \times 0.0005 V_t}{m - 0.017225 V_t}$

Where M_w , m , and V_t stand for molecular weight (g/mol) and mass of the compound (g), and burette reading (mL), respectively.

2.2.6 Quantitative antimicrobial assay (*in PBS and High protein medium*):

A bacterial suspension of a different strain of bacteria in Phosphate-buffered Saline (PBS, 0.1 M, pH 7.4) was prepared at a concentration of 10^8 CFU/mL using 0.5 McFarland standard. After 100 times dilution, 20 μ L of diluted suspension was added to 60 mL of different broths based on the bacterial strains, Tryptone Soy Broth in the case of MRSA and Mueller Hinton Broth for *P. aeruginosa* and *E.coli*, followed by 18 hr incubation at 37 °C. After incubation, 50 μ L of bacterial suspension was added to a solution of 5 [Cl]⁺ ppm to 20 mL PBS (0.1 M, pH 7.4) and 15 [Cl]⁺ ppm to a solution of 5% FBS in PBS and incubated on the orbital mixer at 37 °C. At each timepoint (1, 3, 5, 10, 20, 30, 60 min) , 150 μ L of mixture was added to 150 μ L of neutralizer solution (PBS buffer consisting of 1.4% [w/v] lecithin and 10% [w/v] Tween 80) to deactivate antimicrobial agent. 30 μ L of bacterial suspension was diluted, 10^4 - 10^6 times, and plated on TryptonSoya Agar plates and incubated for 24 h, followed by colony counting.

2.2.7 Results and Discussions

2.2.7.1 Chemistry Synthesis

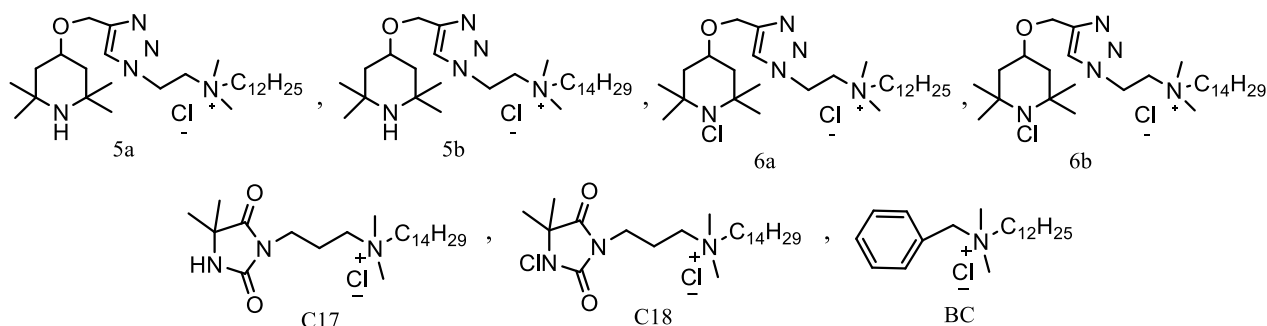


Figure 2-3. Chemical structure of different compounds used as biocide in this work

We previously demonstrated the antimicrobial enhancement effect of covalently binding long alkyl chain QAC moieties to 5,5-dimethyl hydantoin (DMH) *N*-chloramine analog (C17 and C18 in **Figure 2-3**) [206]. In this study, we attached different long chain QACs to 2,2,6,6-Tetramethylpiperidinol *N*-chloramine moieties (5a,5b,6a,6b in **Figure 2-3**) to explore the effect of secondary amine which provides more chloramine stability in high protein media. The chemical reactions pathway is represented in **Figure 2-2**. An overnight reaction between different long chain alkyl bromide compounds led us to have azido functionalized QACs 3a and 3b. 2,2,6,6-tetramethyl-4-(prop-2-yn-1-yloxy)piperidine **4** was prepared using the method utilized by Barnes et al [207] from 2,2,6,6-Tetramethylpiperidinol. *N*-chloramine and different QACs were linked together via a triazole bridge. To have this linkage a click reaction between an azide group on the QACs and the alkyne group on the piperidinol *N*-chloramine took place to get 5a and 5b. By passing 5a and 5b through an ion exchange resin and performing the chlorination reaction using *t*-BuOCl, 6a and 6b were achieved. The list of all the biocide used in this study is represented in **Figure 2-3**.

2.2.7.2 Copper analysis

Copper content of each compound was measured to prevent unwanted antibacterial activity overlapping since the copper ion shows antimicrobial activity. The Cu ^{327.395} content for 5a and 5b were 0.13 and 0.075 ppm, respectively. The amount of Cu ^{324.754} also was measured and appeared to be 0.127 and 0.068 ppm for 5a and 5b. The amount of copper left in each compound found to be very less than the minimum concentration of copper required for antimicrobial activity, reported to be 40 ppm by Gyawali et al [208].

2.2.7.3 Antimicrobial evaluation of new and old biocides

Table 2-1 demonstrates the time needed for the antimicrobial agents to inactivate all the bacteria (total kill) in different solutions (PBS and HPM). It was observed that chlorinated compounds performed better in PBS than non-chlorinated ones which indicate that the introduction of *N*-chloramine boosts the antibacterial activity of the whole molecules regardless of their molecular structure. However, in high protein media (HPM), the antimicrobial efficacy of all biocides (chlorinated and non-chlorinated) deteriorated and the time to achieve total kill for all of them increased, in comparison to PBS. In the case of QAC, this deterioration in activity is the result of ionic interactions between QAC and negatively charged peptides on proteins molecules which resulted in binding of QAC molecules to proteins and deactivation of QAC [209]. On the other hand, chlorine on *N*-chloramine compounds can transfer to proteins or be reduced by sulfur-containing amino acids such as cysteine and methionine, in HPM [210]. However, the consumption rate of chlorine varies from one compound to another.

Table 2-1. Required time to reach the total kill (6 log reduction) for different compounds in PBS and HPM against a different strain of bacteria.

Bacterial strain	Media	Time to total kill (min)						
		5a	6a	5b	6b	C17	C18	BC
MRSA (70065)	PBS	> 60	3	60	5	10	3	10
	HPM	> 60	20	> 60	30	20	30	20
E. coli (25922)	PBS	> 60	3	> 60	3	30	3	3
	HPM	> 60	3	> 60	5	30	20	5
MDR P. aeruginosa (73104)	PBS	> 60	< 1	< 1	< 1	5	5	5
	HPM	> 60	5	60	30	60	30	10
Wild type P. aeruginosa (PA01)	PBS	> 60	3	5	3	5	5	5
	HPM	> 60	20	> 60	> 60	> 60	> 60	30

It was observed that the antimicrobial activity deterioration in the case of piperidine based *N*-chloramine compounds (6a, 6b) was lower than C18 of which the decline in activity was so significant (increase in time to total kill for 6a and 6b is very less, average 13 min increase in compare to 23 min increase in the case of C18). On the other hand, the performance of C18 in PBS was better than C17 but their activity is almost the same in HPM which indicates that [Cl]⁺ on C18 is reduced and C18 is converted back to C17. Antimicrobial performance of 6b decreased in HPM more than 6a due to more protein binding of a QAC with a 14 carbon

chain but less than C18 as a result of less premature loss of $[Cl]^+$ on secondary amine based *N*-chloramines by a redox reaction with proteins than chlorinated amide. These results are in agreement with the redox titration results of chlorinated compounds after contacting with high protein medium which is provided in **Figure 2-4**.

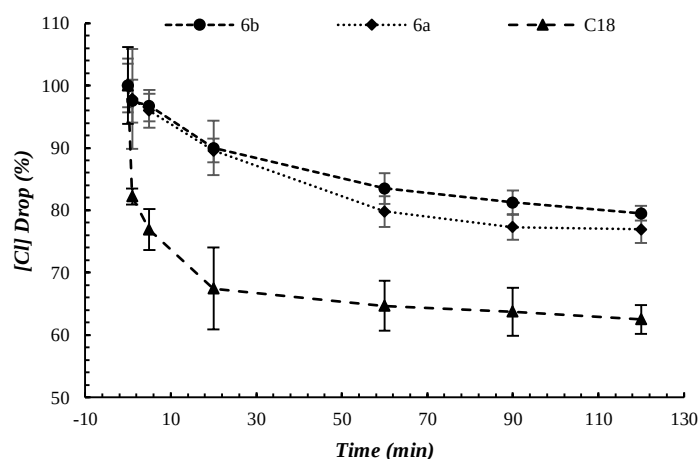


Figure 2-4. Chlorine content of 6a, 6b, C18 in contact with high protein media measured by redox titration

The redox titration assay demonstrated that the chlorine content of C18 (hydantoin-based *N*-chloramine) drops dramatically to 80% within the first 5 min of contact with HPM, and the chlorine content reached a plateau after 30 min and stayed 60%. On the other hand, both 6a and 6b compounds (amine based *N*-chloramine) showed the same behavior and their chlorine content dropped to 80% after 60 min. These results showed that the chlorine on secondary amine (6a and 6b) is more stable than that on amide in HPM. Also, the rate of chlorine consumption in HPM (5% FBS in PBS) is much lower for amine based *N*-chloramine than amide based *N*-chloramine.

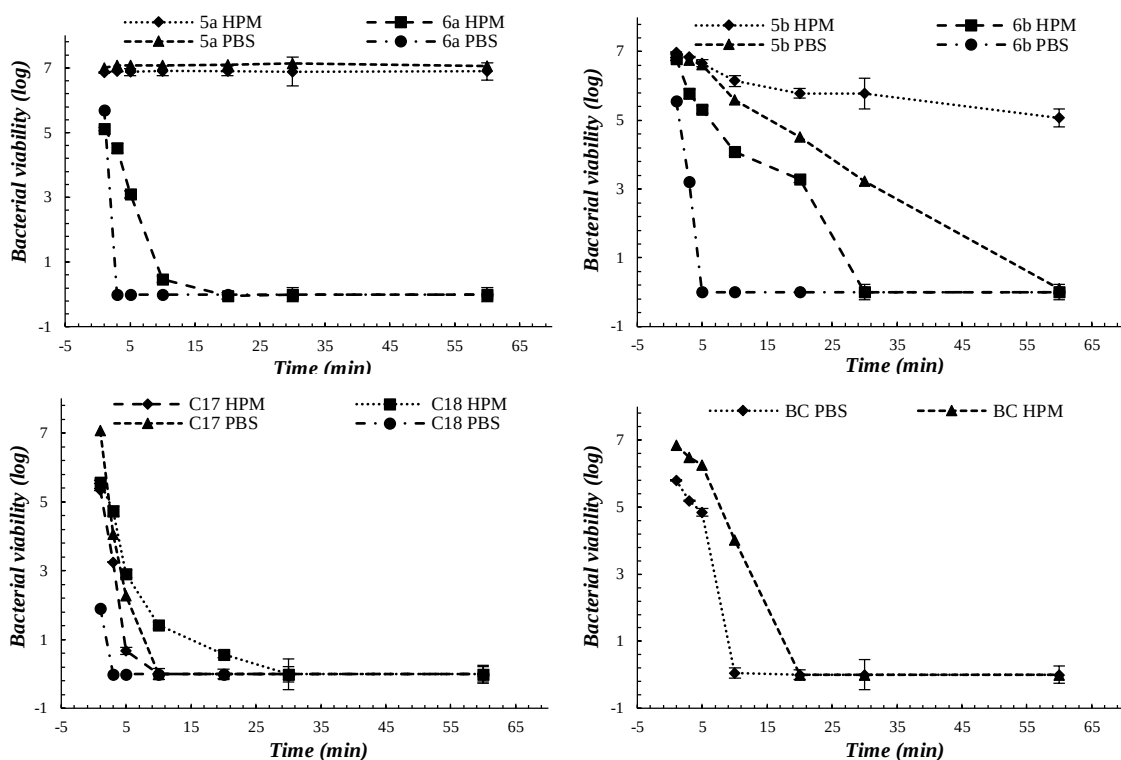


Figure 2-5. Bacterial viability (log) as a function of contact time between biocides and bacteria for MRSA in high protein media (HPM, 5% FBS in PBS) and PBS

Figure 2-5 to **Figure 2-8** provide the quantitative antibacterial results over the contact time with the different bacteria in various media. The composite biocides tended to be more effective against the Gram-negative strains (*E. coli* and *P. aeruginosa*) than Gram-positive one (*MRSA*). Gilbert and Moore [118] have shown that antibacterial activity of QACs is a function of their structure, alkyl chain length, and the strain of bacteria. For example to be effective against Gram-positive bacteria the alkyl chain should consist of 12–14 carbon, however, in the case of Gram-negative bacteria this number is 14–16 alkyls.

Against *MRSA* (**Figure 2-5**), 6a and C18 showed the best performance in PBS. As it was expected their activity deteriorated in HPM but the rate of the antimicrobial activity reduction was different when it came to different antimicrobial agents. C17 and BC showed the least amount of change in antimicrobial activity among all other biocides used in this study against

MRSA.

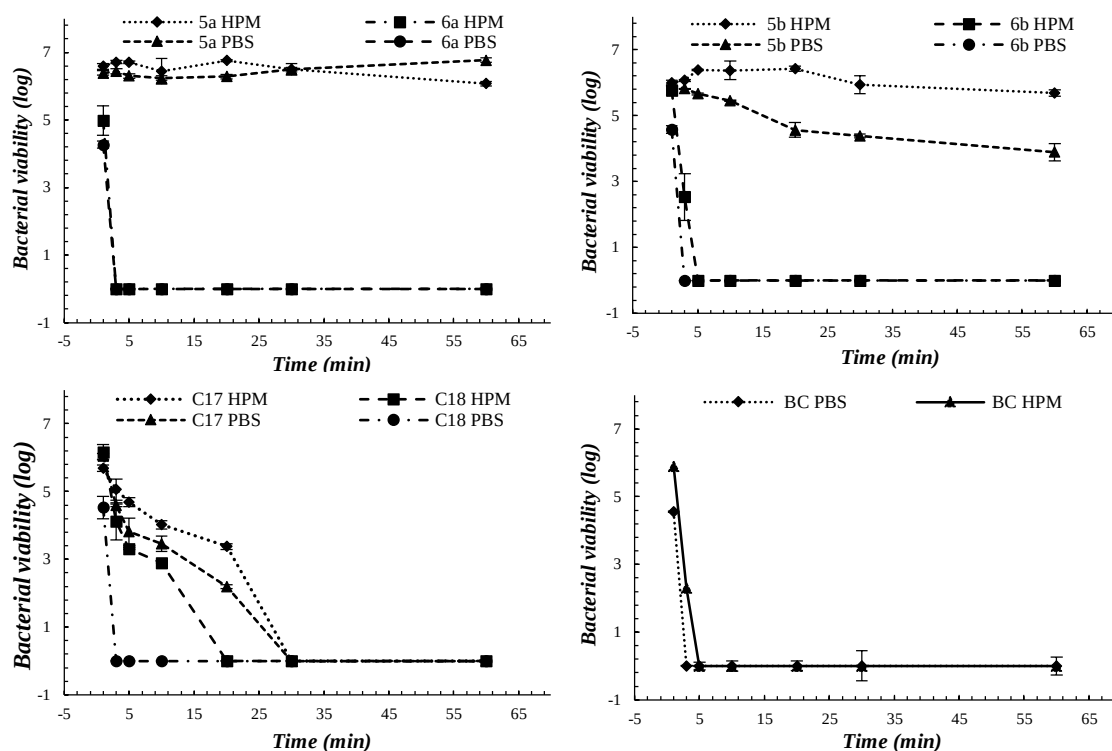


Figure 2-6. Bacterial viability (log) as a function of contact time between biocides and bacteria for *E. coli* in high protein media (HPM, 5% FBS in PBS) and PBS

The activity of C18 dramatically dropped against MRSA in high protein media (**Figure 2-5**). 6a and 6b showed a decline in their activity, but still better than C18. In addition, the antimicrobial property of 6a in the HPM was equal to BC and the best among all the chlorinated compounds which showed superior activity in the PBS.

Biocides demonstrated different antibacterial behavior against *E.coli* and *P. aeruginosa*, Gram-negative bacteria. 6a, 6b, C18, and BC showed almost the same activity in PBS. However, in the case of *P. aeruginosa*, 6a, and 6b illustrated superior killing than others. In HPM, 6a and 6b displayed no changes or the least decline in their activity among the chlorinated biocides.

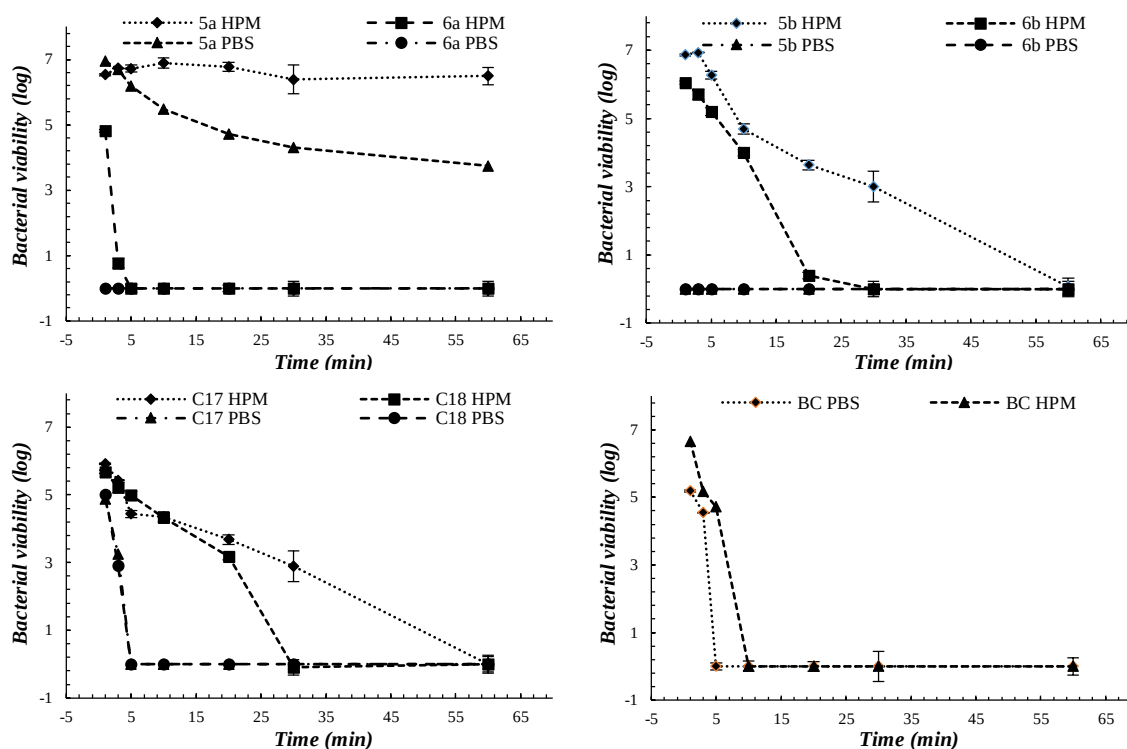


Figure 2-7. Bacterial viability (log) as a function of contact time between biocides and bacteria for *P. aeruginosa* in high protein media (HPM, 5% FBS in PBS) and PBS

On the other hand, C17 and BC did not show any changes in their behavior in different media against *E. coli*. However, C17 lost its antimicrobial activity in HPM against *P. aeruginosa*.

Wild-type *P. aeruginosa* was the hardest strain of Gram-negative bacteria to treat. All the biocides, except 5a, were effective against this strain in PBS, and they reached total kill within the first 5 min of contact. However, only 6a (in 20 min) and BC (30 min) reached total

kill of PA01 in HPM, and all other biocides failed to be effective in HPM. This tough bacterial strain revealed the high efficiency and superior antimicrobial activity of 6a in both PBS and HPM.

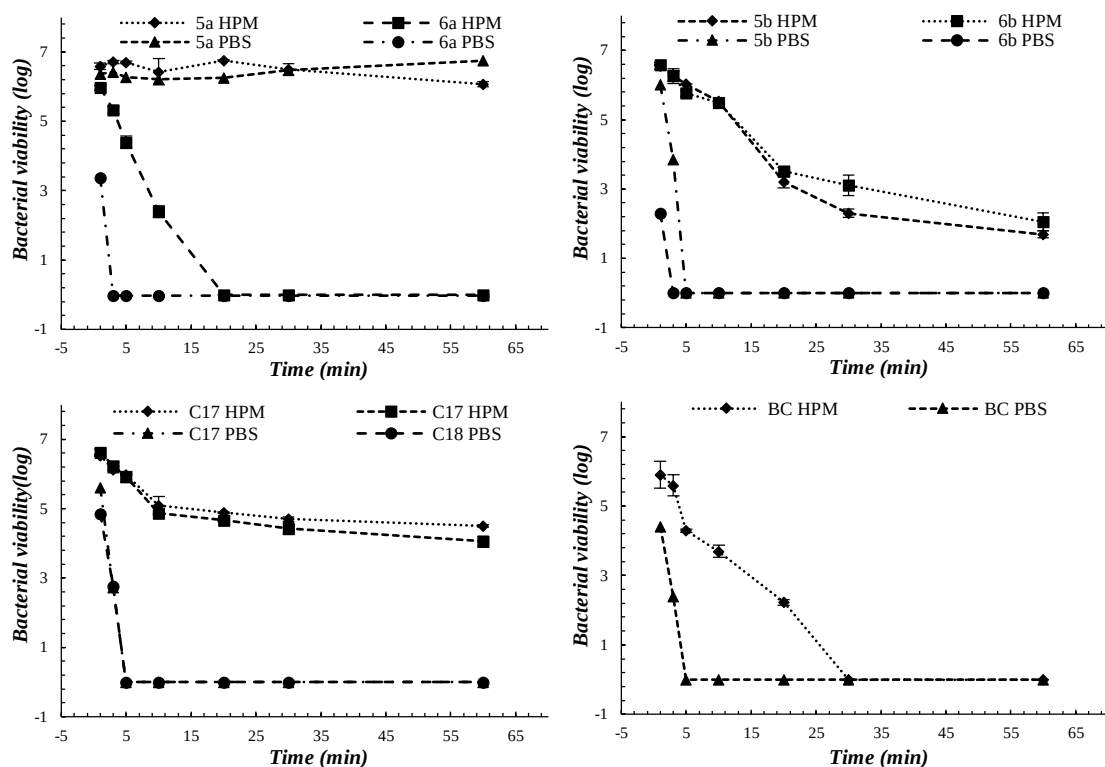


Figure 2-8. Bacterial viability (log) as a function of contact time between biocides and bacteria for wild PA01 in high protein media (HPM, 5% FBS in PBS) and PBS

2.3 Conclusions

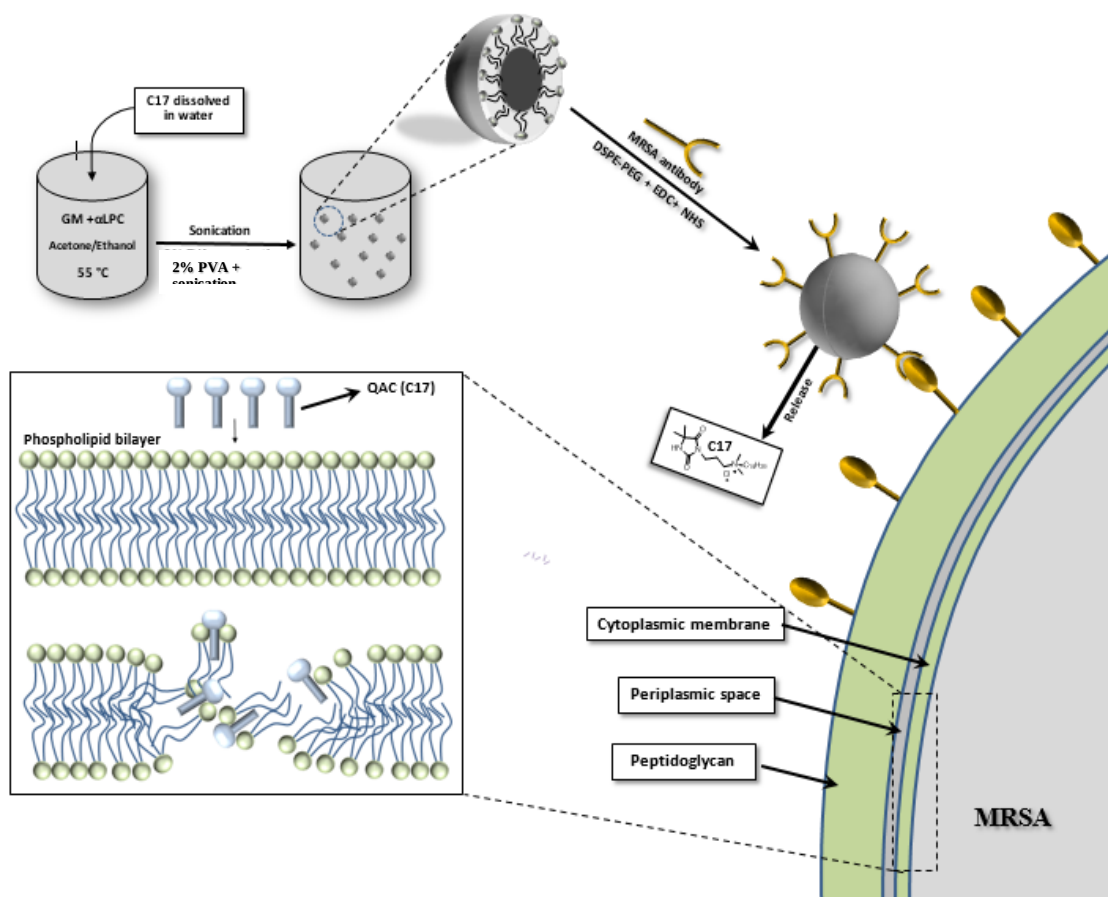
Nowadays, prevention of bacterial contaminations in food industries has become so difficult due to antimicrobial resistance, and high volume and large production [211]. On the other hand, the number of effective antimicrobial agents with a good performance in high protein containing environment is limited [212].

In this study, different amine based *N*-chloramine/QAC composited biocides synthesized using click chemistry to link two moieties. These synthesized biocides were challenged with

various bacterial strains including Gram-positive and Gram-negative bacteria such as MRSA, *E.coli*, multi-drug resistant *P. aeruginosa* and wild-type *P. aeruginosa* (PA01) in both PBS and HPM. Results indicated when an amine, especially secondary amine, was used as *N*-chloramine moiety the chlorine consumption in high protein loaded fluids decreased which resulted in less antimicrobial activity deterioration. This finding can help to design new superior antimicrobial agents for different conditions, especially in food industries. Our highly active composite biocide (6a) demonstrated better antimicrobial efficacy in both PBS and HPM than other biocide utilized in this study. The new strategy can decrease the volume of biocide used in food industry and facilitates healthy food production.

3 Chapter 3: A new strategy for battling bacterial resistance: turning potent, non-selective and potentially non-resistance-inducing biocides into selective ones

To combat the problem of antibiotic resistance, scientists have been working hard to search for antibiotic alternatives. In the present study, selective delivery of a potent and potentially non-resistance-inducing biocide ([3-(4,4-Dimethyl-2,5-dioxo-imidazolidine-1-yl)-propyl]-dimethyl-tetradecyl-ammonium chloride, C17) to superbug methicillin-resistant *Staphylococcus aureus* (MRSA) was achieved by encapsulating it in biocompatible solid lipid nanoparticles (SLNs) conjugated with a MRSA-specific antibody (termed as "Ab"). The C17-loaded Ab-conjugated SLNs (C17-SLN-Ab) have an average size of 272 nm and a C17 encapsulation efficiency of 67%. C17-SLN-Ab demonstrated superior antimicrobial activity than its antibody free counterpart (C17-loaded SLN, "C17-SLN") and C17-SLN conjugated with a nonspecific IgG antibody (C17-SLN-IgG). To examine the selectivity of SLNs, we developed an assay in which toxicity of the SLNs towards MRSA was determined in the presence of fibroblast cells. In this assay, C17-SLN-Ab demonstrated selective toxicity towards MRSA than fibroblast cells. C17-SLN-Ab possesses double selectivity, showing higher toxicity to MRSA than to *Pseudomonas aeruginosa*. We evaluated the interaction of SLNs with MRSA and *Pseudomonas aeruginosa* by TEM and found that SLN-Ab bound to MRSA but not to *P. aeruginosa*. This same strategy was used to successfully increase C17's selectivity against *E. coli* K12 by simply switching the conjugated anti-MRSA antibody to an anti-*E. coli* antibody (termed as "eAb"), demonstrating the versatility of this new strategy. This proof-of-concept research can be extended to other potent antimicrobial agents, against which bacterial resistance is unlikely to develop, to generate a new group of promising antibiotic alternatives.



3.1 Introduction

The growing antimicrobial resistance presents a pressing global healthcare problem. Current worldwide mortality from antibiotic-resistant infections is around 700,000 and it has been predicted that the number of deaths caused by antimicrobial resistance will reach 10 million by 2050, which is more than deaths caused by cancers (8.2 million) [29].

Increased awareness of this global crisis has fueled intensive research targeting antimicrobial resistance, especially the development of antibiotic resistance. Medicinal chemists have been working on several combating strategies. One strategy is to use combination therapy [192] to potentiate the efficacy of existing antibiotics. Randhawa and co-workers formulated cell-penetrating peptides (CPPs) P3 and P8 with four

antibiotics (erythromycin, oxacillin, norfloxacin, and vancomycin) and found that the CPPs at low concentrations ($<10\ \mu\text{M}$) can dramatically decrease the maximum inhibitory concentration (MICs) of oxacillin, norfloxacin, and vancomycin against several clinical isolates of Methicillin-resistant *Staphylococcus aureus* (MRSA). A step further was to covalently link a CPP with an antibiotic to boost its efficacy against persistent bacteria [213].

Another strategy is to genetically target the resistance and design new biocides to thwart the resistance mechanism. It is also called “structure-based design.” New biocides are rationally designed based on the structural understanding of the binding between genetic targets and biocides, as well as the co-crystal structure of the mutated targets. Modest success has been achieved. For example, bacteria can mutate to manifest conformational change at the binding pocket for a protein synthesis inhibitor – linezolid. A new analog of linezolid, tedizolid, was creatively designed to adapt to the structural mutation at the binding site [214], [215]. While the structure-based strategy enables medicinal chemists to synthesize new antibiotics to defeat the existing resistance, there is no guarantee that new resistance won’t occur since it might be an evolutionary inevitability. Also, the development of new antibiotics is a costly and lengthy process.

We intend to approach this problem from the perspective of material science by converting potent broad spectrum non-selective biocides into selective ones. The idea is to reduce the toxicity of potent broad-spectrum biocides by encapsulating them in biocompatible solid lipid nanoparticles (SLNs) and increase the selectivity of these SLNs by conjugating a bacterium-specific antibody onto the surface of the SLNs. A quaternary ammonium compound (QAC) and a “composite” biocide with both QAC

and *N*-chloramine moieties newly developed in house were found to not select for resistant mutants of *P. aeruginosa* despite strenuous efforts of isolating *P. aeruginosa* strains with increased tolerance to those biocides [216]. This might be ascribed to their non-specific interaction with vital proteins in bacterial cells. When used on topical wounds to remove bioburden or taken orally for systematic infection control, the potent broad-spectrum biocides, however, can also compromise skin cell viability or exert toxicity to endothelial cells in the esophagus. Thus, we intend to turn the potent non-resistance-inducing biocides into selective antibacterial agents using nanotechnology, adding a new tool to the shrinking toolbox for combating antibiotic-resistant bacteria.

Encapsulation of drugs into nanoparticles can help improve the pharmacokinetics, therapeutic and metabolic index of the drugs [63]. Various nanoparticles have been used as carriers in the pharmaceutical industry namely: liposomes, dendrimers, SLNs and polymeric nanoparticles [152]. SLNs have documented advantages such as biocompatibility, high encapsulation efficiency for hydrophobic compounds, tissue tolerance and easy production in large quantities [217]. Encapsulation of antibiotics has been employed recently as a means to enhance antibiotic efficacy, and achieve lower MIC and lower probability of antibiotic resistance [61], [63], [152], [218]–[220]. Also, studies have been carried on encapsulating antimicrobials other than antibiotics such as antibacterial peptides [221], nano-metals [222], and bacteriophage [223]. Although the nanotechnology methodology is proven to increase the efficacy of existing antibiotics and slow down the resistance development, it does not intrinsically bypass all the genetic resistances. Bacterial resistance can still develop through overexpression of target enzymes, alteration of the binding sites, and enzymatic degradation of encapsulated antibiotics. Encapsulation of broad-spectrum biocides

such as *N*-chloramines and QACs in SLNs could potentially avoid genetic resistance from bacteria and impart the biocides with selectivity towards bacterial cells than human cells.

To enhance the selectivity of biocide encapsulated SLNs, monoclonal antibodies specific for the bacteria can be conjugated onto the surface of the SLNs. Monoclonal antibodies have been researched as an emerging alternative against bacterial infection and endotoxins. They have also been used for point-of-care diagnostics [224]. Since this kind of antibodies is highly selective to a particular pathogen and does not directly impact on bacteria viability, it is unlikely to induce resistance. This strategy also matches the current trend and efforts of boosting point-of-care diagnosis which is expected to improve clinical outcomes for patients and decrease the chance of antibiotic resistance. In response to the World Health Organization's 2014 global report on worsening antimicrobial resistance [21], the Canadian Institutes of Health Research has launched a series of calls for proposals on point-of-care diagnostics in human health as part of the Canadian Antimicrobial Resistance Federal Action Plan. Similarly, the Infectious Diseases Society of America (IDSA) recommended the US Congress to increase the support for the development of innovative infectious diseases diagnostics [224]. In short, our new strategy of utilizing antibody-conjugated nanoparticles to convert potent broad-spectrum non-resistance-inducing and non-selective biocides into selective antibacterial agents is novel and converges with the development of rapid antibody-based bedside diagnostics to successfully combat the threat of antibiotic resistance.

3.2 Experimental

3.2.1 Synthetic procedures

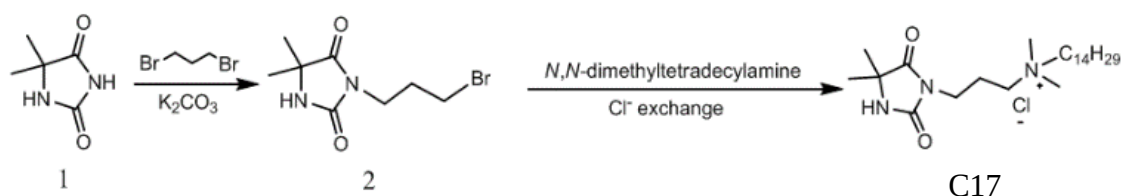


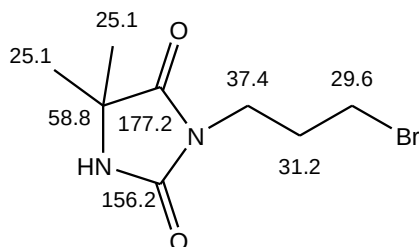
Figure 3-1. Reaction Scheme of the QAC: C17

Synthesis of Bromide 2 (Figure 3-1)

2.05 g (16.0 mmol) of 5,5- dimethyl hydantoin (Sigma-Aldrich, USA) was dissolved in 200 mL acetone (Sigma-Aldrich, USA). 6.63 g (48.0 mmol, 3 equiv.) of potassium carbonate (Fisher Scientific, CA) was added to the solution and reaction continued for 30 min followed by adding 9.69 g (48.0 mmol, 3 equiv.) of 1,3-dibromo propane (Sigma-Aldrich, USA). After 4 h reaction at reflux, solid salt was removed using filtration. The filtrate was evaporated to give clear liquid which was purified employing column chromatography (Silica packed, EtOAc/Hexanes = 1/2–1/1 by volume) to achieve white solid (3.35 g, 84%);

1H NMR ($CDCl_3$, 300 MHz) δ [ppm]: 3.66 (t, $J = 6.9$ Hz, 2 H: $BrCH_2CH_2CH_2N$), 3.40 (t, $J = 6.6$ Hz, 2H: $BrCH_2CH_2CH_2N$), 2.22 (m, 2 H: $BrCH_2CH_2CH_2N$), 1.46 (s, 6 H: $NHC(CH_3)_2$);

^{13}C NMR (CDCl_3 , 75 MHz) δ [ppm]: 177.2, 156.2, 58.8, 37.4, 31.2, 29.6, 25.1; HRMS (MALDI-TOF) m/z : $[\text{M} + \text{H}]^+$ calculated for $\text{C}_8\text{H}_{14}\text{BrN}_2\text{O}_2$: 249.0238, found: 249.0235.

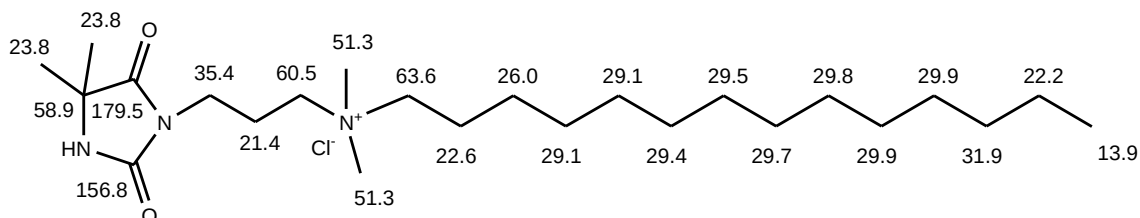


Synthesis of QAC C17 (Figure 3-1)

1.2 g (4.8 mmol) of bromide 2 and 1.74 g (7.20 mmol, 1.5 equiv.) of N, N-dimethyl tetradecyl amine (Sigma-Aldrich, USA) were dissolved in CH_3CN (40 mL, Sigma-Aldrich, USA), and the reaction continued for 24h at reflux. After solvent removal, Br- form of QAC was partitioned between MeOH/Hexane, 1/1 by volume, as a white solid (2.072 g, 88%). The Br- QAC was dissolved in a minimum amount of water and passed through an anion-exchange resin (Amberlite R IRA-900, Cl-) dropwise to get Cl- QAC C17.

^1H -NMR (D_2O , 300 Hz) δ [ppm]: 3.60 (t, $J = 6.7$ Hz, 2H: $\text{NCH}_2\text{CH}_2\text{CH}_2\text{N}^+$), 3.31-3.38 (m, 4 H: $\text{NCH}_2\text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_2\text{CH}_2$), 3.15 (s, 6 H: $\text{NCH}_2\text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_2\text{CH}_2$), 2.09-2.14 (m, 2H: $\text{NCH}_2\text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_2\text{CH}_2$), 1.71-1.73 (m, 2H: $\text{CH}_2\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2$), 1.44 (s, 6 H: $\text{CONHC}(\text{CH}_3)_2\text{CO}$), 1.31-1.37 (m, 22 H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$), 0.91 (t, $J = 6.7$ Hz, 3 H: $\text{N}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$);

^{13}C -NMR (CDCl_3 , 75 Hz) δ [ppm]: 179.5, 156.8, 63.6, 60.5, 58.9, 51.3, 35.4, 31.9, 29.9, 29.8, 29.7, 29.5, 29.4, 29.1, 26.0, 23.8, 22.6, 22.2, 21.4, 13.9; HRMS (MALDI-TOF) m/z : $[\text{M}-\text{Cl}]^+$ calculated for $\text{C}_{24}\text{H}_{48}\text{N}_3\text{O}_2^+$, 410.3741; found: 410.3746.



3.2.2 Fabrication of C17 encapsulated SLNs

Preparation of SLNs was based on the emulsion solvent diffusion method with slight modifications whereby a water/oil/water (w/o/w) double emulsion was introduced into the method [225]. Briefly, glyceryl monostearate (molecular weight 358.56 g/mol, Sigma-Aldrich, USA) and α -L-phosphatidylcholine (Soy-95%) (Molecular weight 770.123 g/mol; Avanti Lipids, USA) were dissolved thoroughly in a mixture of equal volumes (1 mL) of acetone and ethanol (HPLC grade; EMD Serono, Canada) at 55°C. C17 was dissolved in deionized water and added to the lipid mixture under continuous sonication for 30 sec by a microtip probe sonicator (Branson sonifier 150D; QSonica, USA) to form a w/o primary emulsion. The resultant w/o emulsion was then emulsified with an aqueous phase solution containing 2% polyvinyl alcohol (PVA; molecular weight 31-50 kDa; Sigma-Aldrich, USA) for 50 sec under continuous sonication to form a w/o/w secondary emulsion. After adding DSPE-PEG-2000 COOH (Avanti Lipids, USA) (final concentration of 100 μg PEG in the formulation), the resultant dispersion was then stirred overnight at 4°C to evaporate the organic solvent. C17 encapsulated SLNs (C17-SLN) were collected by centrifugation (Sorvall RC6+; Thermo Fisher Scientific) and washed twice with autoclaved Milli-Q water.

3.2.2.1 Antibody conjugation to SLNs

The SLN suspension in 2-(N-morpholino)ethane sulfonic acid (MES) (Sigma-Aldrich, USA) was incubated with 1-ethyl-3-(3-dimethylaminopropyl) solutions obtained from G-Biosciences (MO, USA) at 4 °C for 1 hour on a rotary shaker for activating the carboxylic group. The activated SLNs were incubated with NYR MRSA 16 antibody (SC-73327 LOT # F1708 purchased from Santa Cruz USA) or rabbit anti-*E. coli* IgG type antibody (ab137967 from Abcam Inc, Canada) at 4 °C for 2 hours on a rotary shaker to yield C17-SLN-Ab or C17-SLN-eAb. Both SLNs were then washed and collected using a centrifuge to remove excess NHS, EDC, and unconjugated antibodies. The resultant antibody conjugated SLNs were lyophilized and stored at 4 °C until further use. As a control, a non-specific immunoglobulin G antibody (normal mouse IgG1, SC-3877, Santa Cruz, USA) was also used to conjugate C17-SLN to yield nanoparticles termed as “C17-SLN-IgG”.

3.2.2.2 Antibody-conjugation efficiency (ACE%)

QuantiPro™ BCA assay (QPBCA; Sigma-Aldrich, USA) was performed to determine ACE%. Briefly, unconjugated antibody recovered in the supernatant was quantified by mixing with an equal volume of BCA reagent and incubating it at 60 °C for 60 min as per the manufacturer's instructions. The samples were then cooled to room temperature and analysis was performed at $\lambda_{562 \text{ nm}}$ and $\lambda_{505 \text{ nm}}$ with BSA used as a standard. ACE% was determined by the following equation:

$$\text{ACE (\%)} = \frac{(\text{Amount of total antibody} - \text{Amount of unconjugated antibody})}{(\text{Amount of total antibody})} \times 100$$

3.2.2.3 Lyophilization of SLN dispersion

SLNs were resuspended in a 25% pearlitol® DC-mannitol, stored at -80°C for 5 h and then freeze-dried for 12 h (FreeZone 2.5-L freeze-dry system; Labconco, USA).

Particle Sizing and Zeta Potential Measurements: Lyophilized SLNs were resuspended in autoclaved Milli-Q water, and its size was measured by dynamic light scattering (ZetaPALS, Brookhaven Instruments, USA). Measurements were obtained at a 90° fixed-angle, and scattering intensity data were analyzed by a digital correlator and fitted by the method of inverse Laplace transformation. Measurements were made in triplicate at room temperature for three runs of 2 min each. Zeta potential measurements determined the net surface charge of SLNs (100 µg/mL; Smoluchowski mode) (ZetaPALS, Brookhaven Instruments, USA). Blank-SLN, C17-SLN, and C17-SLN-Ab, C17-SLN-eAb and C17-SLN-IgG were resuspended in autoclaved Milli-Q water (pH 7). Measurements were performed in triplicate at room temperature for three runs of 2 min each.

3.2.2.4 C17 encapsulation efficiency (EE%)

To determine the EE% of C17 in C17-SLN, the amount of un-encapsulated C17 in the SLN supernatant was quantified using the Orange II dye assay. Briefly, the supernatant was mixed with 0.5 mL Orange II dye (Sigma-Aldrich, USA), extracted with CHCl₃, and the absorbance was measured at $\lambda = 485$ nm.

Encapsulation efficiency was determined using the following equation:

$$\text{EE (\%)} = \left(\frac{\text{Amount of total drug added} - \text{Amount of unconjugated drug}}{\text{Amount of total drug}} \right) \times 100$$

3.2.3 *In vitro* drug release profile

The release rate of C17 from C17-SLN, C17-SLN-Ab, and C17-SLN-IgG was determined in PBS (pH 7.4) purchased from Amresco, USA. Briefly, 80 mg post-lyophilized C17-SLN, C17-SLN-Ab, and C17-SLN-IgG were resuspended in 5mL release media and maintained at 37°C on an orbital shaker at a speed of 100 rpm (VWR, Canada). The samples were centrifuged at different time points (20,000 g, 40 mins, 4°C) and 2 mL of release medium was removed, replenishing with equal volumes of fresh medium 3. The SLNs have resuspended again for further investigation. For quantifying the concentration of drug released, samples were analyzed using the Orange II dye assay as previously described.

3.2.4 Quantitative antimicrobial assay

A bacterial suspension of MRSA in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) was prepared at a concentration of 10^9 CFU/mL using 0.5 McFarland standard. After 100 times dilution, 20 μ L of diluted suspension was added to 60 mL tryptic soy broth (Oxoid Inc, CA) and incubated for 18 h at 37 °C. Afterward, 50 μ L of bacterial suspension was added to the suspended SLNs in 4.95 mL PBS (0.1 M, pH 7.4) and incubated on an orbital mixer at 37°C. At each time point (10, 30, 60, 90, and 120 min), 60 μ L of the mixture was added to 240 μ L of neutralizer solution (PBS buffer consisting of 1.4% [w/v] lecithin (Sigma-Aldrich, USA) and 10% [w/v] Tween 80 (Fisher Scientific, CA) to deactivate antimicrobial agent. Afterward, 30 μ L of bacterial suspension was diluted and plated on tryptic soy broth. After a 24 h incubation period, the colonies were counted.

3.2.5 Selective killing

To show the targeted activity of C17-SLN-Ab and C17-SLN-eAb, a previously described method was used [226]. Briefly, 10^9 CFU/mL bacterial suspension of MRSA and *P. aeruginosa* were prepared in tryptic soy broth and Moulin-Hilton broth (Oxoid Inc, CA) as mentioned above, respectively. Afterwards, 25 μ L of each bacterial suspension added to 4.95 mL suspension of 20 mg of SLNs in PBS. After proper contact time, 60 μ L of mixture was transferred to 240 μ L of neutralizer solution in a 96-well plate followed by dilution and plating on mannitol salt agar (MSA) (Fisher Scientific, CA) plates to isolate MRSA and Eosin Methylene Blue Agar (EMB agar) (Fisher Scientific, CA) to isolate *P. aeruginosa* and inhibit MRSA, respectively. Plates were incubated for 24 h following by counting colonies.

3.2.6 Co-culture assay of MRSA and Fibroblast cells:

Antimicrobial activity and cytotoxicity of antimicrobial agents are usually determined using separate tests which do not accurately mimic *in vivo* conditions. As a result, we have used a coculture system which will better mimic *in vivo* conditions.[227] Briefly, Dermal Fibroblast Normal (Human, Neonatal, ATCC® PCS-201-010™, ATCC, USA) were cultured in culture flasks maintained at 37 °C in a humidified atmosphere and contains 5% carbon dioxide for 48h. 5% trypsin solution (Fisher Scientific, CA) was used to harvest cells followed by seeding cells in 96 well plates at a density of 200,000 cells/mL (20,000 cells/well). After 24h, if the confluency of cells is more than 90% the fibroblast growth medium (ATCC, USA) was replaced with fresh medium containing 10^5 CFU/mL MRSA. After different contact times with NPs (10, 30, 60, 90, 120 min), the medium was removed and transferred to another 96 well plate followed by several

dilutions and plating to count the number of survived bacteria. Fibroblast cells were washed several times with PBS and the cell viability measured using MTT assay.

3.2.7 Sample preparation for TEM

For sample preparation, the method described by Tamer et al.[228] was utilized. Briefly, 10 mg of SLN-Ab and SLN were washed with Milli-Q water following by centrifugation. Afterwards, 2.5 mL of 10^6 CFU/mL bacterial suspension in PBS was added to NPs followed by 24 h incubation in 37 °C. To fix the bacterial membrane, formaldehyde (make the final concentration 4%) and glutaraldehyde (make the final concentration 2%), which has shown best fixation efficiency among other chemical inactivation and fixation methods [229], were added to the previous solution to fix the membrane and the mixture was then incubated at 37 °C for 4h. Then a small droplet of the prepared suspension was placed on the formvar-carbon coated copper grid followed by negatively staining with 2% (w/v) phosphotungstic acid solution.

3.2.8 Results and discussion

It was hypothesized that highly potent but non-selective, cytotoxic biocides can be converted into selective, noncytotoxic antibacterial agents by being encapsulated in antibody conjugated SLNs. To test this hypothesis, a potent QAC [3-(4,4-Dimethyl-2,5-dioxo-imidazolidine-1-yl)-propyl]-dimethyl-tetradecyl-ammonium chloride, C17], [194], [216] was adopted as a model biocide and a α -L-phosphatidylcholine based SLN was employed as a nanocarrier for C17. C17 encapsulated SLNs is termed as “C17-SLN”. As a proof-of-concept, a monoclonal IgG specific to MRSA (NYR MRSA 16 antibody) was chosen to be conjugated onto C17 containing SLNs (denoted as “C17-SLN-Ab”) to enable selective delivery of C17 to MRSA. For comparison, a

nonspecific immunoglobulin G antibody was also used to conjugate C17-SLN to yield nanoparticles termed as “C17-SLN-IgG”.

Table 3-1. Particle size, Zeta potential and EE%

	Particle size (nm)	Zeta potential (mV)	Encapsulation efficiency (%)	loading of C17 (%)
Blank-SLN	195.8 ± 1.6	-25.10 ± 3.40	-----	-----
C17-SLN	237.9 ± 3.9	-27.00 ± 4.20	67.6 ± 4.1	1.69 ± 0.10
C17-SLN-Ab	272.3 ± 5.4	3.82 ± 1.30	67.2 ± 2.6	1.68 ± 0.07
C17-SLN-IgG	268.0 ± 3.7	5.87 ± 2.14	65.9 ± 3.5	1.65 ± 0.09
C17-SLN-eAb	278.5 ± 6.2	4.12 ± 0.93	66.8 ± 4.8	1.68 ± 0.11

n=3; Data represents ± Standard Deviation.

Abbreviations: SLN- Solid Lipid Nanoparticles.

Particle size, zeta potential, and encapsulation efficiency of Blank-SLN, C17-SLN, C17-SLN-Ab, and C17-SLN-IgG are presented in **Table 3-1**. Encapsulation of C17 into the SLNs increased the particle size by 20%. The effect of drug characteristics and drug entrapment on SLNs particle size has been reviewed by Ekambaram et al. [230] in which they showed the drug encapsulation increases the size of SLNs depending on the nature of drug and the method used to prepare SLNs. Also, antibody conjugation increased the size by 15-20 nm which agrees with Hainfeld’s work [231] that observed

15 nm change in particle size of gold NPs after antibody conjugation. Encapsulation efficiencies of C17 in C17-SLN, C17-SLN-IgG, and C17-SLN-Ab were all higher than 65%. The surface charge is negative in the case of Blank-SLN and C17-SLN due to the carboxylic acid groups of PEG-COOH on the surface. However, conjugation of antibody on SLNs using the carboxylic groups changes the surface charge to positive for C17-SLN-Ab and C17-SLN-IgG. Antibody conjugation efficiency (ACE%) of C17-SLN-Ab was determined to be $63.5 \pm 6.0\%$.

As shown in **Figure 3-2**, the release of C17 from C17-SLN, C17-SLN-Ab, and C17-SLN-IgG occurred in a controlled and sustained manner throughout the study period. C17 release profile followed a biphasic pattern. The end point of the initial burst release occurred at 4 hours for C17-SLN with a release of $3.60 \pm 0.41\%$ ($48.3 \pm 3.1 \mu\text{g}$) and at 8 hours for both C17-SLN-Ab (release of $5.03 \pm 0.10\%$ ($71.44 \pm 2.20 \mu\text{g}$)) and C17-SLN-IgG (release of $5.85 \pm 0.43\%$ ($76.13 \pm 4.8 \mu\text{g}$)). The initial faster release of C17 may be from these biocidal molecules that loosely entrapped in the lipid layer of SLNs. The following slower release of C17 can be ascribed to the diffusion of C17 located in the core of the SLNs. 48h cumulative release % of C17 from the three assemblies is around 6.14-7.44%. Release results showed that C17 is well protected from premature release and the release trend is very similar for different nanoparticles regardless of antibody conjugation.

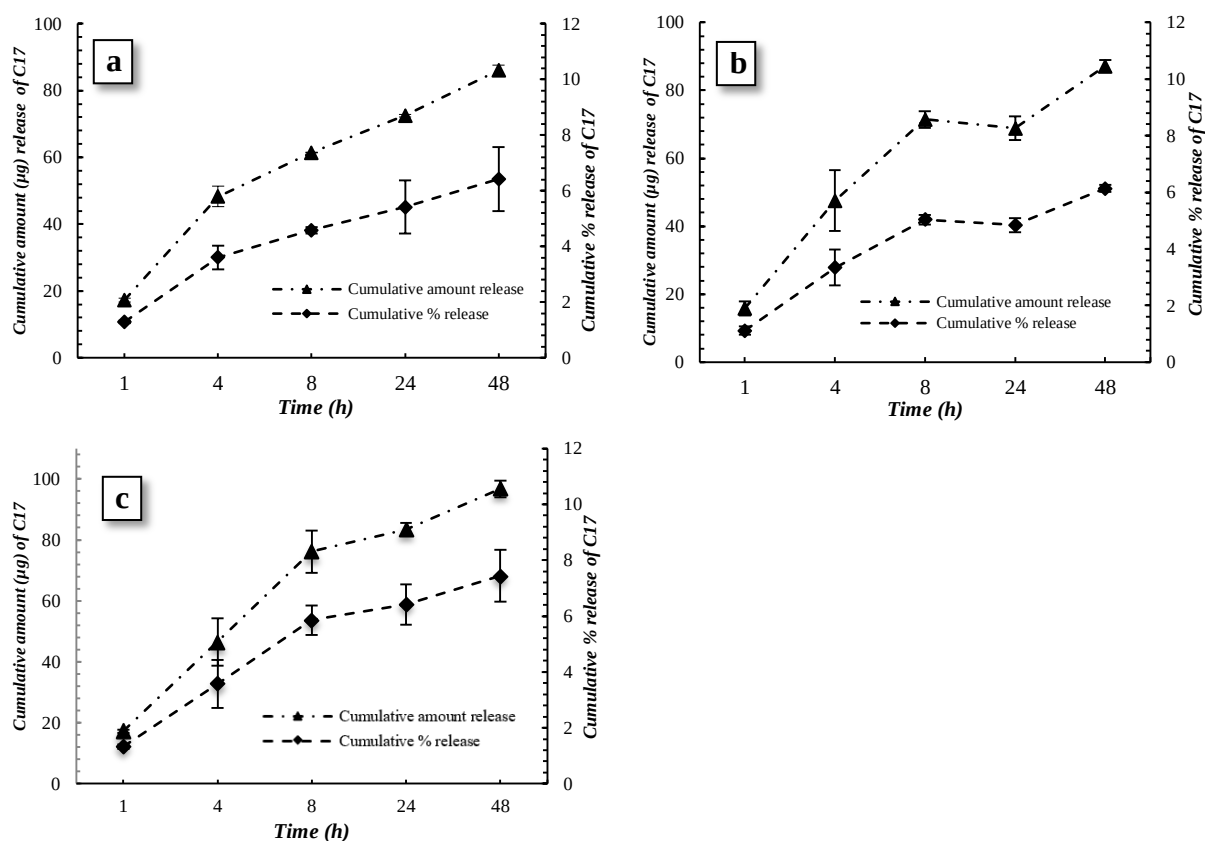


Figure 3-2. In vitro release profile of C17 from C17-SLN (a), C17-SLN-Ab (b) and C17-SLN-IgG (c). Studies were performed at 37 °C in 5 mL of PBS (pH 7.4). Values represent the mean±S.D; n=3 (for C17-SLN) and n=2 (for C17-SLN-Ab).

Figure 3-3 represents the quantitative killing assay for 2 mg/mL and 4 mg/mL of C17-SLN-Ab and C17-SLN against MRSA. Also, C17-SLN-IgG was included to evaluate the effect of the non-specific antibody on antibacterial efficacy of SLNs. Results showed that SLNs with specific anti-MRSA antibody (C17-SLN-Ab) are significantly more effective against MRSA than SLNs without antibody or IgG conjugated SLNs.

4 mg/mL of C17-SLN-Ab showed total kill (no viable bacteria) after 2 h while the other SLN formulations displayed no detectable antibacterial effect at the same concentration. 2 mg/mL of C17-SLN-Ab was partially effective (viable bacteria

decreased from the initial $10^{7.41}$ to $10^{5.7}$ CFU/mL after 2 h of contact) which was the result of the low concentration of SLNs.

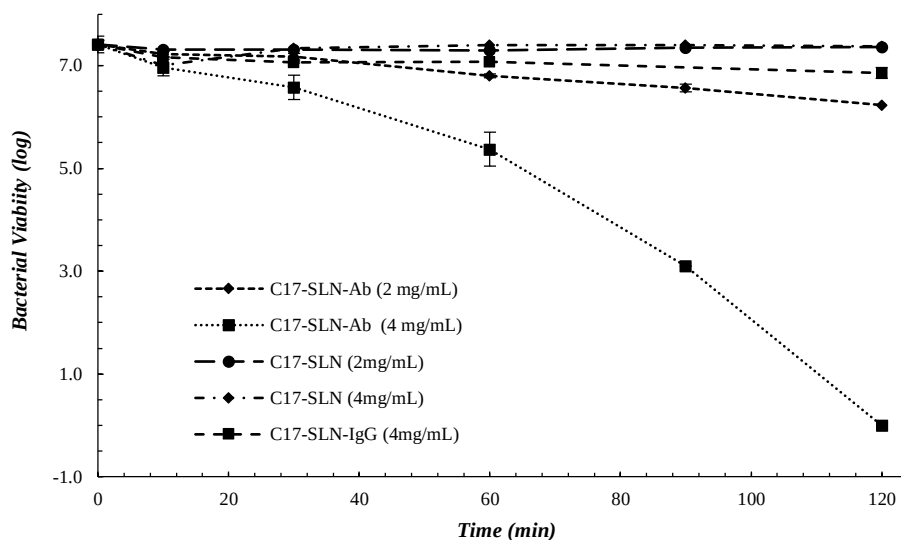


Figure 3-3. Quantitative killing assay for C17-SLN-Ab, C17-SLN in two different concentrations (2 mg/mL and 4 mg/mL) and 4 mg/mL of C17-SLN-IgG against MRSA. Values represent the mean $\pm$ S.D of antimicrobial activity of each SLN series over 2 h; n=3 for all of the compounds. C17-SLN (2 mg/mL), C17-SLN (4 mg/mL), and C17-SLN-IgG are not significantly different ($p>0.05$), however, C17-SLN-Abs (2 and 4 mg/mL) are significantly different ($p<0.05$). The starting inoculum: $10^{7.41}$ CFU/mL.

C17-SLN-IgG at 4 mg/mL exhibited stronger antibacterial property (0.4 log reduction after 2 h) than 4 mg/mL C17-SLN (No detectable reduction). We carried out the same assay on blank-SLNs and SLN-Ab to investigate the effect of non-loaded SLNs and antibody itself and no antimicrobial activity detected. It has been seen by Xu et al [232] that antibody conjugation changes the surface charge of nanoparticles to positive values which our results are in agreement with that finding. Also, the positive charge

has been known to contribute to electrostatic interactions between the antibacterial agent and negatively charged bacterial cell membrane. C17-SLN-IgG has similar, if not higher, amount of positive surface charge than C17-SLN-Ab. But it is not as effective. So, the enhanced anti-MRSA efficacy of C17-SLN-Ab is ascribable to effective binding of the anti-MRSA on C17-SLN-Ab and antigen on the MRSA cells.

The antibacterial assay was carried out with two different bacteria to examine the targeted killing (**Figure 3-4**). A cocktail mixture of MRSA and *P. aeruginosa* was used to challenge the synthesized SLNs to examine the selective targeting on MRSA in the presence of another bacterium. C17-SLNs did not show any antibacterial activity which was expected due to the low concentration of C17 released from these NPs. The concentration of released C17 (free compound) from C17-SLNs after 2 h was calculated to be 6.6 ppm which did not demonstrate any antibacterial activity (**Figure 3-6**). However, C17-SLN-Ab displayed higher antibacterial activity against MRSA than *P. aeruginosa*. No viable MRSA cells could be detected after 2h incubation with 4 mg/mL C17-SLN-Ab, indicating a 7.38 log reduction.

In the case of *P. aeruginosa*, the bacterial viability (log) was 4.82 after 2h which is significantly higher than MRSA. Ning et al. [194] showed that C17 has almost equal killing efficacy towards MRSA and *P. aeruginosa*, meaning the selectivity of C17-SLN-Ab against MRSA over *P. aeruginosa* is due to the conjugation of anti-MRSA antibody.

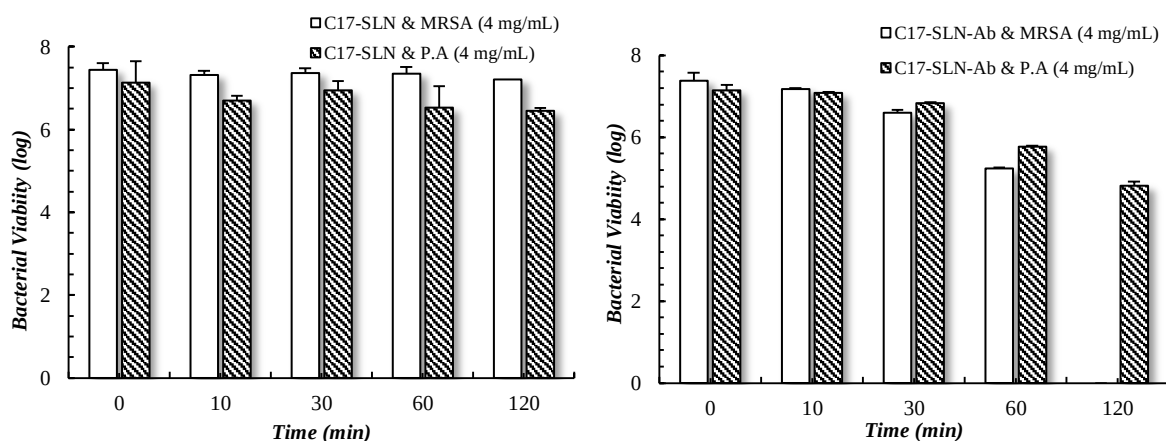


Figure 3-4. Selective antibacterial assay: bacterial reduction (log) as a function of contact time between C17-SLN-Ab and C17-SLN against a mixture of MRSA and *P. aeruginosa* (P.A). Values represent the mean $\pm$ S.D of antimicrobial activity of each SLN series over 2 h in the presence of two different bacterial strains; n=3 for all of the compounds. C17-SLN are not significantly different while contacted with various bacteria ($p>0.05$).

The pressing need of new biocides in wound infection control dictates the need of effective eradication of pathogens in the presence of skin cells. As a result, it is vital to test the cytotoxicity of the nanoparticle assembly. In many studies of new biocides, antibacterial tests and cell cytotoxicity assay were generally conducted separately. Separate assays poorly simulate the real situation and sometimes can mislead researchers. For example, silver NPs are quenched by protein in medium (bovine serum albumin (BSA) enriched medium) to appear to be non-cytotoxic to RAW 264.7 and intestine 407 cells and exist in the unbound form to exert effective antibacterial activity against pathogens in a separate antibacterial assay in PBS [233]. The biocide might high likely be ineffective against the pathogen in a coculture assay where the cell culture medium is enriched to quench it. Therefore, to accurately evaluate the

selectivity of the assembled SLNs against MRSA, a coculture assay was established. **Figure 3-5** represents the co-culture results of free biocide C17, C17-SLN, C17-SLN-Ab. It can be observed that free C17 (**Figure 3-5a**) at the concentration of 66 ppm, which is equivalent to the concentration of encapsulated C17 in the solutions of 4 mg/mL C17-SLN, C17-SLN-Ab and C17-SLN-IgG, displayed severe cytotoxicity to fibroblast cells (<5% viability) after 10 min of contact. Total kill of MRSA was achieved after 60 min (zero percent of bacterial viability) whereas all the fibroblast cells were essentially dead (viability <2%). C17-SLNs (**Figure 3-5b**) did not show significant antibacterial activity and toxicity to fibroblast cells which are consistent with the results of **Figure 3-4**.

On the other hand, anti-MRSA antibody conjugated SLNs showed good antibacterial properties (in different concentrations, **Figure 3-5c** and **Figure 3-5d**) and total kill of MRSA was achieved after 2h. At the same time, fibroblast cells showed almost 50% cell viability for 2 mg/mL of C17-SLN-Ab and more than 20% viability for 4 mg/mL of C17-SLN-Ab after 2h. 2 mg/mL C17-SLN-Ab showed a clear selectivity against MRSA in the presence of fibroblast cells. After all MRSA cells were inactivated after 2h, there were still 50% viable cells which can regrow and heal the wound.

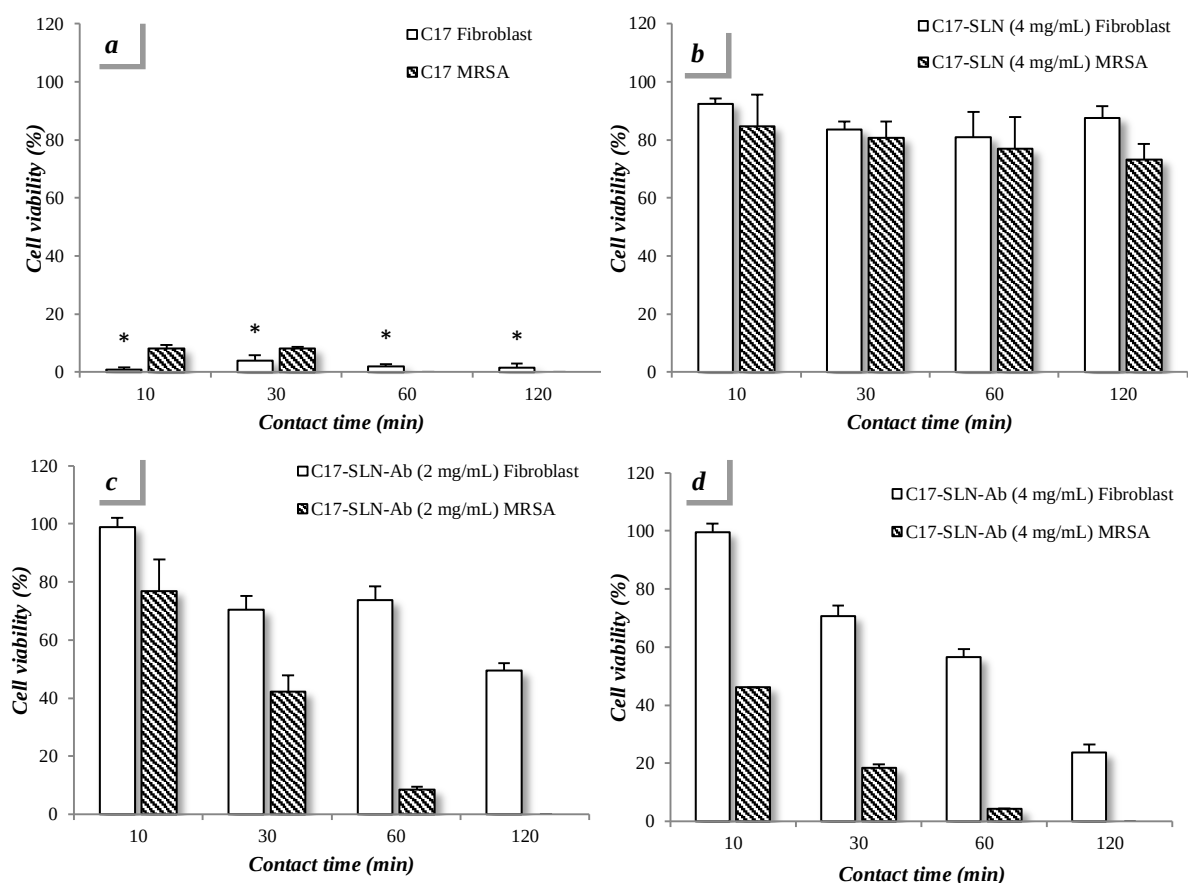


Figure 3-5. Selective toxicity assay between bacteria and Fibroblast cells: bacterial viability (%) as a function of contact time between free C17 (a) , C17-SLN (b) and C17-SLN-Ab in two different concentrations (2 mg/mL (c) and 4 mg/mL (d)) against a mixture of MRSA and fibroblast cells. Values represent the mean $\pm$ S.D; n=3 for all of the compounds. The starting inoculum for MRSA and Fibroblast cells were $10^{4.33}$ CFU/well and 10^4 cells/well. * means no significant difference ($p > 0.05$).

Co-culture results for SLNs (**Figure 3-5c, Figure 3-5d**) and C17 (**Figure 3-5a**) showed that encapsulation of biocide in SLNs conjugated with an anti-MRSA antibody reduces the toxicity of biocides to fibroblast cells.

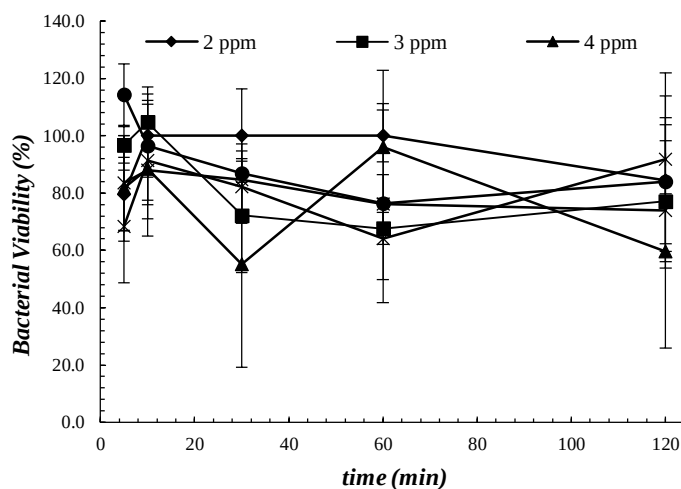


Figure 3-6. Bacterial viability (%) as a function of the concentration of free C17 in the range of released concentration of C17 from 4 mg/mL C17-SLN-Ab. Values represent the mean $\pm$ S.D; n=3. There is no significant difference between the tested concentrations of C17 ($p>0.05$).

Interaction of bacteria and various SLNs was observed under TEM. The study was carried out using the unloaded SLNs (blank SLNs and blank SLN-Ab) to prevent the membrane disruption due to antimicrobial effect of C17. The TEM images are presented in **Figure 3-7**. A MRSA cell is surrounded by many anti-MRSA antibody conjugated SLNs (SLN-Ab, 113.2 $\pm$ 30.9 nm) as denoted by red arrows in **Figure 3-7a**. Few SLN-Ab particles can be seen on or around *P. aeruginosa* (**Figure 3-7d**). The interaction between MRSA and bare SLNs or SLNs conjugated with non-specific antibody (SLN-IgG) can hardly be observed (**Figure 3-7 b,c**). This effective binding between SLN-Ab and MRSA cells might well contribute to the selectivity of C17-SLN-Ab for MRSA over fibroblast

cells. The concentration of released C17 after 2 h release from 4 mg/mL of C17-SLN-Ab has been measured to be 6.6 ppm, which was shown to be nontoxic to MRSA within 2 h of contact (**Figure 3-6**).

The effective reduction of MRSA by 4 mg/mL of C17-SLN-Ab within 2 h of contact as depicted in **Figure 3-5c-d** could arise from the selective binding of the antibody to the target MRSA cells. Although the exact antibacterial mechanism by C17-SLN-Ab is unknown at this time, it is likely to involve effective binding between C17-SLN-Ab with MRSA followed by possible internalization of C17-SLN-Ab by MRSA and release of C17 inside MRSA cells. This binding is likely due to the combination of two different interactions: (i) electrostatic attraction between positively charged C17-SLN-Ab and negatively charged cell membrane; (ii) the antibody and bacterial cells binding which are the major contributing factor of the effective binding.

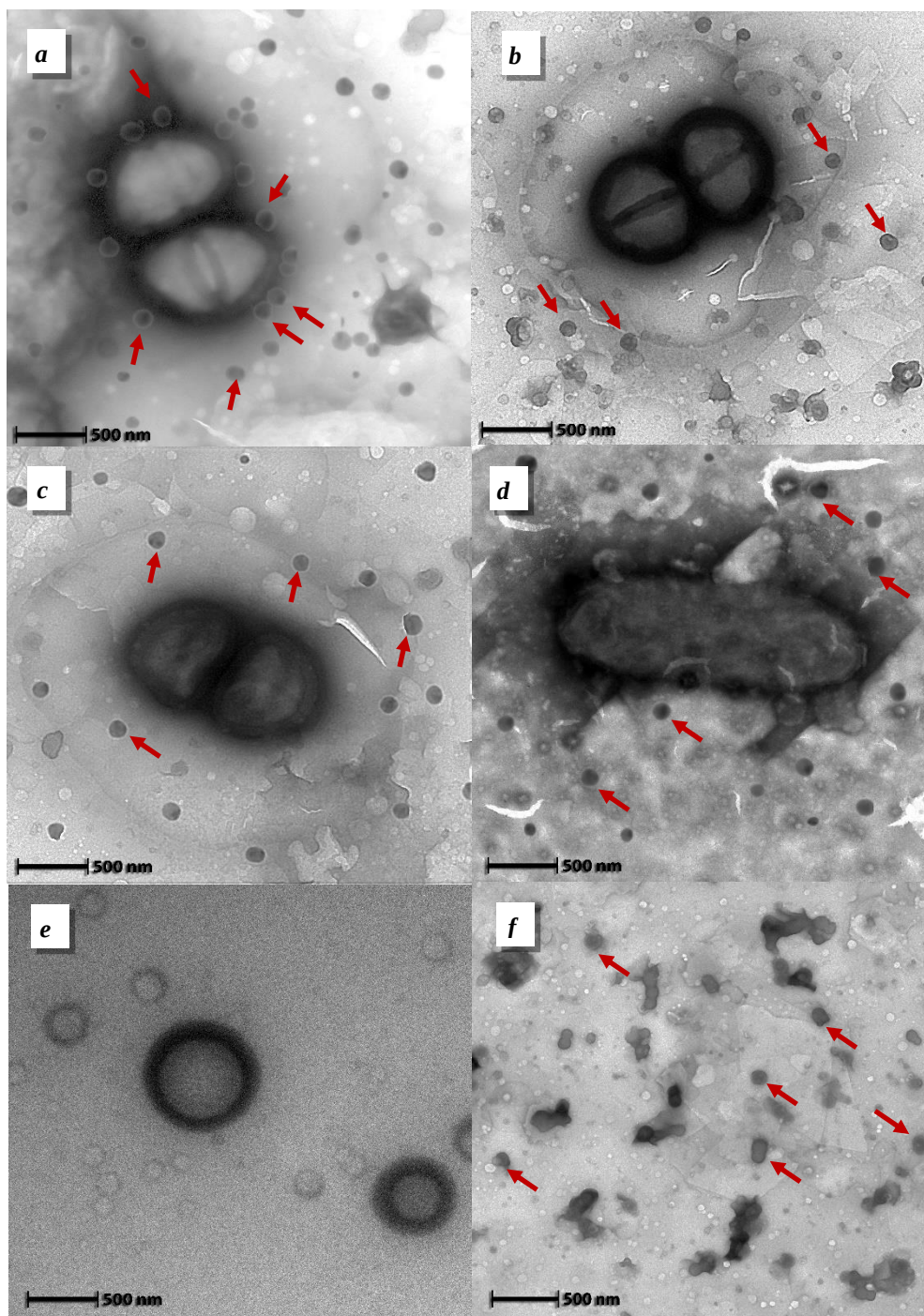


Figure 3-7. TEM images of (a) SLN-Ab and MRSA, (b) SLN and MRSA, (c) SLN-IgG and MRSA, (d) SLN-Ab and *P. aeruginosa*, (e) MRSA, and (f) SLN-Ab. Red arrows represent SLNs. The magnification is 10,000X.

To demonstrate the versatility of this nano-strategy, we switched the anti-MRSA antibody in C17-SLN-Ab to a rabbit anti-*E. coli* IgG type antibody and tested the selectivity against *E. coli* K12 over fibroblast. C17-SLN conjugated with this anti-*E. coli* IgG is termed as “C17-SLN-eAB”. The physical characteristics of C17-SLN-Ab such as particle size and surface zeta-potential value are similar to that of C17-SLN-Ab (Table 1). As shown in **Figure 3-8a**, C17-SLN showed minimum toxicity to fibroblast after 2 hours of contact and non-detectable anti-*E. coli* activity in the tested period. C17-SLN-eAB, however, showed a progressive kill of *E. coli* K12, reaching 5.97 log reduction at 2 hours (**Figure 3-8b**). In this co-culture study, the viability of fibroblast remained as $48.0 \pm 4.1\%$ in the same timeframe of 2 hours, suggesting selective inactivation of the target bacterium *E. coli* K12 in the presence of fibroblast cells.

At the same time, 66 ppm free C17 (concentration or a total amount equivalent to encapsulated C17 in 4 mg/mL C17-SLN-eAb) wiped out unselectively all *E. coli* as well as fibroblast cells in the first 10 min. Similarly, C17-SLN-eAB demonstrated double selectivity, i.e. preferential kill of the target bacterium *E. coli* K12 over MRSA as depicted in **Figure 3-8c**. Total eradication of *E. coli* K12 was attained after 2 hours of contact whereas no significant reduction of MRSA was manifested in the same contact duration and only one log reduction was observed after 4 hours of contact. As a comparison, free C17 at the concentration of 66 ppm killed all the *E. coli* K12 and MRSA cells, in an unselective manner, in the first 10 min of contact.

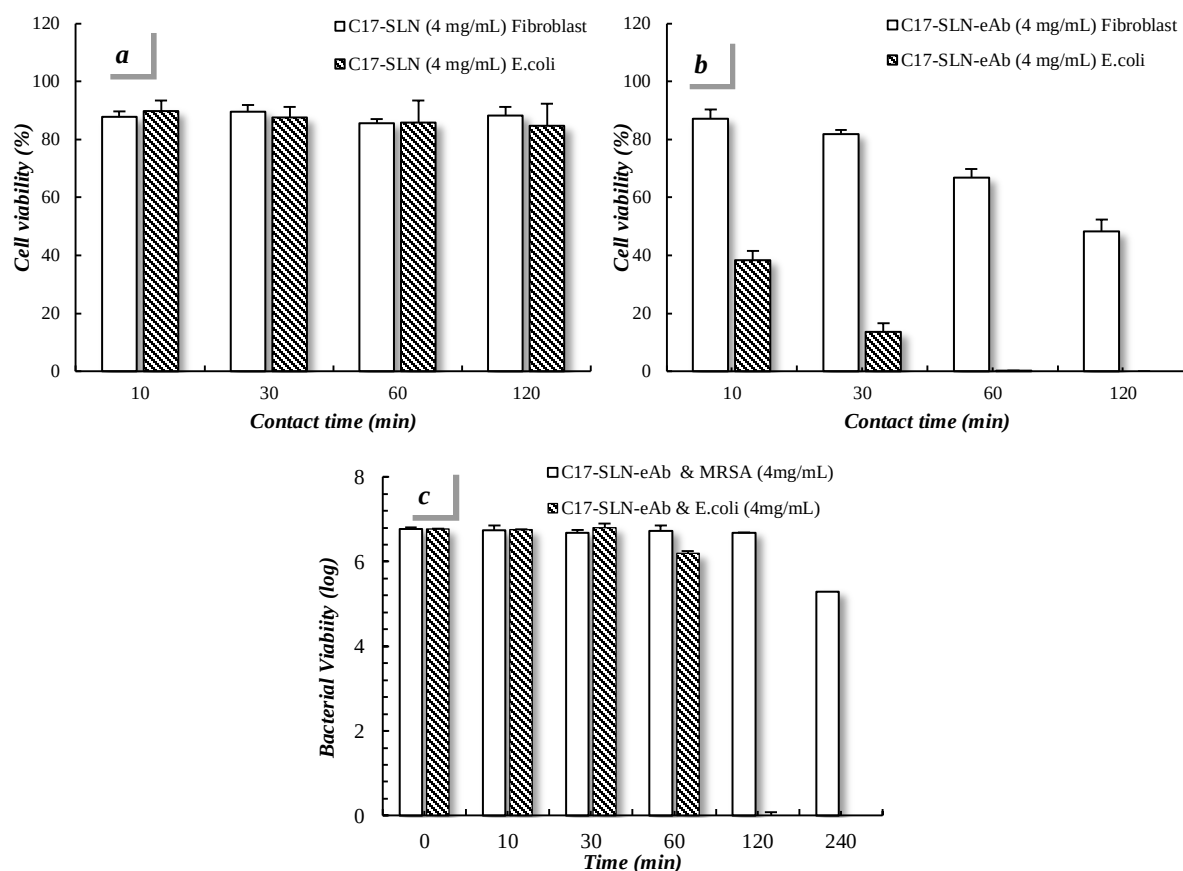


Figure 3-8. Selective toxicity assay (a) bacterial viability (%) as a function of contact time between C17-SLN against a mixture of *E.coli* and fibroblast cells. (b) Bacterial viability (%) as a function of contact time between C17-SLN-eAb against a mixture of *E.coli* and fibroblast cells. (c) Bacterial viability (log) as a function of contact time between C17-SLN-eAb against a mixture of MRSA and *E.coli*. Values represent the mean $\pm$ S.D; n=3 for all of the compounds. The starting inoculum for *E.coli* and Fibroblast cells were $10^{4.88}$ CFU /well and $10^{4.21}$ cells/well.

To show the versatility of this technique to any other biocides benzyldimethyltetradecyl-ammonium chloride (BC) was encapsulated in SLNs (using the same methods) conjugated to MRSA and *E.coli* antibodies. Results for co-culture assay against a mixture of MRSA and P.A (**Figure 3-9**) showed that the similarity in the behavior of BC-SLN-Ab and C17- SLN-Ab in the same situation since the nature and mode of action of BC and C17 are the same. BC-SLN-Ab and BC-SLN-eAb against a mixture of MRSA and fibroblast cells and *E.coli* and fibroblast cells, respectively, demonstrated similar activity and their behavior followed the same pattern (**Figure 3-10**).

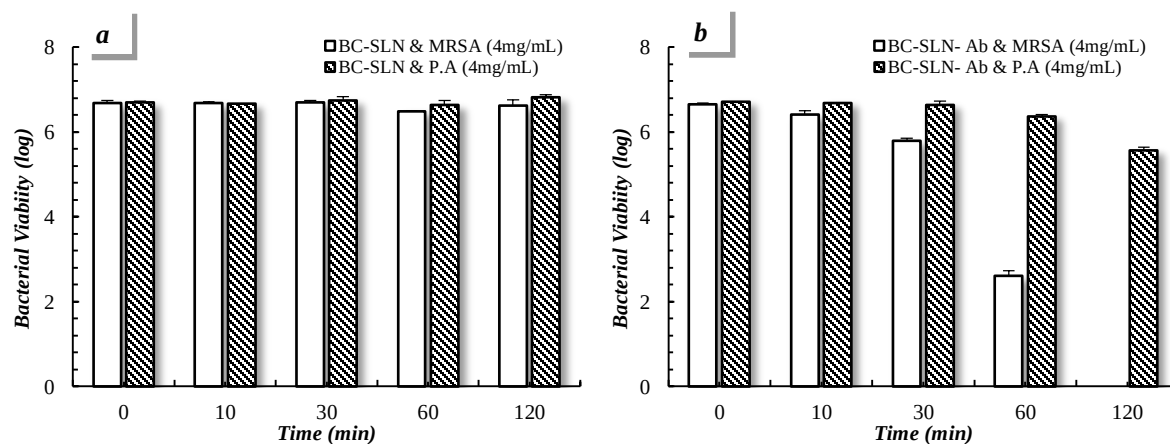


Figure 3-9. Selective antibacterial assay (a) bacterial viability (log) as a function of contact time between BC-SLN against a mixture of MRSA and P.A. (b) bacterial viability (log) as a function of contact time between BC-SLN-Ab against a mixture of MRSA and P.A. Values represent the mean $\pm$ S.D; n=3 for all of the compounds. The starting inoculum for *E.coli* and Fibroblast cells were $10^{4.88}$ CFU /well and $10^{4.21}$ cells/well.

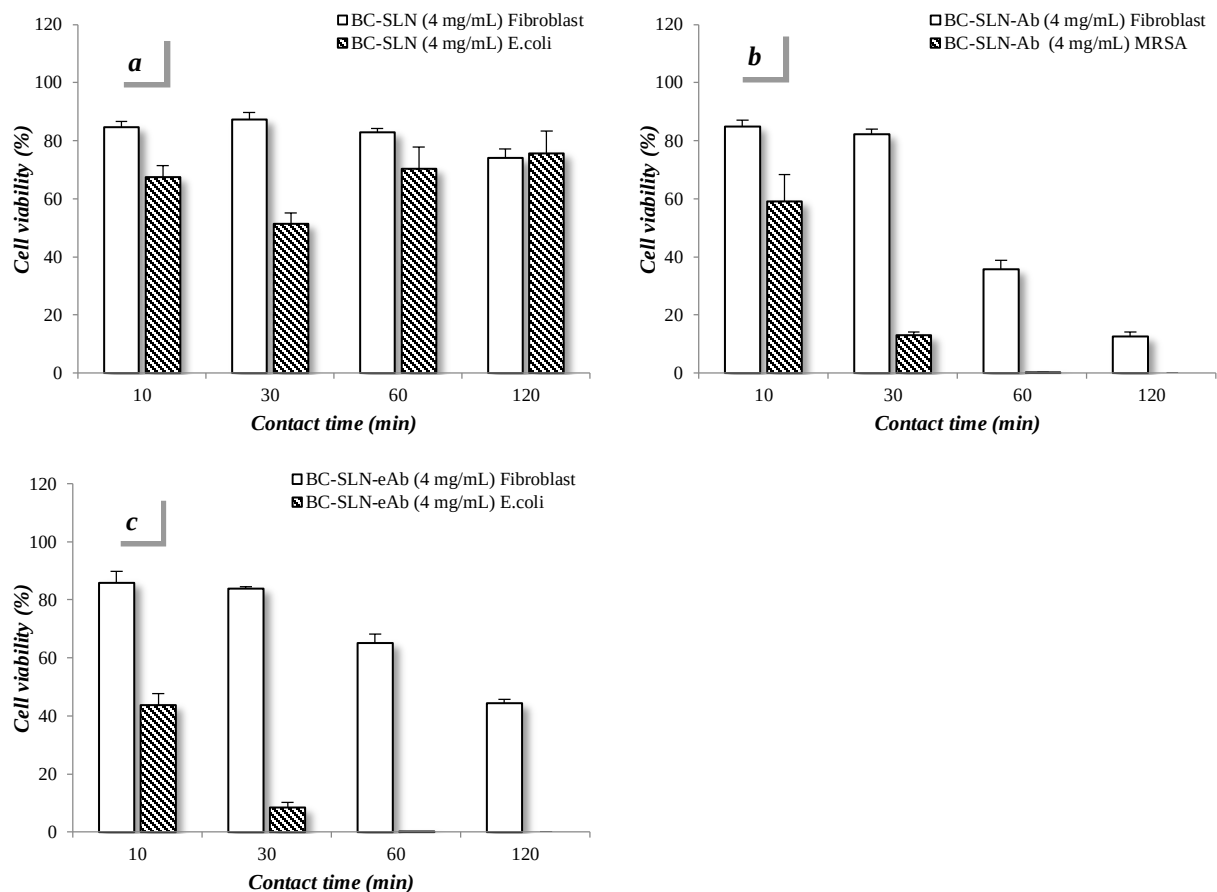


Figure 3-10. Selective toxicity assay (a) bacterial viability (%) as a function of contact time between BC-SLN against a mixture of *E.coli* and fibroblast cells. (b) Bacterial viability (%) as a function of contact time between BC-SLN-Ab against a mixture of MRSA and fibroblast cells. (c) Bacterial viability (%) as a function of contact time between BC-SLN-eAb against a mixture of *E.coli* and fibroblast cells. Values represent the mean $\pm$ S.D; n=3 for all of the compounds. The starting inoculum for *E.coli* and Fibroblast cells were $10^{4.88}$ CFU /well and $10^{4.21}$ cells/well.

3.3 Conclusions

The worldwide concern of rapid emergence of antibiotic resistant pathogens has motivated researchers to develop new effective and non-resistance-inducing antimicrobial agents/materials. To this end, we designed and synthesized a potent antimicrobial agent, C17, which is potentially lethal to bacteria, 6 log reduction of MRSA and *P. aeruginosa* within 10 min at the concentration of 188 ppm [206], and appears not to induce resistance of *P. aeruginosa*. However, even 66 ppm C17 can wipe out >95% of fibroblast cells within 10 min. This cytotoxicity limits application of this effective and potentially non-resistance-inducing biocide. Encapsulation of C17 in SLNs made it non-toxic to bacteria as well as skin cells. Conjugation of a specific antibody, anti-MRSA antibody, onto these C17 containing SLNs (C17-SLN-Ab) resulted in selective toxicity of C17 toward MRSA than fibroblast cells.

In short, a novel concept of turning non-selective potent antimicrobials into selective ones is presented. Antibodies specific to a certain pathogen are conjugated onto antimicrobial-carrying SLNs to impart selectivity. In comparison to free C17, C17-SLN-Ab shows much-improved selectivity against MRSA over fibroblast. Besides the improved selectivity between the target bacterium and fibroblast, double selectivity has also been demonstrated by C17-SLN-Ab. The nanoassembly is more effective in inactivating MRSA than *P. aeruginosa*.

By switching the conjugated antibody, we successfully tuned the nanoassembly to be specific to a Gram-negative target bacterium *E. coli* K12. Because the antibody itself does not impact on the viability of bound bacterium and the encapsulated biocide kills bacteria

in a non-specific way and hence is intrinsically less likely to induce bacterial resistance, this nanoassembly is not foreseen to select bacterial mutants. This concept converges with the trend of developing fast and accurate point-of-care diagnostic techniques. Once specific pathogens are identified by a reliable point-of-care diagnosis, nanoassembly conjugated with corresponding antibodies can be applied topically to treat the infection.

4 Chapter 4: Conclusions and future work

4.1 Conclusions

The worldwide concern of rapid emergence of antibiotic resistant pathogens has motivated researchers to develop new effective and non-resistance-inducing antimicrobial agents/materials. To this end, in this thesis, different composite biocides with the ability to be used in high protein fluids were synthesized and nanotechnology was utilized as a tool to improve their *in vivo* applications. In the first part of this thesis, we showed that using a secondary amine based *N*-chloramine in a composite biocide can significantly improve their antimicrobial activity in high protein media. N-(2-(4-((1-chloro-2,2,6,6-tetramethylpiperidine-4-yloxy)methyl)-1H-1,2,3-triazole-1-yl)ethyl)-N,N-dimethyldodecan-1-aminium chloride, 6a, demonstrated a superior antimicrobial effect over amide based biocides (C18) and commercial QAC (BC) in PBS and HPM. In both cases, 6a wiped out the bacteria in the first 5 min of contact time where other biocides reached the total kill after 10 min of contact with bacteria. Also, redox titration showed that the chlorine on the secondary amine based biocides (6a and 6b) is more stable than amide based biocide (C18). In this part of the project different strains of bacteria were used and the synthesized biocides showed higher effect on Gram-negative bacteria than Gram-positive one. This study lays a strong foundation for practical use of quaternized *N*-chloramines as potent disinfectants in food processing facility and/or hospitals where organic matter might be present.

In the second part of the thesis, for the first time, one of the most commonly used broad-spectrum biocides, quaternary ammonium compound as a model disinfectant, was encapsulated into solid lipid nanoparticles and became selective towards MRSA using specific MRSA

antibody. These SLNs displayed high antibacterial activities (more than the actual biocide) which were caused by proper binding of SLNs to the bacteria and biocide released on the membrane. It was observed that encapsulation of the biocide C17 in SLNs enhanced its ability and reduced its toxicity to fibroblast cells due to a proper bacterial cell – antibody binding. Also, we developed a novel method to determine the toxicity of antimicrobial agent in the presence of MRSA. This method can help to mimic the *in vivo* and provide a better understanding of the antimicrobial activity and toxicity at the same time. It showed that C17-SLN-Ab is more active towards targeted bacteria than eukaryotic cells since after killing all the bacteria there are still alive fibroblast cells left. At the same time, we used another antibody (anti *E. coli* antibody) and biocide (BC) to demonstrate the versatility of this new strategy of converting non-selective biocides to selective ones. Not only selectivity against bacteria over human cells can be imparted to a different non-selective biocide (BC) but also specificity towards bacteria can be tuned by switching the conjugated antibodies (anti-MRSA and anti-*E. coli* antibodies). This study underlines the importance of nanotechnology in the war against superbugs by turning a potent but cytotoxic antimicrobial agent to a non-cytotoxic one. This proof-of-concept research can be extended to other potent antimicrobial agents, against which bacterial resistance is unlikely to develop. Also, because the antibody itself does not impact on the viability of bound bacterium and the encapsulated biocide kills bacteria in a non-specific way and hence is intrinsically less likely to induce bacterial resistance, this nanoparticle assembly is not foreseen to select bacterial mutants. In other words, this study opens a new door of killing bacterial pathogens while potentially bypassing bacterial resistance mechanisms by using nanotechnology.

4.2 Future work

The antibacterial efficacy of composite biocides is related to different parameters such as the stability of the chlorine on the *N*-chloramine moiety and hydrophilicity of the biocide molecule. The hydrophilicity of the molecule is determined by the type of *N*-chloramine, alkyl chain length, and the linker. The effect of the linker can be examined in future works.

Also, the responsive release of the biocide from SLNs as well as the binding efficacy of an antibody to the target protein can affect the antimicrobial activity of SLNs. To improve the responsive release, nano-micelles made of block polymers with two hydrophobic (polymeric biocides) and hydrophilic (biocompatible polymers) blocks linked via a linker which is sensitive to pH change or enzyme degradation can be employed. On the other hand, other targeting ligands like aptamer, which is more efficient than antibodies, can be utilized to improve the binding.

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