The production and engineering of antimicrobial peptides and their application in the coating of urinary catheters

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**THE PRODUCTION AND ENGINEERING OF
ANTIMICROBIAL PEPTIDES AND THEIR
APPLICATION IN THE COATING OF URINARY
CATHETERS**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

A thesis submitted to the Nanyang Technological University in partial fulfilment of
the requirement for the degree of Doctor of Philosophy

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List of Publications

- **Li X.**, Li P., Saravanan R., Basu A., Mishira B., Leong S.S.J.*, 2013, “Antimicrobial functionalization of silicone and catheter surfaces with newly engineered short peptides having broad spectrum antimicrobial and salt resistant property”; Biomacromolecules, Submitted.
- Saravanan R., **Li X.** (Co-first author), Lim K.Y., Basu A., Mishira B., Leong S.S.J.*, 2013, Design of short membrane selective antimicrobial peptides containing tryptophan and arginine residues for improved activity, salt-resistance and biocompatibility; Biotechnology and Bioengineering, Submitted.
- **Li X.**, Saravanan R., Tay D.K.S., Basu A., Leong S. S. J.*, “Engineering and molecular characterization studies of a novel salt-resistant human beta defensin variant”; Chemical Engineering Science, *in press*.
- Li P., **Li X.** (Co-first author), Saravanan R., Li C.M. and Leong S.S.J.*, 2012, “Antimicrobial Macromolecules: Synthesis Methods and Future Applications”; RSC Advances, 2, 4031-4044.
- **Li X.**, Leong S.S.J.*, 2011, “A chromatography-focused bioprocess that eliminates soluble aggregation for bioactive production of a new antimicrobial peptide candidate”; Journal of Chromatography A, 1218: 3654-3659.

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- Tay D.K.S., Gobinath A.K.R., **Li X.**, Chen Y., Lua L.H.L., Leong S.S.J.*,2011, “A new bioproduction route for a novel antimicrobial peptide” Biotechnology and Bioengineering, 108: 572-581.

List of Abbreviations

Human beta defensins (hBDs)
Antimicrobial peptides (AMPs)
Human β -defensin 25 (hBD25)
Human β -defensin 28 (hBD28)
Maltose Binding Protein (MBP)
Size exclusion chromatography (SEC)
Minimal inhibitory concentration (MIC)
Methicillin-resistant *Staphylococcus aureus* (MRSA)
Multi-drug resistant (MDR)
Vancomycin-resistant *Enterococci* (VRE)
US Food and Drug Administration (FDA)
Lipopolysaccharide (LPS)
Human β -defensin 2 (hBD2)
Human β -defensin 3 (hBD3)
Thioredoxin A(TrxA)
Glutathione-S-transferase (GST)
Amidophosphoribosyltransferase (Purf)
Ketosteroid isomerise (KSI)
Tobacco Etch Virus protease (TEVp)
Isopropylthio-D-galactoside (IPTG)
Tris(hydroxymethyl)aminomethane (Tris)
Ethylenediamine-tetraacetic acid (EDTA)
Sodium chloride (NaCl)
Dithiothreitol (DTT)
Reduced glutathione (GSH)
Oxidized glutathione (GSSG)
Hydrochloric acid (HCl)

Fast Protein Liquid Chromatography (FPLC)

Size exclusion chromatography (SEC)

Column volume (CV)

Mueller Hinton Broth (MHB)

Sodium dodecyl sulphate poly-acryl amide gel electrophoresis (SDS-PAGE)

Hexa histidine-tag (6His)

Immobilised metal affinity chromatography (IMAC)

CFU (colony forming unit)

Field Emission Scanning Electron Microscopy (FESEM)

Large Unilamellar Vesicles (LUVs)

1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1-rac-glycerol) (POPG)

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)

Smooth muscle cells (SMCs)

Dulbecco's modified eagle medium (DMEM)

Reverse phase (RP)

Circular dichroism (CD)

Discovery Studio (DS)

Discrete optimized protein energy (DOPE)

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Abstract

Antimicrobial peptides (AMPs) are a promising class of molecules that can curb microbial infections without easily evoking microbial resistance. To employ AMPs as coating agents for biomedical materials and devices, obstacles which include (i) the lack of pure AMPs for detailed structure-function characterization and systematic AMP-based coating studies, as well as (ii) susceptibility of AMPs to salt need to be overcome. In this research project, human beta defensins 25 (hBD25) and 28 (hBD28) were chosen as AMP models to be studied for development into antimicrobial coating agents. hBD25 and hBD28 were discovered in the male genital tract, and thus render them very suitable peptide candidates for immobilization on urinary catheters.

To produce sufficient AMPs for this research project, microbial platforms for the biosynthesis of hBD25 and hBD28 at preparative scale were developed. hBD25 was expressed predominantly as soluble aggregates despite being co-expressed with the solubility-enhancing MBP fusion tag in *E. coli*. This outcome necessitated the development of a refolding based bioprocess for hBD25 production, where the peptides were first subjected to denaturation-reduction, followed by an *in vitro* refolding step to yield bioactive hBD25 at high purity. hBD28, on the other hand, was expressed as a soluble and monomeric fusion protein, which was readily recovered at 79±5% yield and 90±2% purity using a streamlined chromatography-based bioprocess. Although both hBD25 and hBD28 completely inhibited the growth of *E. coli* K12 at a peptide concentration of 60 µg/ml in the absence of salt, both peptides became

inactive in the presence of salt (150 mM NaCl). Given that hBD28 could be produced using a more streamlined bioprocess compared to hBD25, hBD28 was chosen as the peptide candidate for detailed engineering and structure-function characterization studies to improve its salt-resistance property. Salt intolerance of wild type hBD28 was overcome by increasing C-terminus cationicity to increase the net positive charge of the peptide. Additionally, the mutant hBD28 also exhibited improved antimicrobial potency compared to wild type hBD28 in the presence of 150 mM NaCl. Zeta potential analysis results indicate that the increased net charge of mutant hBD28 was important to overcome the salt-induced charge-shielding effect, which inhibits peptide activity in the presence of salt. Two short AMP analogs that are rich in tryptophan and positively charged amino acid residues, RK1 and RK2, were generated from the salt-resistant mutant hBD28 which exhibited excellent broad spectrum antimicrobial activity, and were used as peptide candidates for urinary catheter immobilization studies. A coupling chemistry based on covalent grafting of RK1 and RK2 on an allyl glycidyl ether (AGE) polymer brush was developed to stably immobilize the peptides on polydimethylsiloxane (PDMS) and urinary catheter surfaces. The RK1- and RK2-impregnated catheters exhibited broad spectrum biocidal and anti-biofilm activities against Gram positive bacteria, Gram negative bacteria and fungi. The mechanism of killing exhibited by these immobilized peptides was *via* membrane disruption, similar to that observed for their soluble counterparts, as determined by ATP leakage assay and Field Emission Scanning Electron Microscope (FESEM) analysis, but the rate of bacteria killing was observed to be lower for the immobilized

peptides compared to their soluble counterparts.

In conclusion, this thesis presents a proof-of-concept study on the applications of short AMPs as antimicrobial coating agents on urinary catheter surfaces. The successful development of bioprocesses allowed sufficient amounts of hBD25 and hBD28 to be produced, leading to detailed structure-activity characterization studies of these peptides. The outcome of those studies guided the rational engineering of hBD28 for salt-resistant properties, from which peptide analogs were generated for urinary catheter immobilization studies. The outcome of this thesis will pave the way for the development of other AMP-impregnated devices that combines peptide structure-function studies at both the process and molecular levels.

Chapter 1 Introduction

1.1 Introduction

Infectious diseases have accounted for millions of deaths worldwide every year and caused enormous healthcare costs to society in the past century [1]. Since the discovery of the antimicrobial activity of penicillin in the 1940s, antibiotics have been widely used to counter infectious pathogens including bacteria, fungi and multicellular parasites [2, 3]. In recent years, the widespread use of antibiotics has resulted in the rapid growth of drug-resistant bacteria which include the well-known methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) [4, 5]. These obstinate infections often result in high mortality because there are no effective therapeutic agents to address these problems. As such, the need to identify and develop new classes of molecules that can kill pathogens without incurring the development of resistance is extremely urgent. Antimicrobial peptides (AMPs), which are important effector molecules in innate immune system [6] represent the defense function against foreign intruding particles. Although AMPs kill pathogens by many different mechanisms, including inhibition of cell wall, or protein, DNA and RNA synthesis, the main mechanism of antimicrobial behavior has been widely accepted to be the disruption of bacterial or fungal membrane [7, 8]. The advantage of this mode of killing is the difficulty for pathogens to develop resistance as the membrane is a core and integral part of the cell structure that is difficult to evolve over a short period of time. An appealing class of AMPs is the defensins,

which are found in many multicellular organisms, including plants, animals, as well as insects [9, 10]. In humans, defensins are epithelial-derived, and are cationic and cysteine-rich. Recently, interest in human defensins has increased because of their expanding roles in innate and adaptive immunity [11, 12]. By now, more than 30 kinds of human beta defensins (hBDs), i.e. hBD1 to 33, have been isolated and their coding nucleotide sequences identified by genome analysis [13]. hBD25 and hBD28 are recently discovered hBD variants which were first found in the male genital tract [14]. It is therefore possible that hBD25 and hBD28 functions in protection against genital tract infection, a commonly occurring infection in hospitals. To date, two obstacles stand in the way of research and further application of hBDs as therapeutic agents; i.e., (1) the lack of sufficient amounts of pure peptides for detailed structure-function characterization studies; (2) the inhibition of peptide activity at physiological salt concentrations. Resolving problems (1) and (2) would facilitate detailed studies of AMP structure and activity and open up new possibilities for their use as antimicrobial coating agents on urinary catheters, which forms the aims of this thesis.

In this thesis, the application of AMPs as an antimicrobial coating agent for urinary catheters is studied. Due to the widespread use of urinary catheterization, urinary catheters are often associated with urinary tract infection [15-17]. According to recent surveys, more than 35 million urinary and venous catheters are inserted in patients annually, with an infection rate of 10%-30%, where up to 5% of the infected cases lead to death [18]. To prevent urinary catheter infections, many different methods

have been explored such as the use of antiseptic lubricating gels, anti-reflux valves, catheter drainage tubing junction sealing, and antimicrobial coatings based on antibiotics, silver, and nitric oxide [19-21]. However, the efficacy of these solutions has been sub-optimal due to challenges in biocompatibility and resistance development in pathogens [22]. The use of AMPs as a coating agent for urinary catheters is an attractive solution, which is researched in this thesis.

1.2 Aims and objectives

The overall objective of this Ph.D. research project is to design a streamlined and cost-effective bioprocess to produce hBD variants which are resistant to salt and possess broad antimicrobial spectrum and using these variants as antimicrobial coating agents for urinary catheters. Detailed structure and activity characterizations of the hBD peptides, followed by engineering studies to improve selected peptide characteristics such as salt intolerance would be performed. The immobilization of AMPs to urinary catheter surface and its antimicrobial and anti-biofilm properties would be studied. To achieve this overall goal, the specific objectives of this research thesis are as follows:

- Development of an optimum bioprocess for production of hBDs for structure and activity studies.
- Peptide engineering studies to generate salt-resistant hBD analogs and molecular characterization studies of these peptide analogs.

- Development of antimicrobial urinary catheters by immobilization of hBD analogs.

1.3 Motivations

The motivations of this research are as follows;

- The developed bioprocess from this research can serve as a generic platform for efficient bio-manufacture of other full-length AMPs containing cysteine residues, which are difficult to chemically synthesize.
- The AMP engineering studies will contribute to new knowledge with regards to salt inactivation mechanism inherent in many AMPs in relation to peptide structure-function relationship, which will be useful to guide the future design of other salt-resistant AMPs.
- The development of AMP-based coatings on urinary catheters will alleviate bacterial infections without inducing pathogen resistance at the rate of conventional antibiotics, and open the way for the future use of AMPs as antimicrobial coating agents for other biomedical devices.

1.4 Thesis organization

This thesis comprises eight chapters. **Chapter 1** introduces the background and objectives of this research project. **Chapter 2** presents a literature review on the

structure and function of AMPs and reviews factors to consider when employing AMPs as antimicrobial coating agents. **Chapter 3** describes the experimental details used in this thesis. **Chapters 4 and 5** describe the bioprocess development studies for hBD25 and hBD28 production, respectively. **Chapter 6** presents the engineering design and strategy to generate a mutant hBD28 that is salt-resistant, followed by a detailed molecular characterization study of the mutant. **Chapter 7** reports rational design strategies to generate short AMP analogs and the development of immobilization chemistry for tethering these analogs on a urinary catheter surface. **Chapter 8** presents the conclusions of this thesis and prospects for further research of this work.

Chapter 2 Literature Review*

2.1 Antimicrobial peptides (AMPs): function and therapeutic potential

AMPs are present in virtually every life form as nature's antibiotics. The first AMP, cecropin, was found by Hans Boman and co-workers in the immune system of silk moths. Cecropin was reported to have potent and broad antimicrobial spectrum, where the peptide is active against multiple distinct classes of microbes, e.g., Gram-positive bacteria, Gram-negative bacteria and fungi [23, 24]. Since then, many other types of AMPs have been identified in insects, plants, animals and humans in rapid succession [25, 26]. In the last 30 years, 900 such classical peptides have been identified, indicating that they are important molecules in the innate immune system [27]. By now, the roles of hundreds of AMPs in the innate immunity system have been well characterized [28]. In recent years, research on AMPs is fuelled mainly by the rapid emergence of pathogen strains which are resistant to conventional antibiotics. Prolonged antibiotic misuse in human therapeutics, agriculture and veterinary has provided selective pressure favoring the survival and spread of these antibiotic resistant microbes [29, 30]. The rapid rise in drug resistant pathogens is attributed to the accumulation of resistance genes on plasmids that are independently replicated and passed between microbial cells and species [30]. Such multi-drug resistant (MDR) microbes are termed as "*superbugs*", where they exhibit a plethora of antibiotic

resistant mechanisms to strike back and nullify antibiotic actions including reduced drug uptake, active pumping of drugs extracellularly, enzymatic alteration of antibiotics, modification or overproduction of targets, drug sequestering by protein binding, and metabolic bypass of the targeted pathway [31]. Almost 70% of current day infections are suspected to be due to drug resistance [32], where bacterial infection now ranks as the 4th leading cause of death in the U.S. alone. Over 50% of hospital bloodstream infections are incurred by methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococci* (VRE). Among the *Escherichia coli* (*E. coli*) isolates from China and India, less than 50% remain susceptible to commonly used antibiotics (e.g., Cephalosporins and Ciprofloxacin) [33]. Despite the alarming rise in MDR pathogens and the severity of their infections, many major pharmaceutical companies have had to abandon antibiotic discovery research due to high regulatory barriers and low chance of clinical success, leaving only a small number of companies to meet this challenge [34, 35]. Moreover, most antibiotic compounds in the pipeline are still chemical derivatives of existing antibiotics with known resistance [34]. Collectively, the drug resistance problem and the declining number of drugs approved by the US Food and Drug Administration (FDA) in recent years reflect an unresolved crisis for the post-antibiotic era.

AMPs hold promise as an effective new generation antibiotic because they are effective against pathogenic microorganisms that are resistant to conventional antibiotics. AMPs possess potent antimicrobial activity against a wide range of pathogens including gram-positive and gram-negative bacteria, fungi, and viruses [36].

AMPs share a different mechanism of microbe killing from conventional antibiotics, where most AMPs have been found to target the microbial cell membrane, a previously under-appreciated target of conventional antibiotics [7]. Even some studies have reported pathogens developing resistance to AMPs by modifying the membrane composition or causing the AMPs degradation [37, 38], it is undeniable that AMPs are less likely to evoke pathogen resistance because the cell membrane is a core structure that is difficult to evolve over a short span of time. Now many believe that AMPs will still be superior to conventional antibiotics in combatting resistance development, where pathogen resistance development against AMPs is far more difficult than conventional antibiotics [39].

AMP-based drug development has intensified in the last 25 years, which is reflected by the number of AMP candidates advancing into clinical trials (Table 2.1) [26]. Although many of these products demonstrated high efficacy in preclinical trials, they had to be abandoned at various stages of clinical trials owing to failure in meeting stringent requirements by regulatory agencies. These AMP-based drugs include (i) Pexiganan (MSI-78) (Magainin Pharmaceutical Inc), a 22 amino acid synthetic analog based on magainin 2 for the treatment of diabetic foot ulcers; (ii) Omiganan (MBI-226) (Microbiologix Biotech) a synthetic analog of indolicidin, for prevention of catheter linked infections; (iii) Iseiganan (IB-367, Intrabiotics Pharmaceuticals, Inc), a synthetic derivative of protegrin1 from pig leucocytes developed as a local mouthwash for oral mucositis treatment and the only systemic application designed Neuprex (rBPI₂₁), and (iv) N-terminus portion of bactericidal

permeability increasing protein (BPI), with potent endotoxin neutralizing ability.

Despite these high profile failures, AMPs continues to be the “mother lode” of templates for the design of new anti-infective. Table 2.2 lists some examples of AMP development in the last 2-3 years [40, 41]. For successful development of AMPs as anti-infective, major obstacles including (i) susceptibility to salt, protease, and serum, (ii) toxicity to mammalian cells, and (iii) high production costs, need to be overcome. Therefore, research which can address these challenges by various strategies is very important.

Table 2.1: AMP-based first generation antibiotics in clinical trial [26].

Parent AMP	Product name	Clinical phase
Magainin 2	Pexiganan	III
Pig protegrin	IB367 (Iseganan)	III
BPI	NEUPREX (rBPI21)	III
Bovine Indolicidin peptide	MX-226/CP1226	III
Human Lactoferrin peptide	hLF1-11	II
Defensin	PMX30063	I
Indolicidin	Omiganan	III
Human BPI	XOMA 629	II

Table 2.2: Examples of AMP-based therapeutics being developed [40, 41].

Parent AMPs	Product name
Histatin	PAC-113
LL37	DPK-060
Fungal defensin	Plectasin
Lantibiotics	NVB302 NVB333
Synthetic decapeptide	KSL-W
Synthetic AMP	VI3K

2.2 Mechanism of AMP action

AMPs are short peptides comprising 12 to 60 amino acids and are typically cationic and amphiphilic [27]. Their cationic and amphiphilic property is key to their mechanism of action. Previous studies have demonstrated that the main antimicrobial mechanism of most AMPs is the disruption of cell membranes of pathogens [42]. The membrane disruption action is preceded by peptide binding to the cell membrane through electrostatic and hydrophobic interaction, followed by membrane disruption. Several modes have been proposed to describe AMP-induced membrane disruption, such as the *toroidal pore*, *carpet* and *barrel stave* models (Figure 2.1).

- *Toroidal pore* model: the peptides first accumulate on the cell membrane by binding to the head groups of phospholipids. After a threshold peptide/lipid ratio is reached, the peptides can insert into the lipid membrane and then cluster as unstructured bundles which can span the membrane and create channels. The formed channels throughout the membrane cause the leakage of intracellular components. Different from other modes, the trans-membrane channels are short-lived with an undefined nature, thus it allows the peptides to penetrate the membrane without inducing membrane depolarization. When crossing into the cellular compartment, the peptides interact with specific target molecules and exhibit their killing activity [7, 43].
- *Barrel stave* model: peptide binding on the membrane surface is the first step of the *barrel stave* pore formation. Upon binding, with the help of conformational phase transition, the peptide can induce the localized

membrane thinning by forcing the head groups of phospholipids aside to facilitate the insertion of the peptide's hydrophobic domain into the cell membrane. When bound peptides reach a threshold concentration, the peptides will self-aggregate on the surface of cell membrane resulting in the formation of peptide oligomers, thus the peptides can insert deeper into the hydrophobic membrane core. Continued accumulation of monomeric peptides result in the further expansion of the *barrel stave* pore.

- *Carpet* model: a carpet layer is formed when the peptides cluster on the lipid membrane surface and the peptides act like surfactants to disrupt the lipid bilayers. In this mode, a high peptide density accumulated on the phospholipid membrane surface is required. The changes in membrane fluidity and/or reductions in membrane barrier properties caused by phospholipids displacement subsequently lead to membrane disruption [7, 44].

Figure 2.1: Schematic illustration of different membrane disruption mechanisms for AMPs [42].

2.3 Types of AMPs

AMPs are produced by all complex organisms as well as some microbes (e.g., plants, amphibians, birds, fish, mammals, single-celled microorganisms and insects), and have diverse and complex antimicrobial activities [45]. The two main antimicrobial peptide families found in mammals including human are cathelicidins and defensins [10]. Defensins are widely expressed in mammalian epithelial cells and phagocytes, and form the AMP model to be studied in this research thesis. Cathelicidins are different from defensins in structure but are similar to defensins in terms of abundance and distribution.

2.4 Human defensins

Human defensins are rich in sheet structure, cysteine residues and are also highly positively charged. Based on the spatial distribution of cysteine residues and disulfide linkages, human defensins are divided into three classes (i.e. α -, β - and θ -defensins) [46]. However, it has been found that only α - and β - are endogenously present in humans.

2.4.1. Human β -Defensins (hBDs)

As one of the members of the human defensin family, hBDs have attracted the attention of researchers for more than 25 years due to its high antimicrobial performance and important role in the human immunity system. The first hBD identified was hBD1, which was isolated from human plasma by the Forssmann group in 1995 [47]. In 1997 and 2000, two other members of the hBD family, i.e. hBD2 and hBD3, were found in the extract of lesional scales from patients suffering from psoriasis, and their coding nucleotide sequences information were also identified [48-50]. With the development of bioinformatics (e.g. Biological sequence analysis using ‘Profile Hidden Markov Models’ and ‘Basic Local Alignment Search Tool’), new hBDs continue to be discovered, with the recent ones being hBD25 to hBD29 [51].

Like many other proteins, the genomic structure of hBDs consists of exons and introns. In all but hBD5, the genomic structures have two exons and one intron, while

the genomic structure of hBD5 has three exons and two introns. The full hBD gene comprises the sequence of the signal peptide and the proprotein, where the latter is preceded by the sequence of the mature peptide [52, 53]. The mature peptide typically comprises 41 to 50 amino acid residues which encode three disulfide bonds. After translation *in vivo*, the signal peptide and the proprotein sequence would be cleaved as part of the post-translation modification process [54, 55]. The disulfide linkages of hBDs is Cys¹-Cys⁵, Cys²-Cys⁴, and Cys³-Cys⁶, meaning that the first cysteine in the amino acid sequence form a disulfide bond with the fifth, and so on [56]. Although disulfide bonds are important for tertiary structure maintenance of β -defensins, some studies have indicated that disulfide bond is not absolutely necessary for their antimicrobial ability [57-59]. The expression of hBD in human cells has been reported to be constitutive, inducible or both (Table 2.3).

Table 2.3: Regulation pattern of the expression of hBDs.

Defensin	Pattern of Expression	Stimulator	References
hBD1	Constitutive	TNF- α , IL-1 β ,	[36]
hBD2	Constitutive, Inducible	TNF- α , IL-1 β , LPS and bacterial	[38]
hBD3	Constitutive, Inducible	TNF- α , IL-1 β , IFN γ and bacterial	[47]
hBD4	Constitutive, Inducible	PMA, IFN γ , IL-1 α , IL-6, IFN γ and bacterial	[48]

hBDs have a short α -helix structure near the N-terminus followed by three β -strands. The three antiparallel β - sheets form the core of peptide structure, which is stabilized by three intramolecular disulfide bonds (Figure 2.2) [13, 60-64]. Recently, reports have demonstrated that hBDs have the potential to form oligomeric structures [60]. Out of the 30 hBD sequences identified, only the tertiary structures of three hBDs (i.e. hBD 1-3) have been determined by nuclear magnetic resonance (NMR) using standard homonuclear techniques [63]. The crystal structure of hBD2 was the first to be determined, where it was reported that hBD2 possess two crystallographic forms [58]. The first form is held together by non-covalent interaction of the first β -sheet of each monomer, where the monomers aggregate into dimers. In the second form, the monomers form octamers, which are stabilized by the α -helix portions of the peptide. In contrast, unlike hBD2, no high order oligomeric forms were detected for hBD1[61]. hBD3 has also been found to adopt a dimer structure. As shown in Figure 2.2, the hBD3 dimer is proposed to be stabilized by the second β -sheet and the two salt bridges between Glu-28 and Lys-32 (Figure 2.3) [63].

Figure 2.2: Structural comparison of hBD1, hBD2 and hBD3. (a) Amino acid sequences of three hBDs. Numbers in the brackets indicate the net charges. The conserved six cysteine residues are in yellow; the anionic residues are shown in red, the blue represent the basic residues. Above the amino acid sequence is shown secondary structure elements (b) The ribbon representations of monomeric hBDs (left side of the panel) are painted with the electrostatic potential maps projected on the solvent-accessible surfaces shown in the equivalent orientations (center of the panel) and after 180° rotation (right side of the panel) [13].

Figure 2.3: Possible orientations of two hBD3 monomers to form a dimer. The 6-stranded-sheet was yielded from the inter-monomer hydrogen bonds. The electrostatic interaction between Lys-32 and Glu-28 and to inter-monomer hydrogen bonds between the side chains of Gln-29 could stabilize the dimer interface [63].

2.4.2 Antimicrobial and anti-viral activity of hBDs

To date, the multiple biological activities of some hBDs have been characterized both *in vitro* and *in vivo*. hBDs have hence been recognized to exhibit a broad antimicrobial spectrum. Among all the hBDs, only the antimicrobial activities of hBD 1 to 4 have been well characterized. The antimicrobial properties of hBDs are shown in Table 2.4. Besides antimicrobial activity, hBDs have also been reported to play a role in virus inhibition. For example, the expression of hBD2 and hBD3 can be induced by HIV-1 in human oral epithelial cells, resulting in virus inhibition. Recombinant hBD2 and hBD3 have also been found to inhibit HIV-1 replication *in vitro*. One recent report suggested that hBD3 has an inhibitory effect on the influenza virus [65-67].

Table 2.4: Antimicrobial spectrum of hBD1-4.

Defensin	Pathogen	References
hBD1	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>K. pneumonia</i> , <i>S. aureus</i>	[47]
hBD2	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>K. pneumonia</i> , <i>S. aureus</i> , <i>S. pneumoniae</i> , <i>C. albicans</i> , <i>C. parapsilosis</i> , <i>C. krusei</i> , <i>E. faecalis</i> , <i>HIV-1</i>	[68-73]
hBD3	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>K. pneumonia</i> , <i>S. aureus</i> , <i>S. pyogenes</i> , <i>E. faecium</i> , <i>S. pneumoniae</i> , <i>S. carnosus</i> , <i>B. cepacia</i> , <i>S. cerevisiae</i> , <i>C. albicans</i> , <i>C. parapsilosis</i> , <i>C. krusei</i> , <i>HIV-1</i>	[70, 72-76]
hBD4	<i>E. coli</i> , <i>S. carnosus</i> , <i>P. aeruginosa</i> , <i>B. cepacia</i> , <i>S. pneumoniae</i> , <i>S. aureus</i> , <i>S. cerevisiae</i>	[77, 78]

2.5 AMP production methods

Although hundreds of AMPs have been identified, further studies to confirm their roles by detailed understanding of the peptide structure-function and their development as the anti-infectives have been slow due to the difficulty in obtaining sufficient amounts of pure AMPs [79]. AMP production by isolation and purification from natural sources such as epithelial or plants cells is an extremely tedious task, giving low yields and is prohibitively expensive to scale up [49, 50, 80]. To address this problem, chemical synthesis and biological production

methods have been employed to synthesize AMPs at scales and purity that allow their direct use for characterization or structure-function studies. Advancements in these techniques will be critical to extend the widespread use of AMPs to counter infections. The advantages and disadvantages of both types of synthesis methods for AMP production is reviewed in the following sections.

2.5.1 AMP production by chemical synthesis

Solid phase chemical synthesis, invented by Robert Bruce Merrifield [81], is a mature technique widely employed for peptide production including AMPs at small production scales [82]. Peptide production by chemical synthesis has two advantages; first, artificial modifications of peptides such as the incorporation of unnatural amino acids, peptide backbone modification, amidation and acetylation can be readily achieved. Therefore, chemical synthesis methods have facilitated engineering of AMPs for enhanced properties. The second advantage of using chemical synthesis for peptide production is that chemically-synthesized peptides do not have cellular contaminant carry over such as LPS, proteins or DNA fragments which may be difficult to purify, leading to biocompatibility problems. The main disadvantage of chemical synthesis is the tedious protonation and deprotonation steps necessitated in chain synthesis, low efficiency in cysteine oxidation and therefore the need for cysteine-rich peptides having conformations constrained by disulfide bonds to be subsequently refolded *in vitro*. The use of

chemical synthesis for peptide production is therefore most suitable for peptides that are active in the linear and unfolded form. Should the peptides contain disulfide bonds, incubation in a refolding-enhancing environment would be necessary to promote correct oxidation and pairing of the cysteines. Some common obstacles that are often encountered in AMP refolding *in vitro* include (i) low solubility of AMPs which results in peptide precipitation in the refolding buffer, and (ii) the formation of incorrect disulfide connectivity which can significantly reduce peptide yield. Although studies have shown that disulfide connectivity does not affect the *in vitro* antimicrobial activity for many AMPs [11, 83], there may be other undesirable consequence due to disulfide mis-pairing on other peptide functions which cannot be directly measured. To overcome these problems, optimizations of external parameters like peptide concentration, buffer polarity, ionic strength, pH and the ratio of oxidative and reducing agents which can influence peptide refolding must be performed. These optimization measures can be practically tedious.

2.5.2 Biological production of AMPs

Compared with chemical synthesis methods, bio-production of AMPs in microbial cell factories can be more cost-effective, requires a simpler process and is more environmentally friendly [84]. Biological production of AMPs eliminates the need for the multiple protection and de-protection steps which increases

process complexity and solvent usage in the chemical synthesis route. By employing readily available genetic engineering techniques, many AMPs have been successfully produced through recombinant expression in different types of microbial hosts [85]. Both bacteria and yeast account for over 95% of reported studies on biological production of AMPs. *E. coli* is a common microbial platform for AMP production due to its well-characterized genome, easy availability of cloning vectors and cheap medium for cell growth [80, 85, 86]. Several important considerations in developing an AMP production platform in the bacteria expression system are (i) AMP toxicity to the host cell post-expression, (ii) intracellular degradation by proteolytic enzymes, as well as (iii) peptide purity, integrity and bioactivity [55, 79, 87] Sections 2.5.3 and 2.5.4 review bioprocess considerations for biological production of AMPs.

2.5.3 AMP expression by fusion protein technology

Expressing AMPs as fusion proteins by genetically linking the AMPs to fusion protein tags, is effective in overcoming AMP-induced host toxicity problems. The choice of fusion tag will depend on solubility and secondary purification requirements of the expressed fusion protein. By now, more than 30 kinds of fusion tags with different sizes and functions have been used in AMP expression. Careful selection of fusion tags is important to effectively address toxicity, solubility and purification challenges [12, 88, 89]. Thioredoxin A (TrxA) and

glutathione-S-transferase (GST) are commonly employed tags for peptide expression, accounting for 22% and 12% of the fusion protein expression studies reported, respectively [80]. TrxA is a small electron-transfer protein which is highly soluble and contains a dithiol-disulfide active site that reportedly benefits disulfide bond formation during fusion protein folding [90-92]. Furthermore, the relatively small size of TrxA (~12kDa) compared with other tags allows a high peptide mass ratio in the expressed fusion proteins. Different hBD variants including hBD 5, 6, 26, and 27 have been successfully expressed in the soluble and bioactive form at reasonable yields by employing TrxA as the fusion tag [93, 94]. GST is another commonly used fusion tag for AMP expression, with the added advantage of facilitating purification by affinity chromatography [95, 96]. The large size of the maltose binding protein (MBP) molecule promotes its easy capture on affinity chromatography resins, where the fusion protein can be purified without being impeded by steric constraints commonly encountered with smaller tags like poly-histidine. However, the large size of MBP can compromise peptide expression yield, and necessitates extensive optimisation of culture conditions to increase peptide yield. Intein-mediated systems have proven to be effective for fusion protein expression [97, 98], where its inducible self-cleaving capability allows cleavage in a single step by pH, redox or temperature shifts, leading to simplified and cheaper bioprocessing. However, despite its advantages, the intein system has not become a primary choice for laboratory-scale production due to disadvantages such as lack of solubility-enhancing capacity, and non-specific *in*

vivo or incomplete cleavage [99]. Another family of fusion tags comprising amidophosphoribosyltransferase (PurF) and ketosteroid isomerase (KSI) [100, 101], induces insoluble fusion protein expression, and hence protects the host cell from AMP toxicity. The use of these tags requires an *in vitro* refolding step to recover the AMPs in the soluble and active form, which can reduce peptide yield.

There is no universal fusion tag that can effectively resolve solubility, yield or purification issues for all peptides due to the different advantages and disadvantages of each tag which influences expression and purification of different proteins to varying extents. Therefore, the choice of fusion tags must be optimized with respect to individual peptide properties.

2.5.4 Peptide recovery from fusion proteins

After co-expression with the fusion tag, AMPs have to be cleaved to be released from the fusion protein. There are two methods of cleavage: (i) chemical and (ii) enzymatic. The use of chemical reagents such as cyanogen bromide, hydroxylamine or 1-cyano-4-dimethylaminopyridinium tetrafluoroborate to cleave fusion proteins suffers from disadvantages such as being non-environmentally friendly [102, 103], having low cleavage specificity and high possibility of microchemical modification of the target peptide [104]. The use of enzymatic agents which are more site-specific and which can be manufactured by recombinant DNA technology such as Enterokinase, Factor Xa, Thrombin and

Tobacco Etch Virus protease (TEVp) has thus become more popular [105].

Commonly used enzymatic proteases for removal of fusion tags and their advantages and disadvantages are summarized in Tables 2.5 and 2.6, respectively.

Thorough understanding of the characteristics of different proteins in the post-cleavage mixture is important to rationally design AMP purification methods post-cleavage. Chromatography is commonly reported to recover AMPs after cleavage from the fusion protein (Table 2.6). Besides chromatography methods, the use of other methods such as solvent-, acid-, and heat-mediated precipitation has also been effective [106, 107].

Table 2.5: Common proteases for fusion protein cleavage and AMP release.

Protease	Molecular weight (kDa)	Recognition site	Expression host	AMP candidate	References
Enterokinase	~26	DDDK↓	<i>E. coli</i>	hBD4, 26, 27; Adenoregulin; Cecropin	[93, 108-110]
Factor Xa	~43	LVPR↓GS	Plasma HEK 293 cells	Plectasin; Lactoferrin	[111, 112]
Thrombin	~37	LVPR↓GS	Plasma CHO cells	Cecropin; LL-37 variant	[113, 114]
TEVp	~27	ENLYFQ↓G	<i>E. coli</i>	hBD25, 28; Hepcidin	[79, 84, 115]

Table 2.6: Different AMP purification methods and their advantages and disadvantages.

Chromatography purification method	Advantages	Disadvantages	AMP candidate	References
Ion exchange	High purity, concentrated product, high loading capacity	High salt concentration environment is needed (i.e. AMP must be salt-tolerant)	Perinerin; hBD 4, 5, 26, 27	[116], [74],
Affinity	High purity	Low loading capacity	Plectasin; Scygonadin	[111, 117]
Size exclusion	High purity, integration of purification and buffer exchange	Low loading capacity, diluted product, low productivity	hBD25; Cathelicidin; LL-37	[79, 118]

2.6 Engineering AMPs for improved properties

Natural AMPs have limitations such as (i) susceptibility to salt, protease, and serum, (ii) cytotoxicity to mammalian cells, and (iii) high production costs, especially for disulfide-constrained peptides, which pose as roadblocks to their wide usage in clinical applications as anti-infective. These problems could be addressed by engineering natural AMPs through rational methods to overcome these limitations and further enhance AMP activity. AMP engineering strategies which have been recently reported include incorporation of unusual amino acids for increased peptide stability [119], C-terminus amidation and acetylation for

charge modulation [120], addition of acyl chains for lipopeptides for increased hydrophobicity [121], the addition of “branches” to get dual antimicrobial activity [122], narrow spectra pH activation [123], truncation to obtain shorter active domains [124], and the use of non-peptide backbones as peptidomimetics to address the high cost issue [125, 126]. A detailed review of AMP engineering strategies are presented in Sections 2.6.1 to 2.6.3. Other examples of AMP engineering for improved function are summarized in Table 2.7.

Table 2.7: Modified natural AMPs for improved bioactivities.

Modification	AMP candidate	References
Positive charge enhancement by amino acid substitution or addition	Chicken avian beta-defensin-8; hexapeptide PAF26; Gracisin	[127], [128], [129]
Cysteine substitution	hBD1; hBD3; Thanatin	[130], [131], [132]
Truncation to retain short and active domains	BMAP-27; Pleurocidin; Melittin; Defb14-derived peptides; hBD3 C-terminus	[133],[134], [135], [136], [137]
Combination of active domains from different AMPs (Chimeric AMPs)	hBD2 and hBD3; Lactoferricin; Lactoferrampin	[138], [139]
D-amino acid substitution	Chicken cathelicidin-2; BP100;	[140], [141]
Cyclization	Chicken cathelicidin-2; Histatin	[140], [142], [143], [144]

2.6.1 Incorporation of unusual amino acids

The susceptibility of AMPs to proteases in inflammation areas has been addressed by introducing D-amino acids to natural AMPs to render the peptides unrecognizable by these proteases [140, 145]. D-amino acid substitution has also been reported to enhance both the antimicrobial activity and selectivity of AMPs [146-148]. Systematic study of the D-amino acid substitution on AMP secondary structure, stability and activity showed that partial D-amino acid substitution can improve the activity of AMPs due to the difference in membrane interaction between natural and D-amino acid substituted AMPs [149]. Other studies reported that AMPs comprising D-amino acids alone are also active against HIV-1 and possess *in vivo* activity against plant pathogenic bacteria, which expands the potential applications of AMPs [150] [151].

2.6.2 AMP modification by amidation and acetylation

Amidation and acetylation by chemical synthesis have also been reported to modify natural AMPs for bioactivity evaluation. The role of acetylation and charge in hBD3 was recently reported, where acetylation of the C-terminus portion of hBD3 led researchers to conclude that peptide net charge is a major factor in antimicrobial activity determination [152]. The Dennison group also reported the effect of C-terminal amidation on the antimicrobial and anticancer efficiency and selectivity of AMPs [153], where amidation was found to stabilize helix formation

upon binding to the *E. coli* membrane, thus generating higher local concentrations of the peptide at the membrane bilayer surface, and hence greater levels of membrane disruption [154]. Other modification approaches, like backbone modification by cyclization, and the synthesis of multivalent AMPs by linking two or more monomers for activity enhancement were also reported [155, 156].

2.6.3 Design of congeners, fragments, and hybrids of AMPs

The design of AMP congeners, fragments, and hybrids has also been widely studied. In this design, single or multiple replacements, deletions, or addition of one or more residues to the nature AMPs sequences are involved. The fragments are generated by either truncation of the N- or C- terminus or excising internal regions which have been predicted to be the active domain. A good example of sequential modifications by amino acid substitution leading to improved activity by increased surface cationicity and hydrophobicity, is the fungal defensin, plectasin, which is under development as a new antibiotic [157]. Hybrid AMPs are designed by combination of the active regions of several different natural AMPs to combine the potential benefits from each fragment. Hancock's group constructed a family of hybrid peptides comprising fragments from cecropin A and melittin, i.e. cecropin A-melittin (CEME) [158-160]. The CEME analogs are constructed by combination of the 1–8 amino acid residues from cecropin A and 1–18 amino acid residues from melittin; the analogs were found to have an improved ability to

permeabilize the membrane of *P. aeruginosa* by binding to lipopolysaccharide and lipoteichoic acid. Design of shortened AMPs by studying peptide fragments have also led to unaltered or sometimes even improved properties, with respect to the natural AMPs [161]. Another important motivation for generation of short AMP analogs is to reduce peptide production cost.

2.6.4 hBD engineering

As an important member of the AMP family, hBD engineering studies to improve the peptide's antimicrobial potency is widely reported. Reducing the disulfide bonds of hBD1 was reported to increase its antimicrobial activity against several pathogens [162]. Although disulfide linkage in hBD3 is important to maintain tertiary structure, substitution of the cysteines with other amino acids (alpha-aminobutyric acid, serine, tryptophan, tyrosine, phenylalanine) or complete reduction of the disulfide bonds eliminated the chemotactic cytotoxicity of hBD3 but did not compromise the peptide's *in vitro* antimicrobial activity [57, 163-166]. Structure-activity correlation studies of hBD3 analogs showed that the N-terminus of the peptide is not necessary for antimicrobial activity while the C-terminus (R36-K45), which is rich in positively charged amino acids, has higher antimicrobial activity and lower cytotoxicity toward zwitterionic mammalian cells compared to wild type hBD3 [161]. Comparative structure-activity studies of different hBD1 and hBD3 analogs showed that the internal (or middle) region of

hBD1 and the C-terminus of hBD3 are responsible for antimicrobial activity retention at high salt concentration, which is a unique characteristic of hBD3 not found in other wild type hBD variants [167]. It should be stressed that in designing AMP analogs, good understanding of the effect of engineering on the complete biological function of the AMPs is critical. Since many AMPs have multiple modes of antimicrobial activity exhibition and host immune system modulation, variations such as alterations in the natural AMP sequence may cause significant changes in the peptides' physiological properties which would subsequently affect their biological function or cause other side effects. Thus systematic study should be carried out to investigate the biocompatibility of the AMP analogs.

2.7 Antimicrobial coatings

Bacterial infections occurring on the surface of biomedical implants and devices are increasingly common and currently pose as a clinical threat that needs an urgent solution [168-170]. Such infections cause high morbidity and economic burden globally; in the USA alone, infections associated with cardiovascular and urology implants are estimated to cost at least \$ 50,000 per patient and \$ 250 million per year to treat [171, 172]. Due to the widespread use of urinary catheterization, infections associated with urinary catheterization are common and are linked to 80% of urinary tract infections [15-17]. For example, in intensive care units in the United States, the incidence ranges from 3.1 to 7.4 catheter-associated urinary tract infections (CAUTI)

per 1000 urinary catheter days [173]. *E. coli* and *P. aeruginosa* are the most common pathogens associated with CUTI, accounting for 39% of the cases [174, 175]. Due of the severity and universality of bacterial infections on surfaces, designing an antimicrobial surface to prevent microbial infections by reducing the adhesion of inhibiting the proliferation and survival of microbes is critical. Until now, three classes of coating approaches have been utilized: (i) biocide-release, (ii) microbe-repelling and (iii) contact-active surfaces (Figure 2.4) [176].

Figure 2.4: General classes of antimicrobial surfaces [176].

2.7.1 Biocide-release surfaces

The release of biocides is the most traditional way for keeping microbes off a surface. The oldest and still most intensively used biocidal material are the silver compound, which include silver ions, soluble silver salts, silver nanoparticles, and silver nanocomposites. Besides silver, furanones, iodine, triclosan, quaternary ammonium, nitric oxide, hypo chloride and antibiotics have also been used as

biocides in antimicrobial coatings [177-182]. Although releasing biocides from surfaces has been applied to many biomedical implants and devices, it suffers from several disadvantages. First, exhaustion of the biocides with time reduces the sustainability of this approach. Second, many biocides are toxic to humans and pose as potential environmental hazards [183]. For example, the use of silver is limited to biomedical applications because of the low biocompatibility and silver-induced skin discoloration, both of which have been attributed to the interaction between blood cysteine and silver [184]. Likewise, the use of antibiotics is generally undesirable as it promotes the development of microbial resistance, such as the MRSA [22].

2.7.2 Microbe-repelling surfaces

The microbe-repelling surface is used to prevent microbial adhesion to decrease the incidence of infections. The repelling of microbes can generally be achieved by creating a surface that is resistant to protein adhesion. The microbe-repelling surfaces can be obtained using various approaches, like grafting or depositing of fluoropolymers [185], carbon [186], and the protein resistant poly(ethylene glycol) (PEG) [187-192]. The first bacteria-repelling surface was achieved by surface modification using protein resistant PEG-containing block copolymers to create a negatively charged surface that can repel microbial cells due to electrostatic repulsion between the surface and bacterial cells. This surface resulted in over 99% anti-adhesion effect against bacteria [193]. Another way to keep microbial cells off

from attaching to surfaces is to construct a surface that can undergo controlled degradation using degradable polymers [192]. However, such polymeric degradation typically occurs in the range of days to months while the biofilm can be easily built in hours. Although adhesion of microbes can be decreased, microbe-repelling surfaces cannot prevent the microbes present from reproducing [194, 195]. Therefore, these surfaces only can be truly effective when combined with biocide-releasing capability to target planktonic microbial cells.

2.7.3 Contact-active antimicrobial surfaces

Another way to create antimicrobial surfaces is to develop a coating that is functionalized with antimicrobial reagents, like antimicrobial polymers, cellulose derivatives, hydrogel and antibiotics. To date, various techniques have been adopted to immobilize biocides to surfaces such as layer-by-layer deposition [196], or surface grafting [197-200]. For example, the AMP Chromogranin A, was embedded on poly(methylmeth-acrylate) (PMMA) surface and exhibited 65% inhibition of *Candida albicans* growth [201]. The antimicrobial polymer (poly-(dimethylaminomethyl styrene) has been successfully coated onto a fabric surface by vapor deposition which was effective against *E. coli*, with over 99.99% of killing in just 2 min [202]. The grafting of antimicrobial reagents to a surface through covalent binding, is the most frequently used strategy by “growing” a polymer spacer on the surface and using it as the intermediate to covalently

couple biocides. The immobilization of peptides on a commercial class of polymers (Pentagels), showed that the peptides could potentially cross the cell wall with the help of polymeric spacer resulting in the disruption of cell membrane [203].

Compared with the first two approaches, contact-active surfaces possess two advantages: first, the strong binding of antimicrobial reagents to the surface avoids leaching of the reagents to the environment. Second, the non-leaching mode retains the antimicrobial activity for a longer period. Since the current use of biocides to generate antimicrobial surfaces has several disadvantages, such as toxicity, durability and pathogen resistance problems, the emergence of AMPs can overcome some of these problems. AMPs are unlikely to cause pathogen resistance than conventional antibiotics [204]. In addition to this, AMPs have good biocompatibility properties and minimal cytotoxicity against humans.

2.8 Covalent immobilization of AMPs onto surfaces

Covalent immobilization of AMPs to form a contact-active surface is desirable for increased stability and durability on the surface, provided the peptides are resistant against proteolysis [205]. Important parameters that need to be considered and optimized in AMP immobilization on surfaces include the choice of surface chemistry, chemical coupling strategies, spacer length, peptide concentration on surface, peptide orientation, and peptide conformation in the immobilized state, which could all play a synergistic role on the overall antimicrobial activity of the immobilized peptide.

2.8.1 Coupling chemistry for peptide immobilization

Covalent immobilization of peptides on surfaces can be performed in a random or controlled manner (Figure 2.5) [205, 206]. One popular random immobilization strategy is through the formation of covalent bond between the carboxyl groups on surface and amine groups in the peptides. Alternatively, carboxyl groups from the peptides can also react with surface amines. Controlled immobilization strategies will involve tethering peptides in a predictable manner, having a defined mode by chemo-selective formation of a peptide-surface covalent bond. Compared with the random mode, controlled immobilization can maintain peptide structure motifs to allow the tethered peptides to behave more closely to their soluble counterparts. For example, the use of thiol-bearing peptides allows the peptides to specifically react with the thiol-, or maleimide- groups on a surface without interfering with other amino acids in the peptide sequence which may be required to maintain peptide activity and structure/conformation. The addition of a cysteine residue to either the N- or C-terminus of the peptide may, however, compromise the peptide biological activity to a certain extent and the strict specificity cannot be achieved for the cysteine-rich peptides, moreover it is not applicable to the bio-produced peptides [207, 208]. Peptides can also be tethered on surfaces using side-chain-protecting groups to protect the side-chain residue, which results in the chemoselective reaction on the N- or C- terminus. However, incorporation of the protecting group will increase synthesis

cost, which may negatively impact large scale production. Compared to controlled immobilization, random immobilization is simpler to perform, cheaper and does not involve any modification to the peptide itself. If, however, peptide activity is conformation-sensitive, controlled immobilization may be the preferred mode.

Random immobilization

(A) Amine chemistry on NHS-derivatized and Aldehyde-derivatized surfaces

(B) Carboxyl Chemistry Using Carbodiimide Activation

Controlled immobilization

(C) Thiol Chemistry on maleimide-derivatized, disulfide-derivatized, and vinyl sulfone-derivatized surfaces

Figure 2.5 : Coupling strategies of peptide immobilization to material surfaces, black ellipse represents the peptide [209].

2.8.2 Influence of spacer on peptide antimicrobial activity

Although most immobilization protocols employ a spacer such as PEG to link the peptides to a surface to enhance ease of membrane permeabilization, the antimicrobial activity of immobilized AMPs without the use of spacers have also been reported [210]. Comparison studies of AMP immobilization with and without PEG spacers illustrated that, for some immobilized AMPs, the PEG spacer is necessary to exhibit their antimicrobial activity [203, 211, 212]. Bagheri and co-workers analyzed the effect of the length of PEG spacer on bactericidal activity by using the amphipathic model peptides, KLAL, and magainin-derived MK5E [203]. Those studies demonstrated that the antimicrobial activity of these peptides distinctly decreased with the reduction in spacer length, suggesting that the increased flexibility associated with longer spacers maximizes the antimicrobial activity of the immobilized peptide by facilitating the membrane-peptide interaction (Figure 2.6A). However, these results contradict those of Haynie *et al.* in 1995 whose work indicated no difference in bactericidal activity of peptides which were conjugated to the support using either a two-carbon or a six-carbon chain linker [210]. In addition, Hilpert *et al.* reported that short 9-mers covalently linked without spacer showed bactericidal activity [213]. Besides direct peptide penetration, other modes of killing include the replacement of positively charged cations (e.g., Mg^{2+} and Ca^{2+}) or release of vital anions from the

bacterial membrane upon interactions with AMPs (Figure 2.6B), leading to electrostatic imbalance on the membrane surface and structure disassociation. This charge imbalance will alter the Donnan potential across the cytoplasmic membrane which may trigger an autolytic and/or cell death mechanism. Therefore, the influence of spacer length on antimicrobial activity of immobilized AMPs, could therefore vary depending on whether the tethered peptides kill pathogens through membrane permeabilization or via different mechanisms.

(A)

(B)

Figure 2.6: Postulated mechanisms of immobilized AMPs with and without polymeric spacer: (A) peptides could potentially cross the cell wall with the help of polymeric spacer resulting in the disruption of cell membrane, (B) replacement of positively charged cations (e.g., Mg^{2+} and Ca^{2+}) or release of vital anions from the bacterial membrane upon interactions with AMPs, leading to electrostatic imbalance on the membrane surface and structure disassociation [176].

Lately, surface coating by using polymer brushes has attracted great attention from researchers. By comparison with other surface modification methods, polymer brushes exhibit long-term stability, higher mechanical/chemical robustness and excellent biocompatibility [214, 215]. Moreover, such technology can be easily applied to most of the commonly used biomedical materials including plastics, metals and silicone rubber [216-219]. A recent study investigated the influence of brush properties (e.g. graft density and thickness of polymeric brushes) on antimicrobial activity of the functionalized surface [220]; the results indicated that a high polymer brush graft density and thickness resulted in increased antimicrobial activity due to a combination of limited peptide chain flexibility and high peptide density on the surface. As such, it can be concluded that the requirement for a spacer and the effect of the spacer's properties to antimicrobial activity is associated with the mode of action of the specific AMP.

2.8.3 Effect of immobilization on peptide secondary structure

It has been suggested that soluble AMPs adopt well-defined structures (e.g. α -helix and β -sheet) upon interaction with bacterial membranes and these conformations are directly related to their antimicrobial function [205]. Few studies have reported the effect of immobilization on the AMPs' secondary structure, where the results suggest

that the antimicrobial activity of immobilized AMPs is often dependent on the presence of a well-defined secondary structure [205]. The study of Haynie *et al.* (1995) demonstrated that only immobilized peptides that retain their ability to form α -helices had antimicrobial activity [210]. Similarly, Cho's study concluded that the secondary β -sheet conformation is essential for the antimicrobial activity [221]. In 2012, Gao *et al.* immobilized peptides to a quartz surface and employed circular dichroism spectroscopy to investigate the secondary structure changes of immobilized peptides upon interaction with bacterial membrane. Their results showed that the percentage of α -helix conformation of soluble peptides increased from 25% (in PBS) to 92% in the presence of lipid membrane, while the conformation of immobilized peptides changed to a substantially lesser degree. The structural content of polymer brush conjugated peptides in PBS were as follow: α -helix-18.6%, β -sheet/strand -42.3%, β -turn -19.4% and random coil -19.7% while tethered peptides incubated with lipid membranes showed a structural content as follow: α -helix -52.1%, β -sheet/strand-17.2%, β -turn -10.8% and random coil -19.1%. This result indicated that tethered peptides behaved differently from their soluble peptide counterparts and the occurrence of different bacterial killing mechanism for soluble and tethered peptides cannot be ruled out [220].

In conclusion, in order to achieve the maximal antimicrobial activity of immobilized peptides, investigation of the different factors involved in the immobilization strategy, like chemical coupling strategies, spacer length, peptide concentration on surface, peptide orientation, and peptide conformation in the

immobilized state, is important. In addition, good understanding of the antimicrobial mechanism or mode of action of the immobilized peptide will guide the design of the most optimal immobilization strategy.

Chapter 3 Experimental Methods

3.1 Materials

Kanamycin sulfate, chloroamphenicol, isopropylthio-D-galactoside (IPTG), urea, L-arginine, tris(hydroxymethyl)aminomethane (Tris), ethylenediamine-tetraacetic acid (EDTA), sodium chloride (NaCl), dithiothreitol (DTT) and reduced (GSH) and oxidized glutathione (GSSG), hydrochloric acid (HCl), maltose, allyl glycidyl ether (AGE), ethanol, acetone, potassium chloride (KCl), magnesium chloride (MgCl_2) and calcium chloride (CaCl_2), gluteraldehyde, ethanol, 3,3' - Dipropylthiadicarbocyanine iodide (DiSC3(5)), glucose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylene diamine tetraacetic acid (EDTA), Dulbecco's modified eagle medium (DMEM), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), trifluoroacetic acid, acetonitrile, Triton X-100, sodium fluoride (NaF) and Mueller-Hinton broth (MHB) were purchased from Sigma-Aldrich (Singapore). Tryptone and yeast extract were purchased from Becton, Dickson and Co. (USA), and USB Corporation (USA), respectively.

3.2 Plasmids

Five pET-48b(+) plasmids harbouring the gene sequences (N- to C-terminus) of (1)

GST-TEVp-hBD25, (2) MBP-TEVp-hBD25 and (3) TrxA-6His-TEVp-hBD25, (4) 6His-MBP-TEVp-hBD28, (5) 6His-MBP-TEVp-mutant hBD28 were designed and employed in the hBD bioprocess development studies reported in Chapters 4 and 5 of this thesis. The amino acid sequences of hBD25 and 28 are given as follows; hBD25: SFEP QKCWKNNVGH CRRRCLDTER YILLCRNKLS CCISIISHEY TRR; hBD28: ARLKKCFNKVTGYCRKKCKVGERYEIGCLSGKLCCAN.

TEVp represents the tobacco etch virus protease cleavage site. A spacer sequence (ITSLYKSAGSPR) was introduced between fusion tags and the TEVp cleavage site which was deemed necessary to prevent the large fusion tag from blocking TEVp accessibility to the cleavage site. These plasmid constructs were synthesized by the Protein Expression Facility, University of Queensland.

3.3 hBD fusion protein expression in *E. coli*

Following heat shock transformation of the pET-48b(+) vectors containing the hBD25 or hBD28 gene insert into competent *E. coli* BL21-CodonPlus (DE3)-RIL cells, the transformed cells were grown overnight in LB medium (10 g/l Bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl) containing 30 µg/ml kanamycin sulfate and 34 µg/ml chloroamphenicol at 37°C under shaking conditions. 1% (v/v) of the overnight culture was inoculated into 250 ml LB or 2×YT medium, and protein expression of the hBD fusion proteins was induced with IPTG at OD₆₀₀ = 0.7~0.8. After 2 to 4 h post-induction at 37°C or 28°C, the cells were harvested by

centrifugation at 10,000 g for 30 min. The cell pellets were washed with PBS, and centrifuged at 10,000 g for 30 min.

3.4 Cell lysis for hBD recovery

The washed cell pellets were resuspended in 10 ml 50 mM Tris-HCl (pH 8.0) and disrupted by sonication using a digital sonifier (Branson Sonifier Cell Disrupter, Connecticut, USA). Sonication was performed for 30 cycles at 5 s/cycle, followed by 10 s cooling after each sonication cycle. The lysed cells were centrifuged at 10,000 g for 30 min, and soluble protein expression was determined by SDS-PAGE analysis.

3.5 Expression and purification of tobacco etch virus protease (TEVp) [222]

E. coli BL21-CodonPlus (DE3)-RIL harboring a pRK793 plasmid containing the TEV protease sequence kindly provided by Professor Stephen Bottomley (Monash University, Australia), was grown overnight in LB medium containing 30 µg/ml chloramphenicol and 50 µg/ml ampicillin, at 37 °C, 200 rpm. 1% v/v overnight culture was inoculated into fresh LB medium supplemented with 30 µg/ml chloramphenicol and 50 µg/ml ampicillin. The culture was grown at 37 °C, 200 rpm until OD₆₀₀=1 was attained, induced with 1mM IPTG, and grown for another 4 h (30 °C, 200 rpm). Cell pellets were harvested, sonicated and pre-treated using the

same methods described in Section 3.4. The cell lysate was then purified using a Ni SepharoseTM High Performance column pre-equilibrated with Buffer A (50 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, pH 8.0). All chromatography purification in this thesis were performed using an AKTA Explorer 100 Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare, Singapore) at room temperature (24°C).

After washing with Buffer A, TEVp was eluted at 35% Buffer B (50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 8.0) over 10 column volumes (CVs). The TEVp-containing fraction was buffer exchanged to 20% (v/v) glycerol buffer in 20 mM Tris-HCl (pH 8.0) using a desalting column (PD-10, GE healthcare, Singapore) and stored at -80 °C.

3.6 Maltose binding protein (MBP)-hBD fusion protein purification by amylose affinity chromatography (AAC)

The supernatant of the cell lysate was filtered with a syringe filter (0.45µm Acrodisc, Pall Corporation, Singapore) and loaded into a 5 ml MBP Trap column (GE Healthcare, Singapore) which was equilibrated with Buffer C (50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) at a flow rate of 2.5 ml/min. The column was washed with 5 CVs of Buffer C to remove unbound proteins, and two step elutions at 0.4% and 40 % Buffer D (50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM maltose, pH 7.4) were performed to recover the purified MBP-hBD fusion proteins.

The recovered MBP-hBD fusion protein was buffer exchanged into cleavage buffer (50 mM Tris-HCl, pH=8) using a desalting column (PD-10, GE healthcare, Singapore).

3.7 6His-containing hBD fusion protein purification by immobilized metal affinity chromatography (IMAC)

The filtered supernatant of cell lysate containing 6His-hBD fusion protein (i.e. TrxA-6His-TEVp-hBD25, 6His-MBP-TEVp-hBD28, 6His-MBP-TEVp-mutant hBD28) was loaded onto a 5 ml Ni SepharoseTM His Trap column (GE Healthcare, Singapore). The column was pre-equilibrated with Buffer E (50 mM Tris-HCl, 500 mM NaCl, 25 mM imidazole, pH 8.0). After washing with 10 CVs of Buffer E, the hBD fusion proteins were eluted at 80% Buffer F (50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 8.0) over 10 CVs. The recovered fusion proteins were then buffer exchanged to cleavage buffer (50 mM Tris-HCl, 150 mM L-arginine, pH 8.0) using a desalting column (PD-10, GE healthcare, Singapore).

3.8 hBD fusion protein size characterization using size exclusion chromatography (SEC)

SEC performed on the FPLC was operated at a constant mobile phase flow rate of 0.5 ml/min for size characterization of MBP-TEVp-hBD25, TrxA-6His-TEVp-hBD25,

6His-MBP-TEVp-hBD28, 6His-MBP-TEVp-mutant hBD28 fusion proteins. 0.5 ml fractions of purified fusion proteins as described in Sections 3.6 and 3.7 were introduced into a SEC column (Superdex 200 10/300 GL, GE Healthcare, Singapore) which was equilibrated with 2 CVs Buffer G (50 mM Tris-HCl, 150 mM NaCl, pH 8.0). After sample loading, the protein was eluted in Buffer G over 1.5 CVs.

3.9 Minimum inhibitory concentration determination (MIC₁₀₀)

To determine the antimicrobial activities of the peptides, MIC₁₀₀ tests were performed on different pathogens, i.e. (i) Gram negative (*Escherichia coli* (ATCC 8739)), (ii) Gram positive (*Staphylococcus aureus* (ATCC 6538)) bacteria and (iii) fungi (*Candida albicans* (ATCC 10231)). MIC₁₀₀ were determined as the lowest peptide concentration that inhibited cell growth by 100%. Briefly, lyophilized peptides were solubilized in distilled H₂O and filtered through a 0.22 µm filter (Minisart syringe filter, Sartorius, Singapore). Pathogens were first grown overnight in MHB (Bacteria) or YM (fungi) medium for 12 h. 1% v/v of the cells were inoculated into fresh medium, grown at 37 °C (bacteria) or 28 °C (fungi) and 200 rpm until mid-log phase was attained. The antimicrobial assays were conducted in the MHB or YM medium with and without 150 mM NaCl, and stock peptides were added to each well at different volumes to obtain final peptide concentrations ranging from 0 to 100 µg/ml. 10 µl of diluted cell culture (10⁵ CFU/ml) was added into each well to

achieve a final cell concentration of 10^4 CFU/ml. After 12 h growth, 10 μ l samples were taken and diluted to 10^4 CFU/ml for plating. The blank control comprised only the culture medium, while the negative control comprised of bacteria culture without peptides. The antimicrobial activities were determined by viable colony plate counting and expressed as killing of pathogens calculated from Equation 1.

Equation 1:

$$\text{Killing of pathogens (\%)} = \left\{ 1 - \frac{A_p - A_b}{A_t - A_b} \right\} \times 100\%$$

where A_p , A_b and A_t represent the number of colony forming units (CFU) for a known peptide concentration, the negative control and the positive control, respectively. The survival of pathogens was calculated from Equation 2.

Equation 2:

$$\text{Survival of pathogens (\%)} = 100 - \text{killing of pathogens (\%)}$$

3.10 Analyticals

3.10.1 SDS-PAGE analysis

SDS-PAGE analysis was performed using precast 4-12% gradient and 12% polyacrylamide gels (NuPAGE[®] Novex Bis-Tris gels, Invitrogen, Singapore). For the precast 4-12% gradient polyacrylamide gel, electrophoresis was performed at 200 mA for 45 min in the MOPS (3-(N-morpholino)propanesulfonic acid) buffer and for the 12% polyacrylamide gel, electrophoresis was performed at 200 mA for 60 min in the MES (2-(N-morpholino)ethanesulfonic acid) buffer. The gel bands were visualized by

staining with Coomassie Blue.

3.10.2 Bradford assay

For protein concentration determination using Bradford assay, 20 μ l of the peptide sample was added to 1 ml of the Bradford reagent (B6916, Sigma, Singapore) and mixed for 5 min, followed by sample absorbance measurement using a UV spectrophotometer (BioPhotometer Plus, Eppendorf, Germany) at 595 nm. Protein concentration was estimated using an absorbance versus mass calibration curve based on bovine serum albumin standards. Protein purity and percentage of target protein in the total protein mixture were determined by densitometry, which was performed by SDS-PAGE gel analysis using the Gel Pro Analyzer software. The target protein concentration is determined using Equation 3 and the yield of each step is determined using Equation 4.

Equation 3:

Target protein concentration = total protein concentration $\times$ percentage of target protein in the total protein mixture

Equation 4:

$$\text{Yield of each step (\%)} = \left\{ \frac{\text{target protein concentration} \times \text{sample volume}}{\text{target protein concentration of initial starting sample} \times \text{initial starting sample volume}} \right\} \times 100\%$$

3.10.3 RP-HPLC analysis

Peptide concentration was determined from reverse phase high performance liquid chromatography (RP-HPLC) analysis (LC-10AVP, Shimadzu, Singapore) using a C18 silica reverse phase column (Phenomenex, Singapore). Sample absorbance was measured at 214 nm using a UV detector (SPD-20A, Shimadzu, Singapore). The column was first equilibrated with 2% v/v acetonitrile-water containing 0.05% v/v trifluoroacetic acid for 10 min, followed by a 2-80% v/v acetonitrile-water gradient over 78 min.

3.11 Hemolysis assay [223]

Erythrocytes from the blood of a healthy donor were obtained by centrifugation of 5 ml of the blood samples at 1000 g for 10 min. The erythrocytes were then washed thrice with Tris buffer (50 mM, pH 8) before diluting to a final concentration of 5% (v/v). Peptide solution in Tris buffer (50 μ l) at a range of different concentrations were mixed at equal volumes with the erythrocytes stock, added to a 96-well microplate, and then shaken in an incubator at 37 °C for 1 h. After incubation, the microplate well contents were centrifuged (at 1000 g) for 10 min, and the supernatant added to the wells of a new 96-well microplate and diluted with equal volumes of Tris buffer. The absorbance of the solution at 540 nm was measured using a microplate spectrophotometer (Bio-Rad Inc., USA). Tris buffer plus 0.1% Triton X-100 was used as the positive control while Tris buffer served as the negative control. The hemolysis

percentage was determined from Equation 5:

Equation 5:

$$\text{Hemolysis (\%)} = \left[\frac{(\text{Hp} - \text{Hb})}{(\text{Ht} - \text{Hb})} \right] \times 100\%$$

where Hp is the absorbance value for a known peptide concentration, Hb is the absorbance value for the buffer and Ht is the absorbance value for the Triton X-100 positive control.

3.12 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay [204]

Biocompatibility properties of both bare and peptides impregnated PDMS were investigated by testing their cytotoxicity against smooth muscle cells (SMC) using the MTT assay. SMCs were cultured in 96-well plates containing 1×10^5 cells in each well, following which 200 μl Dulbeccos' modified Eagles medium (DMEM) culture medium containing peptides at different concentrations were added to the plates. The cells were incubated with the peptides at 37 °C for 8 h, following which the peptide-containing culture medium was buffer-exchanged with fresh DMEM culture medium and added with 20 μl MTT solution (5 mg/ml in PBS). After 4 h incubation, the MTT medium was removed and 100 μl DMSO was added under shaking conditions for 15 min. Cell viability was determined by absorbance measurement of each well at 490 nm. Percentage cell viability was determined using Equation 6.

Equation 6:

$$\text{Cell viability (\%)} = \frac{M_p}{M_b} \times 100\%$$

where M_p is the absorbance value for a known peptide concentration, M_b is the absorbance value for the 'non-peptide'-treated control.

3.13 Circular dichroism (CD) spectroscopy

The peptide secondary structure, in the presence and absence of 20 mM SDS, 5 mM POPG and 150 mM NaF, were determined using the ChirascanTM Circular Dichroism Spectrometer (Applied Photophysics Limited, UK). A 0.01 cm path length quartz cuvette (Hellma, Germany) was used to contain all samples. The peptide concentration for the CD experiments was fixed at 100 µg/ml in 10 mM phosphate buffer (pH 7.4). The spectra were recorded at 25°C from 185 to 250 nm using a bandwidth of 1 nm, and averaged over five scans. Baseline scans were obtained for the use of buffer alone (and with SDS or NaF), which were then subtracted from the respective data scans with peptides.

Chapter 4 Development of a SEC refolding bioprocess for production of bioactive hBD25*

4.1 Summary

hBD25 is a hBD variant which has been recently discovered in the male genital tract. Since its discovery, hBD25 has been hypothesized to play a key role in protection against genital tract infection, a commonly occurring infection which has significantly increased mortality rates in the last decade [224]. However, further studies to confirm the role of hBD25 are hindered by the lack of sufficient amounts of pure hBD25 for clinical studies. This chapter reports the successful development of an effective chromatography-oriented bioprocess for production of hBD25. hBD25 was expressed predominantly as soluble aggregates despite being co-expressed with MBP fusion tag in *E. coli*. Soluble aggregate formation of the fusion protein was disrupted by denaturation-reduction of the hBD25, followed by an *in vitro* size exclusion chromatography refolding step, which yielded bioactive and purified hBD25 peptides at 90±2% purity. The refolded hBD25 showed antimicrobial activity against *E. coli* K12 at a minimal inhibitory concentration of 60 µg/ml in the absence of salt, but was inactive in 150 mM NaCl. With an overall hBD25 bioprocess yield of 48±5% obtained, this bioprocess will pave the way for detailed clinical studies of hBD25 and

can serve as a generic platform for efficient recovery of other small biomolecules that inevitably exist as soluble aggregates.

4.2 Introduction

hBD25 is one of the recently discovered hBD variants, comprising 47 amino acid residues and was first found in the male genital tract [14]. The possible role of hBD25 in protection against genital tract infection, which is a commonly occurring infection, however, remains to be elucidated and established. The extremely low peptide concentration present in natural sources makes peptide extraction a difficult and costly task. Therefore, to accurately characterize the antimicrobial role of hBD25 and extend its use as an antimicrobial coating agent in biomedical materials and devices, an efficient bioprocess to rapidly and cost-effectively produce purified and bioactive hBD25 is needed.

Among all the biological expression systems, *E. coli* was chosen as the host system for hBD bio-production because it has a well-characterized genome and grows rapidly in cheap medium [225]. In this study, we first screened the possible fusion tags for hBD25 expression to eliminate host cell toxicity. The choice of fusion tag was to be made based on expression and purification yields. Based on the fusion protein constructs chosen, a bioprocess will be developed to recover and purify the peptide for detailed structure-activity studies.

4.3 Methodology

Three plasmid constructs harbouring hBD25 fusion protein sequences, i.e. (i) GST-TEVp-hBD25, (ii) MBP-TEVp-hBD25 and (iii) TrxA-6His-TEVp-hBD25 were used in this study. The methods for hBD25 fusion protein expression and cell lysis were described in Sections 3.2, 3.3 and 3.4. To optimize expression yield, temperature (15°C to 37 °C), media composition (LB, 2×YT), and induction conditions (2 h to 8 h) were varied. The MBP-TEVp-hBD25 fusion protein was purified from the cell lysate by amylose affinity chromatography (AAC) on FPLC as described in Section 3.6. The TrxA-TEVp-hBD25 fusion protein purification was performed by IMAC as described in Section 3.7. The recovered hBD28 peptides were characterized using SEC as described in Section 3.8. The antimicrobial activity of the recovered purified peptides was determined by the MIC₁₀₀ assay as described in Section 3.9. The protein purity and concentration were determined by SDS-PAGE analysis and Bradford assay as described in Section 3.10.

4.3.1 hBD25 recovery by fusion protein cleavage using TEVp

Recombinant TEVp production and purification for fusion protein cleavage was described in Section 3.5.5. Cleavage of the MBP-TEVp-hBD25 fusion protein to release the hBD25 peptide was performed at a TEVp to hBD25 mol ratio of 1:10 in an optimised cleavage buffer (50 mM Tris-HCl, pH 8.0). The post-cleavage mixture was incubated for 8 h at room temperature. MBP-TEVp-hBD25 cleavage efficiency was

determined by SDS-PAGE analysis.

4.3.2 Refolding and purification of hBD25 by SEC

The post-cleavage mixture was denatured and reduced in 8 M urea and 10 mM DTT (50mM Tris-HCl, pH 8) for 2 h and then introduced into a SEC column (Superdex 200 10/300 GL, GE Healthcare, Singapore) using a 0.5 ml sample loop for on-column refolding. SEC refolding is performed on the AKTA Explorer FPLC system. The SEC column was equilibrated with 2 CVs refolding buffer (2 M urea, 0.5 M arginine, 150 mM NaCl, 1 mM to 10 mM GSH and 1 mM GSSG, pH 8.0) at a flow rate of 0.5 ml/min. The hBD25 peptide was eluted in refolding buffer over 1.5 CVs.

4.4 Results and Discussions

4.4.1 hBD25 fusion protein expression

In this study, three different constructs harbouring hBD25 co-expressed with three different fusion tags (i.e. GST, MBP and TrxA) were synthesized and studied for soluble hBD25 fusion protein expression. The choice of fusion tags was directed at eliminating host cell toxicity and enhancing soluble expression of hBD25 [85, 226]. The soluble expression results from the three hBD25 fusion protein constructs were compared after optimization of culture conditions such as temperature, media composition and induction conditions (Figure 4.1). SDS-PAGE analysis showed that

TrxA-6His-TEVp-hBD25 and MBP-TEVp-hBD25 were expressed as soluble fusion proteins, while the GST-TEVp-hBD25 fusion protein was expressed as insoluble inclusion body. Since the use of GST-TEVp-hBD25 necessitates an *in vitro* refolding step which increases bioprocess complexity and the refolding yield of such large protein is very low, TrxA-6His-TEVp-hBD25 and MBP-TEVp-hBD25 were subsequently employed for hBD25 production and their optimized culture conditions are summarized in Table 4.1. The presence of the MBP tag in the MBP fusion protein construct and the 6His tag in the TrxA fusion protein construct facilitated hBD25 purification by amylose affinity chromatography (AAC) using a MBP Trap column, and IMAC using a His Trap column, respectively. Although both MBP-TEVp-hBD25 and TrxA-6His-hBD25 fusion proteins were recovered at >70% purity (Figures 4.2A and 4.2B), there was a huge discrepancy in recovery yields, where 87% of the MBP-TEVp-hBD25 fusion protein was recovered following AAC purification while only 8% of the TrxA-6His-hBD25 fusion protein could be recovered following IMAC purification, with most of the fusion protein lost to the flowthrough fractions. This large difference in hBD25 fusion protein recovery yield was hypothesized to be associated with steric constraints that limit the accessibility of the histidine residues to the Ni^{2+} chromatography resin in the case of TrxA-6His-hBD25 purification. MBP-TEVp-hBD25 was subsequently employed for hBD25 bioprocess development studies.

Figure 4.1: SDS-PAGE analysis showing the expression profiles of different hBD25 fusion protein constructs under optimized culture conditions; lane 1: protein molecular weight marker, lanes 2 and 3: soluble and insoluble fractions of cell lysate after GST-hBD25 expression, respectively, lanes 4 and 5: soluble and insoluble fractions of cell lysate after TrxA-6His-hBD25 expression, respectively, lanes 6 and 7: soluble and insoluble fractions of cell lysate after MBP-TEVp-hBD25 expression, respectively.

Table 4.1: Soluble expression yields of MBP-TEVp-hBD25 and TrxA-6His-hBD25 fusion proteins under optimized culturing conditions.

Expression variable	Medium	Temperature (°C)	Post induction time (h)	Fusion protein concentration (mg/l)
Protein				
TrxA-6His-hBD25	LB	26	4	100
MBP-TEVp-hBD25	2×YT	37	2	120

(A)

(B)

Figure 4.2: SDS-PAGE analysis of (A) MBP-TEVp-hBD25 and (B) TrxA-6His-hBD25 purification by AAC and IMAC, respectively. (A) Lane 1: protein molecular weight marker, lane 2: load (supernatant of cell lysate), lanes 3 to 4: flow through, lane 5: eluted fractions; (B) Lane 1: marker, lane 2: load (supernatant of cell lysate), lanes 3 and 4: flow through, lane 5: eluted fractions.

4.4.2 Characterization of hBD25 fusion protein by SEC

It is important to acknowledge that soluble expression of proteins does not guarantee bioactivity of the expressed protein, as evident in several proteins which

were expressed as soluble aggregates [227, 228]. For example, fusion protein MBP-E6 was found to be predominantly misfolded and aggregated despite being expressed in the soluble form, where the target protein, E6, precipitated following fusion tag cleavage as a result of incorrect folding of E6 *in vivo* [229]. To determine monomericity of MBP-TEVp-hBD25, the AAC-purified MBP-TEVp-hBD25 fusion protein was subjected to SEC analysis. MBP-TEVp-hBD25 eluted at an elution volume which corresponded to a molecular weight of $\geq 600\text{kDa}$ (Figure 4.3B) (from calibration against protein standards of known molecular mass (Figure 4.3C), which indicated the existence of MBP-TEVp-hBD25 fusion proteins as soluble aggregates. IMAC-purified TrxA-6His-hBD25 was also found to be predominantly expressed as soluble aggregates after SEC analysis (Figure 4.3A). It is therefore entirely possible that aggregate formation of the TrxA fusion proteins increases steric shielding of the 6His residues, thus contributing to the low IMAC protein purification yield as discussed earlier. The formation of MBP-hBD25 soluble aggregates, on the other hand, did not appear to have hindered their purification by AAC, presumably due to the relatively larger molecular mass of MBP compared to the 6His tag.

(A)

(B)

Figure 4.3: Size exclusion chromatogram of ‘affinity chromatography’-purified (A) TrxA-6His-hBD25 and MBP-TEVp-hBD25; (B) protein standards of known molecular mass.

4.4.3 MBP-TEVp-hBD25 fusion protein cleavage and characterization

From the purification and recovery results obtained, the MBP-TEVp-hBD25 construct was thus chosen as the model construct for subsequent bioprocess development studies. To recover hBD25 from the fusion proteins, enzymatic cleavage of the fusion protein was performed using TEVp. MBP-TEVp-hBD25 soluble aggregate formation did not hinder the ability of TEVp to cleave the fusion proteins, where 80% cleavage efficiency was readily achieved, as determined by SDS-PAGE analysis and Bradford assay. This outcome suggests that the formation of fusion protein soluble aggregates will not impact the final peptide yield. Post-cleavage SEC analysis of the protein fractions, however, revealed that the cleaved hBD25 peptides continued to exist as aggregates, which is undesirable. To identify the cause of the observed non-specific peptide interaction which leads to soluble aggregation, different additives (i.e. 5 mM DTT, 2 M urea, 0.5 mM EDTA and 150 mM NaCl) were added into the cleavage buffer to study the effects of these additives on cleavage yield. Addition of the reducing agent, DTT, was aimed at reducing intra- and inter-molecular disulfide bonds to eliminate covalent-induced aggregation. 2 M urea was aimed at reducing intra- and inter-molecular hydrophobic interaction between the peptides, while the addition of NaCl was hypothesized to eliminate non-specific electrostatic interaction. The addition of EDTA was aimed at reducing His-mediated assembly of the protein fragments caused by adventitious metal ion chelating. SDS-PAGE and densitometry analyses showed that none of these additives affected

TEVp cleavage efficiency, which remained approximately constant at 80% (Figure 4.4). The post-cleavage mixtures treated with the different additives were each analyzed by SEC. From the SEC chromatograms obtained, none of the additives appeared to reduce hBD25 aggregation, where all the cleaved hBD25 peptides were still eluted from the SEC column as large molecular mass aggregates. Figure 4.5A compares the SEC elution profile of the post-cleavage mixture with and without 150 mM NaCl, while Figure 4.5B shows the protein composition of the eluted fractions. The SEC elution chromatograms of the post-cleavage mixture incubated with other additives showed a comparable profile to that obtained for 150 mM NaCl. Following the ineffectiveness of non-denaturing additives to overcome hBD25 aggregation, high concentrations of urea and DTT were subsequently employed to disrupt the aggregates, which necessitated an *in vitro* refolding step to recover hBD25 in a bioactive form.

Figure 4.4: SDS-PAGE analysis of TEVp cleavage efficiency under different cleavage buffer composition at pH 8; lane 1: protein marker, lane 2: purified MBP-TEVp-hBD25 fusion protein before cleavage, lane 3: post-cleavage mixture in 50 mM Tris-HCl, lane 4: post-cleavage mixture in 50 mM Tris-HCl, 5 mM DTT, lane

5: post-cleavage mixture in 50 mM Tris-HCl, 2 M Urea, lane 6: post-cleavage mixture in 50 mM Tris-HCl, 0.5 mM EDTA, lane 7: post-cleavage mixture in 50 mM Tris-HCl, 10 mM NaCl, lane 8: post-cleavage mixture in 50 mM Tris-HCl, 30 mM NaCl, lane 9: post-cleavage mixture in 50 mM Tris-HCl, 150 mM NaCl.

(A)

(B)

Figure 4.5: (A) Size exclusion chromatogram of post-cleavage mixture in 50 mM Tris-HCl containing no additive and 150 mM NaCl; (B) SDS-PAGE analysis of SEC-eluted fractions in (A); lane 1: protein marker, lane 2: fraction from peak A, lane

3: fraction from peak B.

4.4.4 Purification and refolding of hBD25 by SEC

In order to separate hBD25 from the aggregates and recover its activity, a chromatography refolding step was performed. Following denaturation-reduction of the post-cleavage mixture in 8 M urea and 10 mM DTT, followed by 12 h of incubation, the cleavage mixture was subjected to refolding in a SEC column equilibrated in refolding buffer. The use of a SEC refolding platform is advantageous due to its simultaneous purification and refolding capability to simplify downstream bioprocessing [230, 231]. L-arginine was added to the refolding buffer to suppress aggregate formation in the refolding process. The SEC elution profile obtained following SEC refolding is shown in Figure 4.6A, where hBD25 was eluted in a monomeric form and recovered at 90% purity. SDS-PAGE analysis of the protein fractions from different purification steps in the bioprocess is shown in Figure 4.6B and the step and overall bioprocess yields are given in Table 4.2.

(A)

(B)

(C)

Figure 4.6: (A) Size exclusion chromatogram showing the elution of hBD25 following SEC refolding; (B) SDS-PAGE analysis of SEC refolding step: lane 1: protein molecular weight marker, lane 2: second peak in (A), lane 3: third peak in (A); (C) SDS-PAGE analysis of essential bioprocess steps, lane 1: protein molecular weight marker, lane 2: cell lysate, lane 3: fusion protein after AAC, lane 4: post-cleavage mixture, lane 5: 'SEC'-purified and refolded hBD25.

Table 4.2: Overall yields and purity in different production step.

Process step			Yield $\pm$ s.d.	Purity $\pm$ s.d.
Cell lysis ^a			100%	23 $\pm$ 2%
Amylose	affinity	chromatography	87 $\pm$ 4%	45 $\pm$ 4%
purification ^a				
TEVp cleavage ^b			80 $\pm$ 3%	15 $\pm$ 1%
SEC refolding ^b			95 $\pm$ 2%	90 $\pm$ 3%
Overall			48 $\pm$ 5%	90 $\pm$ 2%

^a hBD25 fusion protein^b hBD25 peptide* Data are presented as mean $\pm$ s.d. (n=3)

4.4.5 Antimicrobial activity determination of hBD25

The antimicrobial activity of refolded hBD25 was evaluated by determining MIC₁₀₀ against *E. coli* K12, which was chosen as a model strain. Figure 4.7 shows a hBD25 concentration-dependent bacteria inhibition profile, where the growth of *E. coli* K12 was significantly suppressed with increasing concentrations of refolded hBD25, with 100% growth inhibition attained at hBD25 concentration of 60 μ g/mL. hBDs are cysteine-rich and disulfide bonds have been suggested to be important for tertiary structure stabilisation of β -defensins [57-59]. Therefore, optimizing the refolding buffer with respect to redox environment is important to enhance oxido-shuffling cysteines and hence promote correct formation of disulfide bonds [232, 233]. The effect of varying GSH: GSSG ratio in the refolding buffer on the antimicrobial activity of hBD25 was investigated. From Figure 4.7, variation in GSH: GSSG ratios did not have a big impact on of the MIC₁₀₀ values of refolded hBD25.

Importantly, the antimicrobial activity of hBD25 refolded in the absence of GSH and GSSG was significantly suppressed compared to hBD25 refolded in the presence of GSH and GSSG at different ratios. This result suggests that correct disulfide bond formation could play an important role in hBD25's antimicrobial killing capability. Disulfide-driven stabilization of the hBD25 peptide could be instrumental in rendering an optimum distribution of positive charges on the peptide surface, thus increasing the peptides' electrostatic interaction toward the oppositely charged bacteria membrane.

Figure 4.7: Bacteria inhibition tests of refolded hBD25 in liquid culture. Survival percent is defined as the ratio of cell density (measured by OD₆₀₀) in the presence of hBD25 peptide to the cell density without the hBD25 peptide. Data are presented as mean $\pm$ s.d. (n=3).

As the cellular physiological environment is rich in salt (e.g. 150 mM NaCl 100 mM KCl, 2 mM MgCl₂ and 0.2 mM CaCl₂), evaluating the potency of hBD25 under a salt-rich environment is important to assess its potential to be developed as an

antimicrobial coating agent for use in a physiological environment. The antimicrobial activity of hBD25 in the presence of 150 mM NaCl was therefore evaluated and the result is represented in Figure 4.7. It is clear that the activity of hBD28 is inhibited by salt, where the survival of *E. coli* was higher than 80% at a peptide concentration from 20 µg/ml to 80 µg/ml.

4.5 Conclusion

A chromatography-centered refolding bioprocess for hBD25 production is reported in this chapter. The existence of MBP-TEVp-hBD25 as soluble aggregates was addressed by denaturation-reduction of the post-cleavage protein mixture followed by a SEC refolding step to simultaneously renature and purify the hBD25 peptides. A single SEC step was effective in recovering bioactive hBD25 at 90±2% purity, which demonstrated a MIC₁₀₀ of 60 µg/ml on the model microorganism, *E. coli* K12, while the activity was suppressed in the presence of 150 mM NaCl. The study in this chapter provides a generic chromatography-based platform that can be widely used for bioprocessing of peptides or small molecules which inevitably exist as soluble aggregates, which until now remains a common bioprocessing challenge. However, from a bioprocessing perspective, a refolding-based process increases process complexity and often compromises yield, making it potentially difficult to scale. The bioprocessing complexity of the hBD25 platform motivates the initiation of the study of the bio-production of another recently discovered hBD candidate, which also targets genital tract infections for antimicrobial coating applications and this forms the

aim of the next chapter of this thesis.

Chapter 5 Bioprocess development for production of bioactive hBD28

5.1 Summary

A bioprocess for the production of bioactive hBD28 was successfully developed in this chapter. The solubility and monomericity of the expressed hBD28 fusion protein allows the development of a more streamlined bioprocess for hBD28 compared to the hBD25 bioprocess due to the elimination of an *in vitro* denaturation-reduction and refolding steps. hBD28 at $90\pm 2\%$ purity was obtained at $79\pm 5\%$ yield from the bioprocess. The produced hBD28 showed antimicrobial activity against *E. coli* K12 at a MIC₁₀₀ of 60 µg/ml in the absence of NaCl, while its antimicrobial activity was significantly suppressed in the presence of NaCl. The successful development of the bioprocess facilitated bulk production of hBD28 for detailed structure-function studies. hBD28 characterization studies revealed that the activity hBD28 was inhibited by salt at physiological concentrations.

5.2 Introduction

Chapter 4 reported the development of a refolding-based bioprocess for bioactive hBD25 production. The complexity and low yields inherent in the refolding-based bioprocesses does not favor hBD25 as a candidate for further development into an

anti-infective coating agent. As such, this chapter aims to study the development of another recently discovered hBD candidate as an antimicrobial coating agent. Earlier studies in our laboratory found that hBD28, when co-expressed with the MBP fusion tag [84], was expressed as a soluble monomeric fusion protein. This outcome justified our decision to choose hBD28 as the model peptide for the bioprocess development studies in this chapter.

5.3 Methodology

The hBD28 fusion protein construct was designed to have the 6His tag at the N-terminus followed by the MBP tag which preceded the TEVp cleavage sequence and hBD28 peptide. Modification of the 6His tag in the N-terminus of fusion protein sequence was aimed to allow comparison studies of peptide purification using amylose affinity chromatography (AAC) and immobilized metal affinity chromatography (IMAC). The hBD28 fusion proteins (6His-MBP-TEVp-hBD28) expression and cell lysis were performed as described in Sections 3.2 to 3.4. To increase fusion protein expression levels, culture conditions such as temperature, media composition and post-induction time were optimized. The expression and purification of TEVp for 6His-MBP-TEVp-hBD28 cleavage is as detailed in Section 3.5. The 6His-MBP-TEVp-hBD28 was purified from the cell lysate by AAC on FPLC as described in Section 3.6. The IMAC purification method for 6His-MBP-TEVp-hBD28 purification is as described in Section 3.7. Characterization

of the 6His-MBP-TEVp-hBD28 was performed using SEC, as described in Section 3.8. The antimicrobial assay was determined by MIC₁₀₀ determination in the presence and absence of salt (150 mM NaCl) as described in Section 3.9. Protein purity and concentration were determined by SDS-PAGE analysis and Bradford assay as described in Section 3.10.

5.3.1 hBD28 recovery by 6His-MBP-TEVp-hBD28 cleavage using TEVp

Cleavage of the 6His-MBP-TEVp-hBD28 to release the hBD28 peptide was performed at a TEVp to hBD25 mol ratio of 1:10 in a cleavage buffer (50 mM Tris-HCl, pH 8.0) with different final concentrations of L-arginine (0 to 250 mM). The cleavage reaction time was optimized by incubating the cleavage mixture for 8 to 24 h at room temperature. 6His-MBP-TEVp-hBD28 cleavage efficiency was determined by SDS-PAGE analysis.

5.3.2 Purification of hBD28 by SEC

The cleavage mixture was incubated for 8 h at room temperature. After TEVp cleavage, the hBD28 peptide was purified from the post-cleavage mixture containing 6His-MBP fusion tag and TEVp by SEC using a Superdex 200 10/300 GL (GE Healthcare, Singapore) column with FPLC. The purified fraction was desalted to

de-ionized water in 4 °C for 24 h by using dialysis cassettes (Slide-A-Lyzer G2, Thermo scientific, Singapore) for detailed structure-function studies.

5.4 Results and Discussions

5.4.1 Construct design, cloning and expression of 6His-MBP-TEVp-hBD28

The design of the hBD28 fusion protein construct, i.e. 6His-MBP-TEVp-hBD28, was described in Section 5.3 (Figure 5.1). The construction of a dual tag i.e. MBP and 6His, was intended to allow two different purification strategies to be studied and compared. The original C-terminus residue of the TEVp cleavage site (i.e., glycine) was replaced with the N-terminus residue of hBD28 (i.e., alanine) to generate a native hBD28 peptide sequence, where an earlier study reporting on the effect of different amino acid substitution on TEVp cleavage efficiency indicates that a glycine to alanine substitution will not impact TEV cleavage efficiency [234]. The optimal soluble expression yields achieved using this 6His-MBP-TEVp-hBD28 construct was 180 ± 10 mg/l fusion protein after optimization of culture conditions such as temperature, media composition and post induction time.

Figure 5.1: The 6His-MBP-TEVp-hBD28 fusion protein comprises two fusion tags (i.e. 6His and MBP), a spacer, TEVp cleavage site (ENLYFQ↓) and hBD28. The arrow indicates the site where the cleavage will occur [84].

5.4.2 Platform development for 6His-MBP-TEVp-hBD28 purification

Since the fusion protein construct contained dual purification tags, 6His-MBP-TEVp-hBD28 fusion protein purification was performed using both AAC and IMAC, and the resulting protein yield and purity were compared. Cell lysates produced from the same batch of cell culture were divided into two fractions having equal volumes, and loaded into a 5 ml MBP Trap and 5 ml IMAC column, respectively. IMAC purification yielded a fusion protein purity of $80\pm5\%$ (Figure 5.2A), while the purity of AAC-purified fusion protein was $55\pm7\%$ due to non-specific binding of other host proteins in the eluted fraction (Figure 5.2B). 6His-MBP-TEVp-hBD28 yields from IMAC purification was also higher than AAC (Table 5.1), suggesting that the N-terminus localization of the 6His residues was instrumental in enhancing binding of the fusion proteins on the IMAC resin comprising charged nickel ions compared to the AAC resin which is functionalized with amylose. IMAC was subsequently employed to purify 6His-MBP-TEVp-hBD28 in subsequent studies.

Figure 5.2: SDS-PAGE analysis of (A) IMAC-purified 6His-MBP-TEVp-hBD28; lane 1: protein marker, lane 2: eluted fraction after IMAC purification; (B) AAC-purified 6His-MBP-TEVp-hBD28; lane 1: protein marker, lane 2: cell lysate, lane 3: eluted fraction after AAC purification.

Table 5.1: Yield and purity comparison of IMAC- and AAC-purified 6His-MBP-TEVp-hBD28.

Purification method	Yield $\pm$ s.d.	Purity $\pm$ s.d.
IMAC	93 $\pm$ 3%	80 $\pm$ 5%
AAC	82 $\pm$ 4%	55 $\pm$ 7%

* Data are presented as mean $\pm$ s.d. (n=3)

As discussed in Chapter 4.4.2, it is important to acknowledge that soluble protein expression does not eliminate the possibility of soluble aggregate formation as observed for MBP-TEVp-hBD25 in Chapter 4. To investigate the monomericity of the expressed 6His-MBP-TEVp-hBD28, the IMAC-purified proteins were subjected to SEC analysis. The SEC elution profile for IMAC-purified 6His-MBP-TEVp-hBD28 revealed two peaks at retention volumes of 9 ml (Peak 1) and 16 ml (Peak 2) (Figure 5.3). These retention volumes correspond to proteins with molecular masses of $\sim$ 670 and $\sim$ 47 kDa, respectively, as determined by the SEC calibration curve generated using protein standards of known molecular weights. SDS-PAGE analysis of both peaks revealed that both fractions comprised

predominantly 6His-MBP-TEVp-hBD28 (data not shown). This result suggests that Peak 1 contained 6His-MBP-TEVp-hBD28 that had aggregated while those in Peak 2 were monomeric, where a higher ratio of monomeric fusion proteins over the aggregates was obtained compared to hBD25 fusion protein.

Figure 5.3: Size exclusion chromatogram of purified 6His-MBP-TEVp-hBD28.

5.4.3 Optimization of cleavage conditions

The purified 6His-MBP-TEVp-hBD28 was subjected to TEVp cleavage reaction to release hBD28. However, insoluble protein precipitation was observed in the cleavage mixture. To determine the composition of the precipitate and evaluate whether precipitation will affect cleavage yield, the post-cleavage mixture was subjected to centrifugation and the insoluble fraction was dissolved in 8 M urea. Both the supernatant and solubilized precipitate fractions were analyzed by SDS-PAGE, where

the latter comprised 6His-MBP, hBD28 and some contamination proteins (Figure 5.4, lane 2). This observation indicates that these proteins are not stable in the cleavage mixture, leading to a low hBD28 yield post-cleavage. L-arginine, which has been widely used as a protein stabilizer [235] was added to the cleavage mixture; the concentration and incubation time of L-arginine concentration in the cleavage mixture were varied and their effects on cleavage yield was studied (Figure 5.4). Using densitometry and the Gel Pro Analyzer software for protein concentration determination, the optimal cleavage condition was obtained with the use of 150 mM arginine, which stabilized the proteins in the cleavage mixture without inducing precipitation. After 8 h incubation in the cleavage buffer, a $90\pm4\%$ cleavage yield was attained.

Figure 5.4: SDS-PAGE analysis of post-cleavage mixture at different concentration of L-arginine and reaction time. Lane 1: protein marker; lane 2: insoluble precipitation solubilized in 8M urea without L-arginine; lane 3: supernatant of post-cleavage mixture after 8 h incubation for cleavage reaction without L-arginine; lanes 4 to 7: supernatant of post-cleavage mixture after 8 h incubation for cleavage reaction with 50 mM, 100 mM, 150 mM, 200 mM L-arginine, respectively; lanes 8 to 9: supernatant of post-cleavage mixture with 150 mM L-arginine after 16 h and 24 h incubation for cleavage reaction.

5.4.4 Purification of hBD28 from post-cleavage mixture by SEC

Having hBD28 present in the soluble form post-cleavage eases secondary purification of hBD28 tremendously, where high selectivity separation of hBD28 from other proteins was readily performed using SEC. The purified hBD28 is monomeric as determined from protein standards of known molecular weights for calibration (Figure 5.5A). High selectivity separation of hBD28 was achieved, as determined by SDS-PAGE analysis (Figure 5.5B). The overall bioprocess yield for hBD28 production using the developed platform is given in Table 5.2.

(A)

(B)

Figure 5.5: (A) Size exclusion chromatogram of post-cleavage mixture; (B) SDS-PAGE analysis of SEC-eluted fractions in (A); lane 1: protein marker, lane 2: fraction from peak 1, lane 3: fraction from peak 2, lane 3: fraction from peak 3 after 5 times concentration by lyophilization.

Table 5.2: Overall yields and purity in different hBD28 production step.

Process step	Yield	Purity
Cell lysis ^a	100%	25±1%
IMAC ^a	93±2%	80±3%
TEVp cleavage ^b	90±4%	13±2%
SEC ^b	95±1%	90±3%
Overall	79±5%	90±2%

^a hBD28 fusion protein

^b hBD28

* Data are presented as mean ± s.d. (n=3)

5.4.5 Antimicrobial activity determination of hBD28 with and without salt

The antimicrobial activity of hBD28 was evaluated by measuring the MIC₁₀₀ against *E. coli* K12. The solid line curve in Figure 5.6 shows the growth inhibition effect of hBD28 on *E. coli* K12 in the absence of salt (e.g. 150 mM NaCl), where the

growth of bacteria was suppressed by 100% at a peptide concentration of 60 $\mu\text{g/ml}$, which was comparable to hBD25. As the cellular physiological environment is rich in salt, the antimicrobial activity of hBD28 in the presence of 150 mM NaCl was therefore evaluated and the result is represented by the dotted line in Figure 5.6. It is clear that the activity of hBD28 is inhibited by salt, where the killing of *E. coli* decreased from 100% to 28% at a peptide concentration of 60 $\mu\text{g/ml}$. The salt-sensitive behavior of hBD28 will pose as an obstacle to its development as an antimicrobial coating agent for coating and other applications. To address this roadblock, hBD28 engineering studies will be performed, which forms the basis of the study reported in Chapter 6 of this thesis.

Figure 5.6: MIC₁₀₀ determination by *E. coli* inhibition tests in liquid culture of hBD28 in the absence and presence of 150 mM NaCl. Data are presented as mean $\pm$ s.d. (n=3).

5.5 Conclusion

A scalable bioprocess was developed for the production of hBD28, which opened the way for activity characterization studies. Unlike hBD25 fusion protein which formed soluble aggregates, the hBD28 fusion protein was predominantly monomeric. After TEVp cleavage, hBD28 could be readily recovered using SEC purification without involving any denaturation-reduction and refolding steps, thus rendering a relatively simpler bioprocess compared to that of hBD25. An optimized overall process yield of $79\pm 5\%$ and hBD28 peptide purity of $90\pm 2\%$ was achieved. The antimicrobial assay of hBD28 indicated a MIC₁₀₀ of 60 $\mu\text{g/ml}$ against *E. coli* K12 in the absence of NaCl, while its activity was greatly inhibited in the presence of NaCl which was also observed for hBD25. It is important to recognize that the salt sensitivity of hBD25 and hBD28 presents an obstacle for its future application as an antimicrobial coating agent because its applications will have to be limited to a salt-free environment. This outcome motivated the engineering of hBD28 for salt-resistance properties, which forms the aim of the next chapter.

Chapter 6 Production and molecular characterization studies of hBD28 and its salt-resistant mutant

6.1 Summary

For hBD28 to be effective as an antimicrobial coating agent, the peptide's low salt-tolerant property must be overcome. The aim of this chapter is to address low salt tolerance in hBD28 by rational peptide engineering. Specifically, a salt-resistant hBD variant was engineered by amino acid substitution to increase the C-terminus cationicity of the peptide. The mutant peptide clearly exhibited salt-tolerance behavior and showed improved antimicrobial potency compared to wild type hBD28. Zeta potential analysis of the wild type and mutant peptides confirmed that the increased charged state in the C-terminus region of the mutant was crucial to overcome salt-induced charge-shielding effect, thereby enhancing the efficiency of peptide-membrane interaction compared to the wild type peptide. The mutant peptide did not, however, exhibit differences with respect to hydrophobicity, oligomerization ability, and secondary structure compared to the wild type peptide, as determined by reverse phase HPLC, size exclusion chromatography and circular dichroism spectroscopy analyses. The study in this chapter elucidates the salt inactivation mechanism inherent in many AMPs and presents a simple strategy to design

salt-resistant synthetic beta defensins and other AMP candidates, which will open the way for their use as coating agents in the physiological environment.

6.2 Introduction

A barrier to the development of hBDs as anti-infective agents for clinical applications is their activity inhibition under physiological salt conditions. Although a few studies have investigated this important phenomenon [138, 236, 237], questions pertaining to the existence of a synergistic effect between salt-inactivation and antimicrobial potency remain inconclusive. Amongst all the hBD candidates studied, only hBD3 was reported to display antimicrobial activity against a wide range of pathogens in the presence of salt up to 150 mM NaCl [11, 49], while the antimicrobial activity of all other hBDs is impaired at salt concentrations of ≥ 150 mM [94, 238-240], and by now, the mechanism for hBD3's unique salt-tolerance behavior is not well-understood. Scudiero and his team engineered series hBD variants by the different domain substitution, and the variant of hBD1 engineered by replacing the five residues -AKCCK at the C-terminal with -RKCCRRKK which is derived from the C-terminal of hBD3, exhibited salt-resistant antimicrobial activity [167]. In a separate study, the close proximity of the cationic amino acid residues, Arginine (R) and Lysine (K), for a given AMP was reported to result in an aggregation of positive charges which appeared to also promote salt resistance in AMPs [241].

The study in this chapter aims to address salt-induced inactivation problem by

designing a salt resistant hBD28 mutant with the substitution of a smaller number of residues, which may be more universal than the lengthy substitution and developing a biological platform, which is low-cost and more environmental friendly, to produce these variants for detailed and improved structure-function elucidation. Using hBD28 as a model peptide, this study employed rational amino acid substitution to increase the C-terminus cationicity of wild type hBD28 and hence increase the net peptide charge.

6.3 Methodology

6.3.1 Production of wild type and mutant hBD28 peptides

E. coli BL21 (DE3)-RIL strains harbouring a plasmid containing mutant hBD28 sequences were constructed using the same method as that reported in chapter 5. The amino acid sequences of the wild type and mutant hBD28 are shown below, where the underlined regions indicate the location of amino acid substitution.

Wild type hBD28:	Net charge
ARLKKCFNKVTGYCRKKCKVGERYEIGCLSGKLCC <u>AN</u>	+8
Mutant hBD28:	
ARLKKCFNKVTGYCRKKCKVGERYEIGCLSGKLCC <u>RRKK</u>	+12

The mutant hBD28 was expressed and purified using the same methods as those for wild type hBD8 described in Chapter 5.

6.3.2 Antimicrobial activity assay

The bacteria inhibition tests were performed using the MIC₁₀₀ determination as described in Section 3.9. *E. coli* K12 was used as the model strain to determine the MIC₁₀₀ required to fully inhibit microbial growth in liquid culture. The assays were conducted in MHB medium with and without salt, where the salts used were 150 mM NaCl, 100 mM KCl, 2 mM MgCl₂ and 0.2 mM CaCl₂.

6.3.3 Cell morphology investigation by field emission scanning electron microscopy (FESEM)

E. coli K12 was incubated with wild type and mutant hBD28 at their respective MICs with and without 150 mM NaCl for 60 min, after which the cell integrity was visualized using FESEM. Bacterial cells were fixed with 4% glutaraldehyde in 150 mM sodium phosphate buffer (pH 7.4). The slides were rinsed with 150 mM sodium phosphate buffer (pH 7.4) and dehydrated through a graded ethanol series (30–100%). After dehydration with ethanol, the slides were dried at 60 °C for 5 min. The slides were coated with platinum metal for 80 s at 20 mA and transferred to FESEM (JEOL field electron microscope, Jsm-6700F, Japan) for imaging.

6.3.4 Membrane depolarization assay

The cytoplasmic membrane depolarization activity of wild type and mutant hBD28 was determined using the membrane potential-sensitive dye DiSC3(5) [242]. Briefly,

overnight cultures of *E. coli* K12 were grown to the mid-log phase and harvested by centrifugation. The cell pellets were washed 3 times with buffer (20 mM glucose, 5 mM HEPES, pH 7.4) and resuspended in the same buffer to which was added 100 mM KCl and 150 mM NaCl, to a final OD₆₀₀ of 0.05. 0.1 mM EDTA was added to the mixture, followed by the addition of 20 nM DiSC3(5). The mixture was left to incubate until a stable baseline was obtained, indicating the successful uptake of the dye into the bacterial membrane. After the addition of peptides, membrane depolarization was determined by monitoring the change in fluorescence emission intensity using a spectrophotometer (LS55, PerkinElmer, Singapore) under shaking conditions, at an excitation and emission wavelength of 622 nm and 670 nm, respectively. EDTA and KCl were used to permeabilize the outer membrane to allow dye uptake and equilibrate the cytoplasmic internal and external K⁺ concentrations, respectively, based on a previously reported method [243].

6.3.5 Preparation of Large Unilamellar Vesicles (LUVs)

1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1-rac-glycerol) (POPG) (16:0-18:1) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, Singapore). POPG vesicles were prepared by dissolving the required amounts of phospholipid in chloroform. The solution was stored at -80 °C overnight and lyophilized. The lyophilized cake was dissolved in 10 mM phosphate buffer (pH 7.4) to a final lipid concentration of 5 mM. The lipid solutions were incubated for 1 h in water bath at

temperatures above lipid transition temperatures and subjected to freeze thaw cycles. The solution was later extruded through the Avanti mini extruder syringe device using 100 nm polycarbonate membrane filters according to manufacturer's protocol (Avanti Polar Lipids, Inc, Singapore).

6.3.6 Zeta potential analysis of POPG vesicles

The charge state of the POPG LUVs and peptide-POPG LUV complex was evaluated by zeta potential using the zeta potential analyzer (Brookhaven Instruments Corporation, Singapore). First, POPG vesicles prepared as described in Section 6.3.4, were dissolved in 10 mM phosphate buffer (pH 7.4) to a final concentration of 0.2 mM. Zeta potential measurements were taken immediately as NaCl was added to the sample to varying final salt concentrations (i.e. 0 to 150 mM). Zeta potential measurements were performed in the same way when wild type and mutant hBD28 (0 to 80 µg/ml) were added to POPG vesicles in the presence of 150 mM NaCl.

6.3.7 Hemolysis and MTT assay

The hemolysis and MTT assay were performed as described in Sections 3.11 and 3.12.

6.3.8 Peptide hydrophobicity and monomericity characterization

Peptide hydrophobicity was measured by reverse phase (RP) HPLC analysis as described in Section 3.10. The hydrophobicity of the wild type and mutant hBD28 was compared based on the difference in their retention times on the RP column.

SEC-FPLC was performed using a SEC column (Superdex peptide 10/300 GL, GE Healthcare, Singapore) at a constant mobile phase flow rate of 1 ml/min to determine peptide monomericity. 0.25 ml of wild type and mutant hBD28 dissolved in Tris-HCl buffer (pH 7.4) to a final peptide concentration of 100 µg/ml was introduced into the SEC column which was equilibrated with 2 CVs of equilibration buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). The peptide was eluted in the same buffer over 1.5 CVs, and the FPLC chromatogram was analyzed by comparing the elution volumes to estimate the molecular mass of the eluted peptides. A calibration curve for molecular mass determination was constructed using standard products from a calibration kit (GE Healthcare, Singapore).

6.3.9 Circular dichroism (CD) spectroscopy

The secondary structure of wild type and mutant hBD28, in the presence and absence of POPG vesicles and salt (NaF), were determined by CD spectroscopy. The methodology for CD spectroscopy analysis is detailed in Section 3.13.

6.3.10 Homology modeling

The three dimensional structure of mutant hBD28 was determined using homology modeling based on the previously determined three dimensional structure of hBD3 (PDB ID, 1KJ6), and wild type hBD28 [244]. Sequence alignment of the target (mutant hBD28) to templates was performed using the CLUSTALW program [245] and the models were generated using the MODELLER protocol in Discovery Studio (DS) 2.5 (Accelrys Software Inc.), which is an automated homology modeling software. The loops were refined using the discrete optimized protein energy (DOPE) method and disulfide bridges were constrained in the parameter settings in DS.

6.4 Results and Discussion

6.4.1 Designing salt-resistant mutant hBD28

Amongst all the hBD candidates studied, only hBD3 has been reported to display antimicrobial activity against a wide range of pathogens in the presence of salt [11]. Although the mechanism for this unique salt-tolerance behavior shown by hBD3 is not well-understood, several structure-function studies have attributed hBD3's salt insensitivity to the highly charged C-terminal of the peptide [246]. Scudiero and co-workers successfully engineered a salt-resistant variant of hBD1 by replacing the five residues -AKCCK at the C-terminal with -RKCCRRKK, derived from the C-terminal of hBD3 [167]. In a separate study, the close proximity of the cationic

amino acid residues, arginine (R) and lysine (K), for a given AMP was reported to result in an aggregation of positive charges which appeared to also promote salt resistance in AMPs [241]. Based on these observations, we hypothesize that salt-resistance in AMPs is the result of a charge-shielding effect imposed by competing salt ions, which reduces the ionic-driven attraction between positively charged AMPs and negatively charged microbial membranes. We expect the interaction between charged molecules to be influenced by differences in the extent of their charge states. Therefore, increasing the AMP charge to a “critical” point is expected to overcome this salt-induced charge-shielding, where salt ions bound on the microbial membrane will be sufficiently displaced by the AMPs *via* the ion-exchange mechanism, leading to membrane disruption. Increasing the peptide charge state beyond a critical point may also result in undesired cytotoxicity and/or excessively strong interaction between the cationic residues on the peptide and the phospholipid groups on the microbial membranes, thus disturbing the ease of peptide translocation into the cell. Therefore, a critical peptide charge state which yields balanced antimicrobial activity versus biocompatibility profile will be important.

Based on this hypothesis, the effect of increasing the C-terminus charge of wild type hBD28 on peptide activity was studied, by replacing the last two amino acids on the C-terminus –AN with four cationic amino acids (i.e. -RRKK). The substitution of a hydrophobic and a polar uncharged amino acid residue on the C-terminus of wild type hBD28 with 4 polar charged residues increased the overall peptide net charge from +8 to +12, which approximates that of hBD3 (i.e. +11) and the recently reported

salt-tolerant hBD1 variant (i.e. +12) [167]. It was also considered important that the number of amino acid substitution in this study be kept to a minimum, while maintaining a ‘critical’ charge state, to alleviate any peptide thermodynamic instability due to sequence-associated conformation change. By using the bioprocess platform which was developed for wild type hBD28 production described in Chapter 5, the mutant hBD28 was successfully produced in the same way for further studies in this chapter (Figure 6.1A) and the purified peptide was further analysed by mass spectroscopy to determine the peptide molecular weight (Figure 6.1B).

(A)

(B)

Figure 6.1: (A) SDS-PAGE analysis of mutant hBD28 recovered from each bioprocess step: lane 1: protein molecular weight marker, lane 2: cell lysate after centrifugation (supernatant fraction), lane 3: mutant hBD28 fusion protein after IMAC purification, lane 4: post-cleavage mixture, lane 5: SEC-purified mutant hBD28; (B) Mass spectrometry of purified mutant hBD28.

6.4.2 Antimicrobial activity determination of mutant hBD28

E. coli K12 was used as a model strain to study the effects of an elevated ionic environment on antimicrobial activity. The antimicrobial activity of mutant hBD28 was determined against *E. coli* K12 in the presence and absence of NaCl, at the physiological concentration of 150 mM NaCl. Bacteria growth was significantly suppressed by mutant hBD28 with increasing peptide concentrations (Figure 6.2A), which indicates dose-dependent killing kinetics. Mutant hBD28 showed greater antimicrobial activity against *E. coli* K12 compared with its wild type hBD28 by having a lower MIC₁₀₀ value of 40 µg/ml to achieve 100% cell growth inhibition. Importantly, the mutant peptide exhibited the same MIC₁₀₀ in the presence of 150 mM NaCl, which indicates its newly conferred salt tolerance property that is absent in its

wild type counterpart.

The effect of increasing NaCl concentration on the microbicidal activity of both peptides against *E. coli* at a fixed peptide concentration (i.e. 40 µg/ml) was also investigated. Even at 200 mM NaCl, approximately 80% kill of *E. coli* was still observed when incubated with mutant hBD28 (Figure 6.2B). On the contrary, the antimicrobial activity of wild type hBD28 was significantly more sensitive to the build-up of NaCl concentration, leading to a total impairment of antimicrobial property at NaCl concentration of 100 mM. It is clear that the killing ability of both peptides decreased proportionally with increasing NaCl concentrations, which is in agreement with the reported observation for hBD1 variants [167]. Several mechanisms for hBD killing of bacteria has been postulated; the most widely accepted being the membrane permeabilization mode which is preceded by electrostatic interaction between the positively charged peptide and negatively charged phospholipid bilayer of the cell membrane [247]. The inverse dependence between NaCl concentration and killing, as shown by our results, is thus likely due to the salt-induced charge-shielding effect, leading to lesser effective interaction of the peptide to the bacteria membrane at higher salt concentrations.

Besides Na⁺, other ions like K⁺, Mg²⁺ and Ca²⁺ are also present in the cellular physiological environment. To determine if the mutant hBD28 activity could also tolerate these salts at its MIC₁₀₀ concentration, we performed the *E. coli* inhibitory tests with both wild type and mutant peptides in the presence of K⁺, Mg²⁺ and Ca²⁺ at the respective physiological concentrations of the salts (i.e. 100 mM KCl, 2 mM

MgCl₂ and 0.2 mM CaCl₂) [248]. Figure 6.2C shows that the killing behavior of both hBD28 variants was not strongly affected by the presence of Ca²⁺ and Mg²⁺ in physiological concentration, where both peptides maintained a killing percentage of $\geq 95\%$. Interestingly, when 100 mM KCl was added to the peptide solution, the microbicidal behavior of both hBD28 wild type and mutant decreased, where only 60% kill was achieved at the mutant MIC₁₀₀ concentration of 40 $\mu\text{g/ml}$. This result indicates that KCl has a higher inhibition effect on the peptides compared to NaCl. It has been hypothesized that electrostatic interaction forces increase with increasing atomic number and decreasing hydration radius of the ions (which may affect steric factors) [249]. Therefore, Na⁺ could be competitively displaced more easily than K⁺ from the cell membrane surface by mutant hBD28 due to the weaker interaction between Na⁺ ions with cell membrane compared with K⁺, leading to a stronger inhibitory effect on mutant hBD28 activity by the latter.

(A)

(B)

(C)

Figure 6.2: Antimicrobial assay results: (A) MIC₁₀₀ determination by *E. coli* inhibition tests in liquid culture of mutant hBD28; (B) the effect of NaCl concentration on *E. coli* killing at peptide concentration of 40 µg/ml; (C) the effect of different physiological salts on microbicidal activity of wild type and mutant hBD28

on *E. coli* at peptide MIC₁₀₀ concentrations. Data are presented as mean $\pm$ s.d. (n=3).

6.4.3 Morphological changes and membrane depolarization kinetics

Figure 6.3 shows the characteristic representative single cell FESEM images of peptide treated with *E. coli* in the presence and absence of 150 mM NaCl compared to untreated peptides. The surface of untreated *E. coli* cell appears smooth and rounded, while the mutant-treated cell exhibited obvious wrinkling of the membrane surface compared with the untreated controls both with and without salt. This result indicates that the mutant hBD28 is effective in disrupting the outer membrane of *E. coli* in the presence and absence of 150 mM NaCl. However, the morphological changes of the wild type-treated *E. coli* were only visible in the absence of salt.

(A)

(B)

(C)

Figure 6.3: FESEM images of *E. coli* cells: (A) cells without treatment; (B) cells treated by wild type hBD28 without salt; (C) cells treated by wild type hBD28 with salt; (D) cells treated by mutant hBD28 without salt; (E) cells treated by mutant hBD28 with salt.

The membrane disruption activity of wild type and mutant hBD28 was further studied by investigation of membrane depolarization to study the permeabilization profile of the wild type and mutant hBD28 peptides on the bacterial cytoplasmic membrane. To evaluate the cytoplasmic membrane depolarization activities of the wild type and mutant peptides in the presence of 150 mM NaCl, the membrane potential-sensitive fluorescent dye, DiSC3(5), was added to peptide samples and changes in fluorescence emission were subsequently measured. Figure 6.4 shows the fluorescence changes with time when cells were treated by wild type and mutant

hBD28 in the presence of 150 mM NaCl. In the presence of salt, the addition of mutant hBD28 caused an immediate increase in fluorescence, while no significant change was observed when the wild type peptide was added. From this membrane depolarization results, it was concluded that only mutant hBD28 was effective in disruption the outer and inner membrane of *E. coli*, respectively, in the presence of salt.

Figure 6.4: Fluorescence spectroscopy to study cytoplasmic membrane depolarization of wild type and mutant hBD28 in the presence of 150 mM NaCl.

6.4.4 Study of salt-induced peptide-membrane interaction by zeta potential analysis

Until now, three mechanisms have been postulated to explain the antimicrobial action of AMPs, i.e. the *barrel-stave*, the *toroidal pore* and the *carpet* mechanism. However, regardless of the specific mechanism the AMP abides, the binding of AMPs

to the cell membrane has been well accepted as the first step of killing. Based on the observed MIC₁₀₀ and cytoplasmic membrane depolarization results, it is likely that the salt-induced charge-shielding effect led to lesser binding and penetration of the peptide on to the bacteria membrane, leading to wild type hBD28 inactivation at higher salt concentrations. To validate this hypothesis, we performed zeta potential measurements to understand the effect of salt on changing the charge states of the peptide-membrane complex when they interact. POPG was used as the bilayer model to mimic the anionic bacteria cytoplasmic membrane. Figure 6.5A shows the effect of varying NaCl concentration on the zeta potential changes of samples containing only POPG at a fixed concentration of 0.2 mM. It is clear that the dissociated Na⁺ ions can bind to the negatively charged POPG thus reducing the net negative charge of POPG. Figure 6.5B shows the changes in zeta potential values of systems containing POPG with hBD28 (wild type or mutant) and 150 mM NaCl. The higher zeta potential values obtained in the system containing mutant hBD28 compared to wild type hBD28 suggests that the mutant peptide was more effective in reducing the net negative charge of the POPG compared to wild type hBD28, when interacting with the POPG vesicles. In the presence of salt, we hypothesize that the mutant hBD28 will displace Na⁺ ions surrounding the POPG surface more easily compared to wild type hBD28 due to the more cationic nature of the former, resulting in a lowered MIC₁₀₀ in the presence of NaCl.

(A)

(B)

Figure 6.5: Zeta potential analysis results: (A) the effect of varying NaCl concentrations on POPG surface zeta potential; (B) the effect of incubating POPG with wild type and mutant hBD28 in the presence of 150 mM NaCl on zeta potential changes.

6.4.5 Hemolytic activity and cytotoxicity of wild type and mutant peptides to mammalian cells

Although the increased net charge of the mutant hBD28 contributed to lowered MIC₁₀₀ and conferred new salt-resistant properties to the peptide, it is important to ensure that the peptide's increased cationicity did not increase cytotoxic behavior. Many natural AMPs display selectivity in disruption of microbial membranes over eukaryotic membranes [250, 251]. This selectivity arises from the differences in the composition of bacterial and mammalian cell membranes, where bacterial membranes contain a higher proportion of negatively charged hydroxylated phospholipids such as

phosphatidylglycerol, cardiolipin and phosphatidylserine at the extracellular surface while mammalian membranes are rich in zwitterionic phospholipids [247]. To study if the increased charged state has altered the selectivity for disruption of microbial membranes over eukaryotic membranes of the mutant peptide, the wild type and mutant hBD's ability to induce human red blood cell lysis was evaluated (Figure 6.6A). The hemolysis assay results indicated that at a peptide concentration of up to three times that of mutant hBD28's MIC₁₀₀, the mutant peptide still displayed minimal hemolysis (<8%), indicating the peptide's high selectivity toward the bacteria membrane compared to the mammalian cell membrane.

(A)

(B)

Figure 6.6: Biocompatibility assay results: (A) Hemolytic effects of wild type and mutant hBD28 peptides on human red blood cells, 0.1% Triton X-100 was used as the positive control and buffer was used as the negative control; (B) Cytotoxicity of hBD28 wild type and mutant peptides on SMCs, SDS was used as the negative control and buffer was used as the positive control. Data are presented as mean $\pm$ s.d. (n=3).

The biocompatibility of the mutant peptide was also studied by testing cytotoxicity to smooth muscle cells (SMCs), with the tissue culture polystyrene dish serving as a control. Figure 6.6B shows that the cell viability of the SMCs in contact with both wild type and mutant hBD28 were comparable to the control, indicating that both peptides possess comparable *in vitro* biocompatibility. This result will open the way for further use of salt-resistant AMPs as antimicrobial coating agent for devices used under 'physiological salt'-containing environment such as urinary catheters.

6.4.6 Peptide hydrophobicity and monomericity analysis

The zeta potential and MIC₁₀₀ results pointed to the fact that the salt-resistant property of the mutant peptide was conferred by the peptide's ability to overcome charge shielding effects, where microbicidal activity for both wild type and mutant hBD is reduced, *albeit* to different extents, with increasing salt concentration. To investigate if the improved antimicrobial activity could also be due to increased hydrophobicity of the mutant which has been approved can facilitate binding of the peptide into the lipid bilayer of the membrane [252], reverse phase (RP)-HPLC analysis was performed to determine peptide hydrophobicity. The RP-HPLC chromatogram obtained showed that both wild type and mutant hBD28 eluted at the same retention time, indicating no significant differences in the hydrophobicity of both peptides in the presence and absence of 150 mM NaCl (Figure 6.7A). This result confirms that the C-terminus modification of wild type hBD28 did not change the total hydrophobicity pattern of wild type and mutant hBD28 significantly, which suggests that peptide hydrophobicity is not a factor responsible for activity improvement in the presence of salt.

Some salt-resistant AMPs have been reported to form dimers or undergo multimeric oligomerization at high salt concentrations [58, 156, 253, 254]. Compared to peptide monomers, a dimer or oligomer is reportedly better able to induce lysis of lipid bilayers and is more rapidly bactericidal at physiological salt levels. It is therefore hypothesized that oligomerization may improve antimicrobial activity retention at high salt concentration by enhancing charge and hydrophobic

characteristics of the monomer, where some researchers have successfully used intermolecular disulfide bonding to dimerize two monomers to enhance the peptide's antimicrobial activity [156]. The NMR structure of hBD3 which exhibits salt insensitive antibacterial activity reveals a unique amphipathic dimeric structure associated through the $\beta 2$ strand, and was suggested to be responsible for the peptide's unique salt insensitivity. To determine whether the improved antimicrobial potency and salt resistant property shown by mutant hBD28 could be attributed to peptide oligomerization, the peptides were analyzed by SEC in the presence of 150 mM NaCl. The SEC chromatogram indicates that both the wild type and mutant peptides existed as monomers, as both eluted at the same retention times in the presence of NaCl (Figure 6.7B). We therefore conclude that the improved salt-resistant property of mutant hBD28 was not due to peptide oligomerization in the presence of salt.

(A)

(B)

Figure 6.7: RP-HPLC (A) analysis and SEC (B) analysis to determine hydrophobicity and monomericity of hBD28, respectively.

6.4.7 Peptide structure determination by CD measurements and homology modeling

To determine if the C-terminus amino acid substitution of wild type hBD28 resulted in any changes in the peptide's secondary structure leading to improvement in electrostatic interactions between peptide and membrane, both wild type and mutant hBD28 were subjected to far UV CD analysis. Liposomes or lipid vesicles are small bilayered entities and therefore represent a membrane mimetic environment, which are commonly used to study secondary structural studies of beta-defensins [255]. Consequently, the secondary structure changes of the wild type hBD28 and its mutant upon binding to anionic POPG were analyzed by CD in the presence and absence of 150 mM NaF. NaCl was replaced by NaF to avoid any high contribution of spectra noise by Cl^- at wavelengths below 195 nm [256].

As shown in Figure 6.7, the spectra of wild type and mutant peptides hBD28 in 10 mM sodium phosphate buffer agrees with the occurrence of a “ β -defensin fold” in aqueous solution as accounted earlier for hBD3 [257] and hBD1 [167]. Unlike many helical peptides which undergo structural transition from random coil to helical conformations upon interaction with hydrophobic lipid environments [258, 259], the β -sheeted defensins owing to their disulfide and inter-helical hydrogen bonds rigidity do not readily undergo drastic structural transition when contacted with lipids [63, 260]. After incubating with anionic POPG, the CD spectra of both the wild type and mutant hBD28 did not exhibit any structural changes. It is clear from these CD results that C-terminus modification with the addition of -RRKK did not significantly affect

the peptide secondary structure. A slight shift in the peptide secondary structure was observed, however, when both POPG and NaF were present, as indicated by the shift in peak maxima and minima.

It is plausible that the subtle changes in C-terminus of wild type hBD28 could subsequently affect the peptide tertiary structure, resulting in the reinforcement of the peptide-membrane electrostatic interaction to incur salt resistance activity. Homology modeling of mutant was performed based on previously determined three dimensional structures of hBD3 and wild type hBD28 to predict changes in tertiary structure of the mutant, if any. Comparison of the predicted tertiary structures of wild type and mutant hBD28 (Figure 6.8) showed little structural difference between them.

(A)

(B)

(C)

(D)

Figure 6.7: Secondary structure analyses of wild type and mutant hBD28: (A) in 10 mM phosphate buffer (pH 7.4); (B) in 10 mM phosphate buffer with 150 mM NaF (pH 7.4); (C) in 10 mM phosphate buffer (pH 7.4) with POPG; (D) in 10 mM phosphate buffer with POPG and 150 mM NaF (pH 7.4).

Figure 6.8: A comparison of the predicted three dimensional structures of hBD3, wild type and mutant hBD28. (A)-(C): ribbon diagrams of hBD3, wild type and mutant hBD28 showing the conserved beta-defensin fold and the three disulfide bonds in *gold*. (D)-(F): The side chain disposition of the positive charged residues are marked in blue.

6.5 Conclusions

A salt-resistant mutant hBD28 was designed by amino acid substitution with -RRKK to enhance positive charge accumulation at the C-terminus. The bioprocess developed in Chapter 5 facilitated the bulk production of hBD28 mutant for detailed structure-activity characterization studies. The mutant peptide retained bactericidal activity against gram negative bacteria in the presence of 150 mM NaCl, with improved MIC₁₀₀ of 40 μ g/ml compared to 60 μ g/ml exhibited by wild type hB28.

Biocompatibility studies of the mutant hBD28 indicated its high selectivity for bacteria cell membrane with minimal cytotoxicity against mammalian cells. It was found that factors including peptide hydrophobicity, oligomerization, and secondary structure were not the contributing factors for improved salt-tolerance in mutant hBD28, as determined by RP-HPLC, SEC and CD spectroscopy. Zeta potential results suggest that the increased net charge of the mutant was clearly important in overcoming the charge-shielding effect imposed by salt, and facilitates peptide-membrane interaction in the presence of salt, which was not observed for wild type hBD28. This study explores a new and simple strategy for designing a salt-resistant synthetic AMP. The novel salt-resistant mutant hBD28 developed will guide the future design of salt-resistant AMPs and extend the widespread use of AMPs in physiological environments.

Chapter 7 Engineering short AMP analogs for immobilization on urinary catheter surfaces

7.1 Summary

Catheter-associated urinary tract infections (CUTIs) caused by pathogens colonization on catheter surface are a major health threat gripping hospitals worldwide. Antimicrobial peptides (AMPs) are a class of new antibiotics that hold promise in curbing CUTIs by combatting antibiotic-resistant pathogens. In this chapter, we aim to systematically evaluate the feasibility of immobilizing two newly engineered AMPs on silicone surfaces, to address CUTIs. Two novel short tryptophan- and arginine/lysine-rich AMPs with broad antimicrobial spectrum and salt-tolerant properties were successfully designed from hBD28 and subsequently immobilized on polydimethylsiloxane (PDMS) and urinary catheter surfaces via an allyl glycidyl ether (AGE) polymer brush interlayer, as confirmed by X-ray photoelectron spectroscopy (XPS) and water contact angle analysis. The peptide-coated silicone surfaces exhibited excellent microbial killing activity towards bacteria and fungi. Although both the soluble and immobilized peptides demonstrated membrane disruption capabilities, the latter showed a slower rate of kill presumably due to reduced diffusivity and flexibility as a result of conjugation to the polymer

brush. The AGE-AMP coating was also effective in resisting biofilm formation by repelling cell adhesion due to the synergistic effects of the AGE polymer brush and AMPs. The peptide-coated surface showed no toxicity towards smooth muscle cells. The findings of this study clearly indicate the potential for the development of AMP-based coating platforms to prevent CUTIs.

7.2 Introduction

The urinary catheter was used as a model biomedical device for this study for AMP tethering, where it has been reported that catheter-associated urinary tract infections (CUTI) contributes to a significant percentage of hospital-acquired infections today. Generally, CUTIs are preceded by microbial cell adhesion on the catheter surfaces, leading to the formation of colonies and subsequently biofilms [261]. Due to their matrix chemistry, biofilms are not easily destroyed and can facilitate horizontal gene transfer between resistant and non-resistant microbial strains resulting in 10-1000 higher likelihood of antibiotic resistance development in biofilms compared to planktonic cells [205, 262]. Therefore, biofilms function as reservoirs for pathogen proliferation, infections and resistance development. To eliminate CUTIs, preventing biofilm formation is important and necessary. Antimicrobial strategies employed to combat this problem include the use of antiseptic lubricating gels, anti-reflux valves, sealing of catheter drainage tubing junction [263], and antimicrobial coatings based on antibiotics, silver and nitric oxide [19-21], but these coating methods have

continued to evoke pathogen resistance, and suffer from disadvantages such as durability and biocompatibility problems, as discussed in Section 2.7 of the literature review chapter. The benefits of AMPs render them a potentially effective macromolecule that can overcome the disadvantages of existing antimicrobial strategies for urinary catheters. For AMP-based coating to be truly effective, broad spectrum antimicrobial activity of the AMPs must be retained, and a good coating strategy is important [167, 253, 264]. Toward this end, the aims of this study are to (i) engineer short AMP analogs based on salt-resistant mutant hBD28 as the template, (ii) develop a surface coating strategy based on polymer brush to immobilize these AMP analogs on the catheter surface, and (iii) characterize the antimicrobial and anti-biofilm properties of the AMP-impregnated catheter.

7.3 Methodology

MIC₁₀₀ measurement against different pathogens (i.e. (i) Gram negative (*E. coli* (ATCC 8739)), (ii) Gram positive (*S. aureus* (ATCC 6538)) bacteria and (iii) fungi (*C. albicans* (ATCC 10231)) as well as biocompatibility assays (i.e. hemolysis and MTT assay) of the AMP analogs were performed using the same methods described in Sections 3.9, 3.11 and 3.12. The secondary structure transition of the short analogs upon interaction with the SDS was investigated by CD spectroscopy using the same method described in Section 3.13. Peptide concentration was determined by RP-HPLC as described in Section 3.10. All the peptides used in this chapter, i.e.

SGKLVVRRKK, SGKLYYRRKK, SGKLSSRRKK, SGKLWWRRKK, RGKLWWRRKK, SGKRWWRRKK, KGKLWWRRKK, SGKKWWRRKK, KGKKWWRRKK, RWKKWRRGRK, RGKRWWRRKK, RWKRWWRRKK (RK1), and RKKRWWRRKK (RK2), were chemically synthesized by GL Biochem Co., Ltd (Shanghai, China).

7.3.1 Preparation of polydimethylsiloxane (PDMS) for AMPs tethering

A flat PDMS film was used as the model surface for AMP tethering studies as it mimics the urinary catheter, which is made of silicone elastomers [216-219]. The PDMS pre-polymer and curing agent were obtained from the Sylgard 184 kit (Dow Corning Corporation, USA), mixed at a 10:1 mass ratio and subjected to a polymerization reaction at 60 °C for 12 h. After polymerization, the PDMS was rinsed with acetone and dried in vacuum for 12 h.

7.3.2 AGE Polymer brush synthesis on the PDMS and urinary catheter surfaces

AMP immobilization was performed on both PDMS and actual silicone urinary catheter surfaces (Foley catheter, Unomedical, Singapore) using the same method, as described herein. AGE polymer brush was synthesized on the surface of PDMS by

plasma UV induced surface grafting polymerization of allyl glycidyl ether (AGE). The PDMS slide was pre-treated by radio frequency (RF, 13.56 MHz) argon plasma (March PX-500, Nordson MARCH, Germany) at 300W, 100 sccm for 5 min. The treated PDMS slide was immediately submerged into ethanol solution containing 10% (v/v) AGE and exposed to UV (365nm, 100 mW/cm²) for 60 min. The polymer brush grafted slide (PDMS-AGE) was then rinsed in ethanol and water to remove any ungrafted oligomer or homopolymer.

A urinary catheter segment was cut and halved into two pieces to expose the inner and outer surfaces for coating as shown in Figure 7.1. The surface area for each catheter sample piece is ~1 cm². AGE polymer brush was synthesized on the inner and outer surfaces of the catheter respectively using the same method as described for PDMS.

Figure 7.1: Inner and outer surface areas of urinary catheter segments.

7.3.3 AMPs immobilization on PDMS and urinary catheter surfaces

The AMP analogs were immobilized onto the polymer brush grafted PDMS and

urinary catheter surfaces *via* reaction between the amine or amide groups on the peptides and the epoxy groups present on the surfaces which were functionalized as described in Section 7.3.2. The AMP analogs were dissolved in 50 mM phosphate buffer (pH 5.5) at 500 µg/ml. A PDMS-AGE slide (~1 cm² of surface area) or urinary catheter segment (~1 cm² of surface area) functionalized with AGE polymer brush was immersed in 2 ml peptides (RK1 or RK2) solutions at 37 °C for 8 h. The peptide concentrations before and after reaction were determined by RP-HPLC, and the AMP-immobilized PDMS slides (i.e. PDMS-AGE-RK1 or PDMS-AGE-RK2) and urinary catheter segments (i.e. cat-AGE-RK1 or cat-AGE-RK2) were rinsed with de-ionized water several times to remove unbound AMPs until no AMPs can be detected by RP-HPLC. The immobilized AMP concentration was determined using Equation 7.

$$\text{AMP concentration on the surface} = \frac{(C_i - C_a) \times V}{S}$$

where C_i (µg/ml) is the initial peptide concentration in solution before immobilization, C_a (µg/ml) is the peptide concentration in solution after reaction, V (ml) is the solution volume, and S is the surface area of the slide or catheter segment (cm²).

7.3.4 Water contact angle measurement

The static water contact angle on PDMS, PDMS-AGE, PDMS-AGE-RK1 and PDMS-AGE-RK2 surfaces were measured using a goniometer (Firsttenangstroms (FTA32), ALV Technologies Pte Limited, Singapore). Digital images of a 2 µl water

droplet on surface were taken and analyzed using Fta32 Video software. Three different spots on the surface were tested for each sample and the average value was reported.

7.3.5 X-ray photoelectron spectroscopy (XPS)

The elemental composition on the material surface was characterized by XPS. The XPS analyses were conducted using Thetaprobe X-ray photoelectron spectrometer equipped with a monochromatic Al ka X-ray source (hv) (1486.68ev) and a hemispherical analyzer (Thermo Fisher Scientific, USA). The survey spectra were collected from 0 to 1300 ev with pass energy of 200 ev and 1 ev of energy step size. The spectrometer was operated at a photoelectron take off angle of 50°C. Binding energies were calibrated with respect to C 1s hydrocarbon bond at 285.0 eV.

7.3.6 Antimicrobial activity determination of AMP-immobilized PDMS and urinary catheter surfaces

Two antimicrobial testing methods, i.e. (i) “sandwich” and (ii) “immersion” methods were employed to assay the antimicrobial property of the AMP-functionalized PDMS and urinary catheter surfaces, respectively.

The “sandwich” method was adapted from the *ISO 22196* protocol [204, 265]. Briefly, bacteria and fungi cells harvested at mid-log phase were diluted to $1-1.5 \times 10^6$

CFU/ml in (i) medium (MHB or YM), (ii) PBS, or (iii) urine from a healthy donor, respectively. 20 μ l cell suspensions were pipetted on the 1 cm² surfaces of PDMS-AGE-RK1 or PDMS-AGE-RK2, which were then covered with another identical slide and gently pressed to spread the inoculums over the entire slide surface. The slides were placed into a Petri dish and incubated at 37 °C for 3 h. After incubation, 2 ml medium broth (MHB or YM) was used to wash the slides to rinse the cells from the peptide surface, and the recovered cells in the broth were diluted to different concentrations (10³ to 10⁴ CFU/ml) for plating and colony forming unit (CFU) counting. 20 μ l of each cell suspension dilution was plated on an agar plate and CFU determination was performed after 12 h. The percentage killing of bacteria or fungi was calculated from Equation 8, where PDMS slides without immobilized AMPs were used as control.

Equation 8:

$$\text{Killing of pathogens (\%)} = \frac{(\text{CFU of control}) - (\text{CFU of survivor on sample surface})}{(\text{CFU of control})} \times 100\%$$

The “immersion” method aims to determine the antimicrobial activity of the AMP-tethered urinary catheter while it is immersed in (i) medium (MHB or YM), (ii) PBS, or (iii) urine. The rounded surface of the urinary catheter prevents the use of the “sandwich” method for antimicrobial tests. Briefly, the AMP-tethered catheter piece (~1 cm² surface area) was submerged in 1.5 ml tubes containing 1 ml medium, PBS or urine respectively, and then 20 μ l cell suspensions (1-1.5×10⁶ CFU/ml) were added into the tube. The percentage of bacteria or fungi killed after 5 h incubation was calculated from Equation 8. Every experiment was repeated three times and the

average value was reported. For the antimicrobial kinetics study, 10 μ l cell suspensions were removed every hour and diluted to 10^3 to 10^4 CFU/ml for plating and CFU counting (catheters without immobilized AMPs were used as negative controls).

7.3.7 Determination of biofilm formation on AMPs-immobilized PDMS and urinary catheter surfaces

E. coli, *S. aureus*, or *C. albicans* biofilm formation on PDMS, PDMS-AGE, PDMS-AGE-RK1 and PDMS-AGE-RK2 surfaces was studied. First, the pathogens were grown for 24 h and subsequently diluted to 10^7 CFU/ml in MHB or YM medium. Each of the PDMS, PDMS-AGE, PDMS-AGE-RK1 and PDMS-AGE-RK2 slide was placed in a 15 ml sterile tube, and 1 ml cell culture at a concentration of 10^7 CFU/ml was added to each tube. After incubation at 37 °C (bacteria) or 28 °C (fungi) for 3 days, the slides were recovered from the tubes and non-adhering cells were gently washed off by immersing the slides in PBS for 5 s. Biofilm formation on the slides was studied using a confocal laser scanning microscope (LSM510, Zeiss, Thornwood, NY), where the cells were stained with a fluorescence dye, SYTO 9, (Invitrogen, Singapore) according to the manufacturer's protocol. The excitation and emission wavelengths were set at 488 nm and 530 nm, respectively, for image capture [266].

E. coli, *S. aureus*, or *C. albicans* biofilm formation on cat-AGE-RK1 or cat-AGE-RK2 were also studied. The uncoated catheter and cat-AGE-RK1 or

cat-AGE-RK2 segments ($\sim 1 \text{ cm}^2$ area) were each submerged into a 15 ml tube containing 1 ml cells at a concentration of 10^7 CFU/ml. Only one surface of the catheter was used to determine biofilm formation, while the other surface was covered with masking tape. After incubation at 37 °C or 28 °C for 3 days, the masking tape was removed and the catheter pieces were washed in PBS to remove non-adhering cells. Biofilms were quantified using crystal violet staining [267, 268]. Briefly, the catheter was stained with crystal violet (CV) for 1 h and rinsed with water to remove any CV residues. The remaining CV on the surface was extracted by ethanol for 15 min and the amount of biofilm was determined by absorbance measurement at 595 nm using a microplate reader (xMark, Bio-rad, Singapore).

7.3.8 Stability investigation of AMP immobilization on PDMS surface

The antimicrobial activities of fresh prepared PDMS-AGE-RK1 and PDMS-AGE-RK2 were first investigated as described in section 7.3.8. After that, the slides were immersed in PBS. The short term stability of AMP immobilization on PDMS was investigated by comparing the antimicrobial performance after being immersed in PBS for 1 day, 2 days and 3 days, respectively. The long term stability was investigated by comparing antimicrobial performance of PDMS-AGE-RK1 and PDMS-AGE-RK2 after immersing them in PBS for one week.

7.3.9 Biocompatibility determination of AMPs-immobilized PDMS

The biocompatibility properties of PDMS, PDMS-AGE-RK1 and PDMS-AGE-RK2 were investigated by testing their cytotoxicity towards smooth muscle cells (SMCs) [204] using the MTT assay. First, PDMS, PDMS-AGE-RK1 and PDMS-AGE-RK2 were placed at the bottom of each well in a 24 well plate. SMCs were seeded at a density of 1×10^5 cells in each well, followed by the addition of 500 μ l Dulbecco's modified eagle medium (DMEM) into the wells. The cells were incubated at 37 °C for 72 h, and the culture medium was buffer-exchanged with fresh DMEM culture medium, followed by the addition of 250 μ l MTT solution (5 mg/ml in PBS). After 4 h incubation, the MTT medium was removed and 1 ml DMSO was added under shaking conditions for 15 min. Cell viability in each well was determined by absorbance measurement at 490 nm.

7.3.10 Antimicrobial mechanism investigation by cell morphology analysis and ATP leakage assay

E. coli K12 was used as the model strain to investigate the antimicrobial mechanism of soluble and immobilized RK1 and RK2. Field emission electron scanning microscopy (FESEM) analysis was employed to visualize *E. coli* cells before and after AMP treatment to assess cell morphological variation. *E. coli* was incubated with soluble and immobilized RK1 and RK2 in PBS for 60 min before

FESEM analysis was performed. Bacterial cells were fixed with 4% glutaraldehyde in PBS on a silicon wafer slide. The slide was rinsed with PBS, dehydrated with a graded ethanol series (30–100%) and dried at 60 °C for 5 min. The slide was then coated with platinum for 80 s at 20 mA condition and transferred to FESEM (JEOL field electron microscope, Jsm-6700F, Japan) for imaging.

ATP release from the intracellular compartment of the cell was analyzed using the BacTiter-Glo™ Microbial cell viability assay kit (10ml, Promega, USA) and a luminometer (GloMax™ 20/20, Promega, USA). Briefly, *E. coli* was first grown to mid-log phase, and the harvested cells were washed with PBS. 20 µl cell suspensions in PBS at $1-1.5 \times 10^6$ CFU/ml cell concentration were diluted into 1 ml PBS. AMP-immobilized slides (PDMS-AGE-RK1 or PDMS-AGE-RK2) having an estimated immobilized peptide concentration of $\sim 20 \mu\text{g}/\text{cm}^2$ as determined by RP-HPLC, were immersed into the 1 ml cell suspension in PBS. Soluble RK1 or RK2 were also added to the 1 ml cell suspension in PBS to a final concentration of 20 µg/ml as a control. After incubation of peptides and cells at 37 °C for 2 h and 4 h, the peptide and cell suspension was centrifuged, and 100 µl filtered supernatant was mixed with 100 µl luciferase reagent for 5 min, and the luminescence value was recorded.

7.4 Results and discussions

7.4.1 Rational design and engineering of short AMP analogs

The salt-resistant mutant hBD28 developed in Chapter 6 was used as a template to design shorter analogs for urinary catheter immobilization. The rationale for using shorter AMP analogs is motivated by structure-activity constraints and also scale-up cost. Previous studies which compared the antimicrobial activity between soluble and immobilized AMPs showed that the activity of the latter was largely compromised due to immobilization [203, 210, 211]. As different coupling chemistries and AMP models were employed in those studies, the reason for reduced activity of immobilized AMPs could not be directly ascertained. It is expected that peptide immobilization on a surface would inevitably affect peptide structure and flexibility, and hence peptide activity to a certain extent. In addition, the urinary catheter's physicochemical environment which contains urea, salt and other inorganic or organic compounds can also affect peptide structure and activity. For example, steric effects associated with immobilization can result in incomplete exposure of the active or binding domain of the peptide, and thus hinder peptide-bacterial membrane interaction. Therefore, the use of short analogs as coating agents is hypothesized to be able to minimize some of these effects, where short peptides are usually characterized by relatively simpler structures. The last 10 C-terminus amino acid residues of the salt-resistant mutant hBD28 developed in Chapter 6, i.e. – SGKLCCRRKK, was used as a parent template for generation of 10 amino acid residue AMP analogs for this

immobilization study.

We first chose to substitute the two cysteine residues in the parent template with other two valine (V), tyrosine (Y), serine (S) or tryptophan (W) residues to simplify analog synthesis by eliminating the need for correct cysteine oxidation for disulfide bond formation. Previous studies on hBD3 engineering have demonstrated that cysteine substitution with hydrophobic amino acid residues significantly improved peptide antimicrobial activity while minimizing the cytotoxicity [152, 164, 166, 254, 269]. MIC₁₀₀ determination of the V, Y, S and W substituted analogs showed that only the W substituted analog showed broad spectrum antimicrobial activity against Gram positive, Gram negative and fungi, while the other analogs only showed activity against *E. coli* (Table 7.1). This result is in agreement to the postulated role of W for antimicrobial activity improvement by increasing peptide-‘lipid bilayer’ interaction reported in earlier studies [252, 270]. However, the activity of the W substituted analog was inhibited in the presence of 150 mM NaCl. To confer both salt-resistant and broad spectrum antimicrobial activity properties on the analogs, we set out to increase peptide net charge by substituting other neutral residues in the sequence with arginine (R), lysine (K) and W to increase the peptide charge while gradually increasing the number of W to obtain an optimal charge-hydrophobicity balance for optimum antimicrobial activity. Out of the 13 analogs generated, two analogs, i.e. RWKRWWRRKK (RK1) and RKKRWWRRKK (RK2), were found to exhibit both broad spectrum activity and salt-tolerance (Table 1), and hence were selected as the peptide candidates for the urinary catheter immobilization studies; these two analogs

are referred to as RK1 and RK2, respectively, throughout this thesis from this point forward. From this design exercise, it is clear that both hydrophobic and charge effects are important to render broad antimicrobial activity spectrum to the hBD28 analogs under physiological salt conditions.

Table 7.1: Antimicrobial activity of rationally engineered AMP analogs.

Peptide sequence	Number of W residue	Charge	MIC ₁₀₀ (µg/ml)					
			<i>E. coli</i>		<i>S. aureus</i>		<i>C. albicans</i>	
			without NaCl	with NaCl	without NaCl	with NaCl	without NaCl	With NaCl
SGKLVVRRKK	0	+5	10	inactive	inactive	inactive	inactive	inactive
SGKLYYRRKK	0	+5	10	inactive	inactive	inactive	inactive	inactive
SGKLSSRRKK	0	+5	10	inactive	inactive	inactive	inactive	inactive
SGKLWWRRKK	2	+5	10	inactive	20	inactive	20	inactive
RGKLWWRRKK	2	+6	10	inactive	20	inactive	20	inactive
SGKRWWRRKK	2	+6	10	inactive	20	inactive	20	inactive
KGKLWWRRKK	2	+6	10	inactive	20	inactive	20	inactive
SGKKWWRRKK	2	+6	10	inactive	20	inactive	20	inactive

KGKKWRRKK	2	+7	10	20	20	inactive	20	inactive
RWKKWRRGRK	2	+7	10	20	20	inactive	20	inactive
RGKRWWRRKK	2	+7	10	20	20	inactive	20	inactive
RWKRWWRRKK	3	+7	10	20	20	20	20	20
RKKRWWRRKK	2	+8	10	20	20	20	20	20

* Antimicrobial activity at different peptide concentrations (5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 60 µg/ml, 100 µg/ml, 150µg/ml, 200 µg/ml) were tested.

* Inactive indicates that MIC₁₀₀ is higher than 100 µg/ml.

* The NaCl concentration used was 150 mM.

7.4.2 Biocompatibility studies of RK1 and RK2

The ability of RK1 and RK2 to induce human red blood cell lysis was studied to investigate the effect of charged amino acid substitutions in RK1 and RK2 on their biocompatibility properties (Figure 7.2A). The required concentration of RK1 and RK2 to incur 20% hemolysis on red blood cells was 200 $\mu\text{g/ml}$, indicating that the HC_{50} of RK1 and RK2, i.e. peptide concentration which can induce 50% hemolysis on red blood cells, was higher than 200 $\mu\text{g/ml}$. Determination of $\text{HC}_{50}/\text{MIC}_{100}$ of RK1 and RK2 each yielded a value of 20, which indicates that the peptide selectivity for the *E. coli* membrane is 20 times greater than that for the mammalian membrane.

The cytotoxicity of RK1 and RK2 against smooth muscle cells (SMCs) was also investigated, with the tissue culture polystyrene dish serving as a control. Figure 7.2B shows that the viability of SMCs which were incubated with RK1 and RK2 was largely uncompromised up to 80 $\mu\text{g/ml}$ (i.e. 4 times of MIC_{100} against *E. coli*). These results show that both RK1 and RK2 exhibited good selectivity and biocompatibility properties, which opens the way for their use as coating agents for urinary catheters.

(A)

(B)

Figure 7.2: Biocompatibility studies of RK1 and RK2: (A) Hemolysis of human red blood cells, 0.1% Triton X-100 was used as the positive control and buffer was used as the negative control; (B) Cytotoxicity on SMCs, SDS was used as the negative control and buffer was used as the positive control. Data are presented as mean $\pm$ s.d. (n=3).

7.4.3 Secondary structure characterization of RK1 and RK2 by CD spectroscopy

The secondary structure of RK1 and RK2 was characterized by CD spectroscopy, in the presence and absence of 20 mM SDS, which is a membrane-mimicking detergent commonly used for AMP-membrane interaction studies [271]. As shown in Figure 7.3, the minima at ~200 nm of both RK1 and RK2 suggested their probable existence as a mixture of random coil and β -hairpin structures [272]. There was very subtle change in the far UV CD spectra between RK1 and RK2 in the presence and absence of SDS, unlike many peptides which undergo distinct structural transition from the unstructured form to helical conformations upon interaction with lipid environment [258, 259]. For example, hBD3 showed a structural transition to adopt a helical conformation when SDS is present, where the helical structure is found to play an important role in the antimicrobial activity [131]. In the ~225 nm region, where the ellipticity contribution is from the superimposition of peptide bonds and aromatic residues, the changes of both RK1 and RK2 upon interacting with SDS can be attributed to restriction in the backbone structure and reorientation of the side chains [252]. These CD results suggest that the secondary structure may not have strong influence on the peptide antimicrobial activity due to the minimal change in secondary structure both in the aqueous buffer and during interaction with SDS. For coating applications, a low dependence of activity on peptide structure is desirable,

where immobilization-induced peptide structural changes can be ignored.

(A)

(B)

Figure 7.3: Far UV CD spectra of RK1 (A) and RK2 (B) in buffer (10 mM phosphate, pH 7.4) and in the presence of 20 mM SDS.

7.4.4 Immobilization of RK1 and RK2 on PDMS slides and silicon urinary catheters

The excellent antimicrobial and biocompatibility properties of RK1 and RK2 merit further studies to evaluate their performance as peptide candidates for urinary catheter immobilization, which forms the basis of this study. RK1 and RK2 immobilization studies were first performed on PDMS, which is formed by dimethylsiloxane polymerization. PDMS mimics the chemical composition of silicone urinary catheters, and is readily amenable to chemical functionalization [216-219]. The use of flat PDMS slides allows characterization studies such as water contact angle measurements to be performed, which cannot be performed on rounded surfaces inherent in silicone catheters.

The use of polymer brush linkers to facilitate peptide immobilization have gathered much interest because of the advantages of polymer brush in terms of their high mechanical and chemical robustness, excellent biocompatibility and anti-cell adhesion properties, which can prevent or reduce biofilm formation. Microbe repelling properties is generally achieved by surface modification using polyethylene glycol (PEG) [176], where the hydrophilicity of PEG helps to minimize hydrophobic interactions between bacteria and surfaces [266]. In this study, AGE was employed as the monomer for polymer brush formation because the abundant -C-O- groups in the AGE backbone is expected to reduce surface hydrophobicity, and hence prevent

biofilm formation. Also, the availability of unsaturated double bonds and epoxide groups in the AGE monomer facilitates peptide coupling to the silicone surface and eliminates the need to introduce other functional groups for coupling, thus simplifying the coupling chemistry. Other advantages of AGE include low cost, ready availability, and resistance of AGE interlayer against many wash cycles [265].

The immobilization of RK1 or RK2 on PDMS slides were performed *via* three steps: (1) Argon plasma treatment of PDMS surface; (2) induction of carbon-carbon double bonds in AGE to form the polymer brush backbone by UV-induced polymerization, and (3) reaction of the epoxide groups in AGE with peptide-derived amine or amide groups by second-order nucleophilic substitution (Figure 7.4).

Figure 7.4: The schematic representation of RK1 and RK2 grafting to PDMS.

7.4.5 Chemical composition and surface wettability determination

XPS and static contact angle measurements were employed to characterize the chemical composition and wettability of PDMS, PDMS-AGE, PDMS-AGE-RK1 and PDMS-AGE-RK2, respectively. The static contact angle of uncoated PDMS surface was $120.4^{\circ} \pm 2.6^{\circ}$, indicating a hydrophobic surface (Table 7.2). After AGE functionalization, surface hydrophilicity increased, as indicated by a significantly reduced contact angle ($79.2^{\circ} \pm 1.6^{\circ}$) due to the deposition of the hydrophilic -C-O- group from AGE. The static contact angle of PDMS-AGE-RK1 ($71.6^{\circ} \pm 1.1^{\circ}$) and PDMS-AGE-RK2 ($71.7^{\circ} \pm 1.8^{\circ}$) further decreased slightly upon incorporation of AMPs.

AGE and AMP deposition on PDMS was also verified using XPS analysis. Firstly, the grafting success of the AGE polymer brush on the PDMS surface was confirmed by the decreasing Si and increasing C content on the PDMS-AGE surface compared with the untreated PDMS surface (Table 7.2). Figure 7.5A shows the presence of C1s, O1s, N1s, Si2s and Si2p peaks. New peaks at ~ 399.7 eV which represent the presence of nitrogen (N1s) were observed in the XPS spectra of PDMS-AGE-RK1 and PDMS-AGE-RK2 (Figure 7.5B), which were not present in the spectra of the untreated PDMS and PDMS-AGE samples. This result confirms the successful coupling of RK1 and RK2 to AGE-PDMS, where an increase in nitrogen content from 0% to 3.4% was obtained (Table 7.2). In the high resolution C1s spectra (Figure 7.5C

and 7.5D) of PDMS-AGE-RK1 and PDMS-AGE-RK2, the presence of peaks at 288.53 eV and 287.72 eV which are correlated to the peptide bond formation (-C(=O)NH-) further confirms the success of AMP immobilization. RK1 and RK2 concentrations on the PDMS surface were estimated by RP-HPLC to be 16 $\mu\text{g}/\text{cm}^2$ and 15 $\mu\text{g}/\text{cm}^2$, respectively. To investigate the possibility of physical adsorption of the peptides to the PDMS surface, a control experiment was performed, by immersing PDMS into RK1 and RK2 peptide solutions, followed by peptide concentration determination by RP-HPLC analysis and XPS characterization. The characterization results demonstrated that there is no change in peptide concentration in solution, and no nitrogen element was detected by XPS on the PDMS surface (data not shown), indicating that immobilization of RK1 and RK2 on the PDMS surface occurred mainly by covalent bonding and not physical adsorption.

Table 7.2: Elemental analysis of PDMS surfaces treated with different chemistry.

Surface	N (%)	C (%)	O (%)	Si (%)	Contact angle ($^{\circ}\text{C}$)
PDMS	0	27.03	41.91	31.05	120.46
PDMS-AGE	0	34.46	10.47	28.65	79.20
PDMS-AGE-RK1	3.41	41.43	29.48	24.47	71.66
PDMS-AGE-RK2	2.09	41.31	29.62	26.00	71.71

(A)

(B)

(C)

(D)

Figure 7.5: XPS characterization. (A) XPS survey spectra of PDMS, PDMS-AGE, PDMS-AGE-RK1 and PDMS-AGE-RK2; (B) high-resolution N_{1s} spectra of PDMS-AGE, PDMS-AGE-RK1 and PDMS-AGE-RK2; (C) high-resolution C_{1s} spectra of PDMS-AGE-RK1; (D) high-resolution C_{1s} spectra of PDMS-AGE-RK2.

7.4.6 Antimicrobial and anti-biofilm properties of PDMS-AGE-RK1 and PDMS-AGE-RK2

To evaluate the antimicrobial activity of the PDMS-AGE-RK1 and PDMS-AGE-RK2, the *in vitro* antimicrobial activity against three strains, i.e. *E. coli*, *S. aureus* and *C. albicans*, were determined using the “sandwich” assay described in Section 7.3.6. Both RK1- and RK2-immobilized PDMS samples maintained their antimicrobial properties, and exhibited broad antimicrobial spectrum with higher than 70% killing for all pathogens (Figures 7.6A and B). A stronger antimicrobial activity toward *E. coli* was observed for both PDMS-AGE-RK1 and PDMS-AGE-RK2 surfaces, which was also observed for soluble RK1 and RK2 from MIC₁₀₀ determination. Importantly, the antimicrobial activity of immobilized RK1 and RK2 was maintained when the antimicrobial activity test was repeated in urine and PBS, suggesting that the antimicrobial performance of the RK1- and RK2-coated surfaces were not sensitive to the presence of salt, proteases and other ions in urine. No antimicrobial activity was detected when the PDMS-AGE was subjected to the “sandwich” assay, which confirms that the antimicrobial properties of PDMS-AGE-RK1 and PDMS-AGE-RK2 were contributed solely by the RK1 and RK2 peptides.

Preventing biofilm formation is critical to eliminate CUTIs and by improving the surface anti-cell adhesion property, biofilm formation can be effectively inhibited. To evaluate the anti-biofilm formation activity of PDMS-AGE-RK1 and PDMS-AGE-RK2, the slides were incubated in *E. coli*, *S. aureus*, and *C. albicans*

cultures for 3 days. The slides were then stained with SYTO green-fluorescent nucleic acid dyes which will show a large fluorescence enhancement upon nucleic acid binding, and subjected to confocal laser scanning microscopy to investigate biofilm formation on the surface. Figure 7.7 shows that the amount of cells attached to the PDMS-AGE surface was significantly reduced compared to the uncoated PDMS slides. Addition of RK1 and RK2 to the slides appears to further enhance the anti-cell adhesion property of the surface, resulting in almost 100% inhibition of pathogen colonization on the surfaces for *E. coli* and *S. aureus*. These results show that AGE works synergistically with immobilized RK1 and RK2 to prevent biofilm formation on the PDMS surfaces [266, 273, 274]. With only a slight increase in surface hydrophilicity compared to PDMS-AGE after AMP tethering, the corresponding improvement in anti-biofilm property observed is unlikely due to surface hydrophilicity changes, but rather the antimicrobial potency of the peptides.

(A)

(B)

Figure 7.6: Killing of *E. coli*, *S. aureus*, and *C. albicans* on (A) PDMS-AGE-RK1 surface and (B) PDMS-AGE-RK2 surface under different environment. Data are presented as mean $\pm$ s.d. (n=3).

Figure 7.7: Representative confocal scanning laser microscopy images of attached *E.*

coli (A), ; *S. aureus* (B) and *C. albicans* (C) cells on PDMS (1), PDMS-AGE (2), PDMS-AGE-RK1 (3), PDMS-AGE-RK2 (4) surfaces.

The short term stability of AMP immobilization on PDMS surface was investigated by comparing the antimicrobial performance of freshly prepared PDMS-AGE-RK1 and PDMS-AGE-RK2 and the slides after being immersed in PBS for 1 to 3 days. The long term stability of AMP immobilization on PDMS surface was investigated by comparing the antimicrobial performance of freshly prepared AMP-immobilized samples and the slides after being immersed in PBS for 1 week. Table 7.3 shows no significant decrease of antimicrobial performance of the peptide-immobilized slides after 3 days, indicating good stability of immobilized RK1 and RK2 on the PDMS slides. Substantial decrease in antimicrobial performance of the RK1- and RK2-immobilized slides was found after being immersed in PBS for 1 week. Ongoing work is currently underway to improve the long-term stability of the immobilized peptides.

Table 7.3: The antimicrobial performance of freshly prepared PDMS-AGE-RK1, PDMS-AGE-RK2 and after being immersed in PBS for 1 day, 2 days, 3 days and 1 week.

	Killing of PDMS-AGE-RK1 (%)			Killing of PDMS-AGE-RK2 (%)		
	<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>
Fresh	97.4	76.5	74.2	96.1	75.3	72.8
1 day	98.5	75.5	75.3	95.6	70.2	68.2
2 days	97.4	70.9	79.3	93.2	68.3	70.3
3 days	93.8	79.3	78.2	92.4	60.9	65.4
1 week	90.1	61.2	58.7	90.4	51.3	52.4

7.4.7 Biocompatibility determination of PDMS-AGE-RK1 and PDMS-AGE-RK2

The increased cytotoxicity of biomedical implants and devices after surface modification with polycationic and silver-based coating has been previously reported [207, 208], which is undesirable. The cytotoxicity of PDMS-AGE-RK1 and PDMS-AGE-RK2 slides towards smooth muscle cell was evaluated using the MTT assay [275]. PDMS-AGE-RK1 and PDMS-AGE-RK2 showed no statistical difference in affecting MTT cell viability compared to the PDMS control slide after 3 days of cell growth (Figure 7.8), suggesting that the incorporation of polymer brush and AMPs has minimal cytotoxic effects and opens the way for this AMP-based coating platform to be used for different biomedical devices .

Figure 7.8: MTT assay to evaluate the cytotoxicity of PDMS, PDMS-AGE-RK1 and PDMS-AGE-RK2 toward SMCs. Data are presented as mean $\pm$ s.d. (n=3).

7.4.8 Comparison of the antimicrobial performance of soluble versus immobilized RK1 and RK2

E. coli was used as the model strain to investigate the antimicrobial mechanism of soluble and immobilized RK1 and RK2. Membrane disruption is one of the most common mechanisms through which AMPs kill cells, resulting in the leakage of intracellular molecules such as ATP. As shown in Figure 7.9, the release of ATP from *E. coli* cells after incubation with RK1, RK2, PDMS-AGE-RK1 and PDMS-AGE-RK2 were compared. After treatment with both soluble and immobilized RK1 and RK2, an increase in luminescence was observed in the supernatant of the cell suspension, indicating disruption of the cell membrane by both RK1 and RK2. Treatment of the cells with the soluble peptides resulted in significantly higher luminescence than that of immobilized peptides in 2 h, indicating the higher rates of membrane disruption by soluble RK1 and RK2 compared to their immobilized counterparts. After 4 h, comparable luminescence of both soluble and immobilized RK1 and RK2 samples was observed, which indicate that equilibrium in extracellular ATP release was achieved. After 4 h treatment with RK1, RK2, PDMS-AGE-RK1 and PDMS-AGE-RK2, $\geq 90\%$ kill of *E. coli* was reached. This result suggests that except for incurring a slower rate of killing, immobilization of RK1 and RK2 did not have a significant impact on their antimicrobial activity against *E. coli*.

The FESEM images of representative *E. coli* cells that have been treated with soluble and immobilized RK1 and RK2 are shown in Figure 7.10. The surfaces of untreated *E. coli* cells were smooth and rounded. On the other hand, the soluble RK1-

and RK2-treated cells exhibited obvious wrinkling of the membrane surface compared with the untreated controls throughout the cell surface. The immobilized RK1- and RK2-treated *E. coli* cells exhibited a different morphological appearance compared with the 'soluble peptide'-treated cells, where membrane wrinkling and "holes" could only be observed in certain regions of the cell surface. From the results of the ATP leakage assay and morphological observation by FESEM, it is clear that both soluble and immobilized RK1 and RK2 can kill *E. coli* by membrane disruption, resulting in the leakage of intracellular components, like ATP. It is postulated that the relatively different membrane morphology of the 'soluble'- versus 'immobilized peptide'-treated cells is associated to the difference in peptide accessibility to the cells in the soluble and immobilized form. In the soluble form, the peptides can freely diffuse to the cell membrane, as driven by electrostatic and hydrophobic interaction, resulting in the membrane disruption. However, the diffusivity and flexibility of the immobilized peptides are sterically restricted by conjugation to the polymer brush resulting in a slower rate of killing compared to their soluble counterparts, where the peptides can only interact with isolated regions on the cell membrane.

Figure 7.9: ATP efflux from *E. coli* cells determined by measuring the relative luminescence of the supernatant of the cell suspension after treatment with RK1, RK2, PDMS-AGE-RK1 and PDMS-AGE-RK2 for 2 and 4 h. Data are presented as mean $\pm$ s.d. (n=3).

Figure 7.10: FESEM images of *E. coli* cells treated with soluble and immobilized RK1 and RK2.

7.4.8 RK1 and RK2 immobilization on silicone urinary catheters and their antimicrobial and anti-biofilm properties determination

To investigate the feasibility of tethering the AMPs to commercial urinary catheters, RK1 and RK2 were subsequently immobilized on the inner or outer surfaces of silicone urinary catheter segments using the same immobilization platform developed for RK1 and RK2 tethering on PDMS. Successful immobilization of RK1 and RK2 on urinary catheter segments was verified by XPS analysis. The successful immobilization of RK1 and RK2 on urinary catheter segments was confirmed by XPS analysis. New peaks at ~399 eV which represent the presence of nitrogen (N1s) were observed in the XPS spectra of cat-AGE-RK1 and cat-AGE-RK2, which were not present in the spectra of the untreated catheter (Figure 7.11).

(A)

(B)

Figure 7.11: XPS spectra of RK1 and RK2-immobilized catheter surface: (A) 1-inner surface of untreated catheter, 2- inner surface of cat-AGE-RK1, 3- inner surface of cat-AGE-RK2; (B) 1-outer surface of untreated catheter, 2- outer surface of cat-AGE-RK1, 3- outer surface of cat-AGE-RK2.

The peptide-impregnated catheter segments, i.e. cat-AGE-RK1 and cat-AGE-RK2, were subjected to the “immersion” antimicrobial assay. Table 7.4 shows that the RK1- and RK2-immobilized catheters showed broad spectrum antimicrobial activity in medium, PBS and urine. The antibacterial activity of the cat-AGE-RK1 and cat-AGE-RK2 were comparable to PDMS-AGE-RK1 and PDMS-AGE-RK2, although the anti-fungal activity of the catheter-impregnated samples was reduced.

The anti-biofilm property of cat-AGE-RK1 and cat-AGE-RK2 was also investigated and compared with non-treated catheters. After incubation of the catheters with bacterial and fungal cells for 3 days, biofilm development on the coated and uncoated catheters was determined by CV dye staining and biofilm quantification was performed by absorbance measurement of the CV dye extracted from the catheter

surface. Comparison of the absorbance measurements for cat-AGE-RK1, cat-AGE-RK2 and the non-treated catheter segment showed that the RK1- and RK2-coated surfaces exhibited significant anti-biofilm formation activity (Figure 7.12).

(A)

(B)

Figure 7.12: Anti-biofilm property assay of (A) PDMS-AGE-RK1 and (B) PDMS-AGE-RK2 as determined by CV staining. Data are presented as mean $\pm$ s.d. (n=3).

Table 7.4: The antimicrobial activities of RK1 or RK2-coated urinary catheters in medium, PBS and urine environments.
Data are presented as mean $\pm$ s.d. (n=3).

	Killing of <i>E. coli</i> (%)			Killing of <i>S. aureus</i> (%)			Killing of <i>C. albicans</i> (%)		
	Medium	PBS	Urine	Medium	PBS	Urine	Medium	PBS	Urine
RK1-tethered (inner surface)	99 $\pm$ 1	89 $\pm$ 6	98 $\pm$ 1	80 $\pm$ 3	86 $\pm$ 9	78 $\pm$ 6	41 $\pm$ 4	25 $\pm$ 2	31 $\pm$ 3
RK1-tethered (outer surface)	97 $\pm$ 1	91 $\pm$ 9	85 $\pm$ 7	70 $\pm$ 6	66 $\pm$ 3	72 $\pm$ 4	39 $\pm$ 5	30 $\pm$ 8	21 $\pm$ 3
RK2-tethered (inner surface)	96 $\pm$ 2	89 $\pm$ 3	87 $\pm$ 4	78 $\pm$ 9	85 $\pm$ 5	64 $\pm$ 6	35 $\pm$ 2	25 $\pm$ 3	37 $\pm$ 5
RK2-tethered (outer surface)	92 $\pm$ 6	79 $\pm$ 4	85 $\pm$ 4	69 $\pm$ 5	73 $\pm$ 5	60 $\pm$ 9	30 $\pm$ 5	27 $\pm$ 5	27 $\pm$ 2

7.5 Conclusions

In this chapter, two short AMP analogs, i.e. RK1 and RK2, which showed broad antimicrobial activity spectrum and salt-resistant activity, were successfully developed as antimicrobial candidates for silicone surface coatings. First, a coupling chemistry for the immobilization of RK1 and RK2 on PDMS surface *via* AGE polymer brush linker was developed. Immobilization of RK1 and RK2 on PDMS improved the antimicrobial and anti-biofilm properties of PDMS surfaces against *E. coli*, *S. aureus* and *C. albicans* in PBS and urine without compromising cytotoxicity towards mammalian cells. The mechanism of kill of immobilized RK1 and RK2 was shown to be *via* cell membrane disruption, which is similar to their soluble counterparts, albeit at different rates. The same coating chemistry was applied to immobilize RK1 and RK2 on silicone urinary catheter surfaces, which also successfully improved antimicrobial and anti-biofilm properties of the catheters, thus opening the way for the development of AMP-impregnated catheters to prevent catheter-associated infections.

Chapter 8 Overall conclusions and future directions

8.1 Overall conclusions

AMPs are a promising class of antimicrobial molecules with a huge potential to be developed into antimicrobial coating agents for biomedical devices. The aim of this research project was to explore the feasibility of immobilizing AMPs on urinary catheters. Three problems that were identified to pose as roadblocks to achieve this aim were systematically studied and addressed in this thesis. Chapter 4 presents the development of a bioprocess for hBD25 production from a MBP fusion protein construct. hBD25 was recovered by TEVp cleavage and a SEC refolding cum purification step which yielded hBD25 peptides at 90% purity. The antimicrobial activity assay of the recovered hBD25 showed that it could completely inhibit *E. coli* growth at 60 µg/ml. This work first reported the production of hBD 25 by using a biological expression system. However, the formation of soluble aggregates necessitated an *in vitro* refolding step using SEC chromatography which increased process complexity and could potentially compromise yield and cost, thereby rendering the process difficult to scale up. For this reason, hBD25 was not pursued as an AMP coating candidate for further studies as the production of the peptide at preparative scale was considered not to be cost-ineffective. In Chapter 5, the

bioprocess development for another recently discovered AMP candidate, i.e. hBD28, was studied. Unlike hBD25, the hBD28 fusion protein was soluble and stable in the monomeric form, which eased bioprocessing of the fusion protein by IMAC and SEC purification. Purified hBD28 (i.e. at $\geq 90\%$ purity) exhibited MIC₁₀₀ of 60 $\mu\text{g/ml}$ against *E. coli*. Comparing the two bioprocesses developed in Chapters 4 and 5 for hBD25 and hBD28, respectively (Figure 8.1), the hBD28 bioprocess was clearly more streamlined. This is a classic example of how product (or construct) design has a direct influence on bioprocess design, i.e. the product determines the process and *vice versa*. Exploration of other chromatography-free process to substitute SEC such as solvent or acid precipitation to substitute SEC purification would be good to further improve process productivity and economics. Now the successful development of a hBD28 production platform that achieved a $79\pm 5\%$ peptide yield, now opens the way for more detailed structure-function studies of the peptide. In particular, to develop hBD28 into an antimicrobial coating agent, we acknowledge that its salt-intolerant property must first be overcome, which was subsequently studied in Chapter 6.

Figure 8.1: Bioprocess flowsheets of hBD25 and 28 productions.

In Chapter 6, a mutant hBD28 was engineered by substituting the C-terminus of wild type hBD28 with positively charged amino acids (e.g.-RRKK). Comparison studies on antimicrobial and structure-activity behavior between wild type and mutant hBD28 were performed to improve understanding on the salt-intolerant mechanism of the peptide. Mutant hBD28 which has a higher net charge than wild type hBD28 retained its antimicrobial activity and even exhibited a lower MIC₁₀₀ compared to wild type hBD28 against *E. coli*. Zeta potential analysis of the wild type and mutant hBD28 suggest that the increased charged state in the C-terminus region of the mutant helps to overcome salt-induced charge-shielding effect to facilitate peptide-membrane interaction. Molecular level characterization studies of wild type and mutant hBD28 using RP-HPLC, SEC and CD showed that both mutant and wild type hBD28 were comparable with respect to structural characteristics such as hydrophobicity,

oligomerization ability and secondary structure, indicating that these were unlikely factors that contributed to the mutant peptide's salt-tolerance property. Therefore, it was concluded that salt-intolerance in hBD28 was associated to charge shielding effect which could be overcome by increasing net charge of the peptide. This study addressed salt-induced inactivation problem by designing a salt resistant hBD mutant with the substitution of a smaller number of amino acid residues compared with very lengthy substitutions report in other earlier studies [167], which makes this design strategy more universal and cheaper for detailed structure-function studies.

In Chapter 7, a proof-of-concept AMP-immobilized urinary catheter prototype was developed. The AMPs used in this study were short 10 amino acid analogs engineered from mutant hBD28 (parent template). Two AMP analogs, i.e. RK1 and RK2, having broad antimicrobial activity spectrum against Gram positive, Gram negative and fungi, salt-resistant activity and low MIC₁₀₀ (e.g. 20 µg/ml) were selected as candidates used for urinary catheter immobilization studies. A tethering chemistry based on covalent grafting of the peptides onto a polymer brush interlayer was developed to immobilize the AMPs on PDMS slides and silicon urinary catheters. X-Ray photoelectron spectroscopy and water contact angle measurements confirmed the immobilization of the peptides by covalent bonding. The RK1- and RK2-impregnated PDMS exhibited broad spectrum killing and anti-biofilm activities against different pathogens including *E. coli*, *S. aureus*, and *C. albicans*. Results from the ATP leakage assay and FESEM analysis suggest that the peptides, either in solution or the immobilized state, both exhibit their antimicrobial activity by cell

membrane disruption. However, the immobilized peptides showed a slower rate of kill, presumably due to immobilization-associated steric restriction. The same coating chemistry was applied to immobilize RK1 and RK2 on silicone urinary catheter surfaces, which also successfully improved antimicrobial and anti-biofilm properties of the catheters, thus opening the way for the development of AMP-impregnated catheters to prevent catheter-associated infections.

The overall findings of this thesis will open the way for establishing an AMP-based antimicrobial coating strategy for urinary catheters, where by using two novel AMPs, RK1 and RK2, as model peptides, it was found that AMP immobilization on urinary catheter surfaces did not compromise broad spectrum antimicrobial activity and salt tolerance of the peptides. This developed AMP-based antimicrobial coating platform is also readily applicable to aerospace systems, biomedical implants and devices (intravascular catheters, artificial heart valves and the stainless steel used in the biomedical implant), food processing equipment, and food and pharmaceutical packaging, which are prone to bacterial infection/contamination. The findings of this research also contribute to new domain knowledge in bioprocess development for AMP production, and engineering strategies for improved AMP performance such as salt tolerance. Such knowledge can also be readily translated to the biopharmaceutical industry for improved capabilities in making functional bio-molecules, which will accelerate the development of novel bio-products to meet health challenges facing the human society.

8.2 Future directions

8.2.1 Simplified chromatography-free bioprocess development for production of short AMPs

A bioprocess for manufacturing 10-residue peptide analogs such as RK1 and RK2 is suggested to be studied since biological production of the peptides will improve process economics at large scale, compared to chemical synthesis. The production of short and unstructured peptides is expected to simplify bioprocessing by opening the way for the use of a chromatography-free recovery and purification process. After the cell disruption and TEVp cleavage steps to release the peptides, protein contaminants are suggested to be separated from the peptides by solvent precipitation. Examples of solvents commonly used for peptide purification include ethanol and acetone. Complex proteins are expected to precipitate in the organic solvent phase due to thermodynamic disruption of their tertiary structure while peptides are likely to remain soluble and stable in the same solvent phase [276]. Additionally, peptide recovery in the solvent phase allows the simultaneous removal of salts and other ions, which is suggested to facilitate the final lyophilization polishing step. Solvents can be recycled to improve process economics and maintain a green process.

8.2.3 Optimization of AMP coating strategy

Optimization of the AMP-based antimicrobial coating using polymer brush is

suggested to be performed to improve antimicrobial performance such as the rate and efficiency of pathogen killing. Controlled covalent immobilization using engineered thiol-bearing RK1 and RK2 (in the N- or C-terminus) that will specifically react with the surface thiol- or maleimide- groups are suggested to be performed, and their antimicrobial performance compared to those achieved by random immobilization, as reported in Chapter 7. In addition, the use of double bond modified PEG polymer brush is suggested to be also studied, and the effects of spacer length on antimicrobial performance is suggested to be evaluated. For example, the effects of varying PEG length by varying PEG molecular weights (e.g. 500 Da, 200 Da, 5000 Da) on peptide surface density and polymer brush thickness are suggested to be studied. The relationship between antimicrobial activity and surface characteristics is suggested to be systematically investigated to improve understanding on the antimicrobial mechanism of immobilized peptides.

Until now, there are few studies reporting the orientation and conformation of tethered peptides and their precise membrane permeation action. The use of biophysical analytical tools such as ellipsometry and Quartz Crystal Microbalance with Dissipation (QCM-D) to non-destructively monitor peptide immobilization at real-time is suggested to be studied. Correlation between peptide orientation, surface density, and flexibility on bacteria activity will be important to further improve peptide immobilization efforts for maximum efficacy.

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