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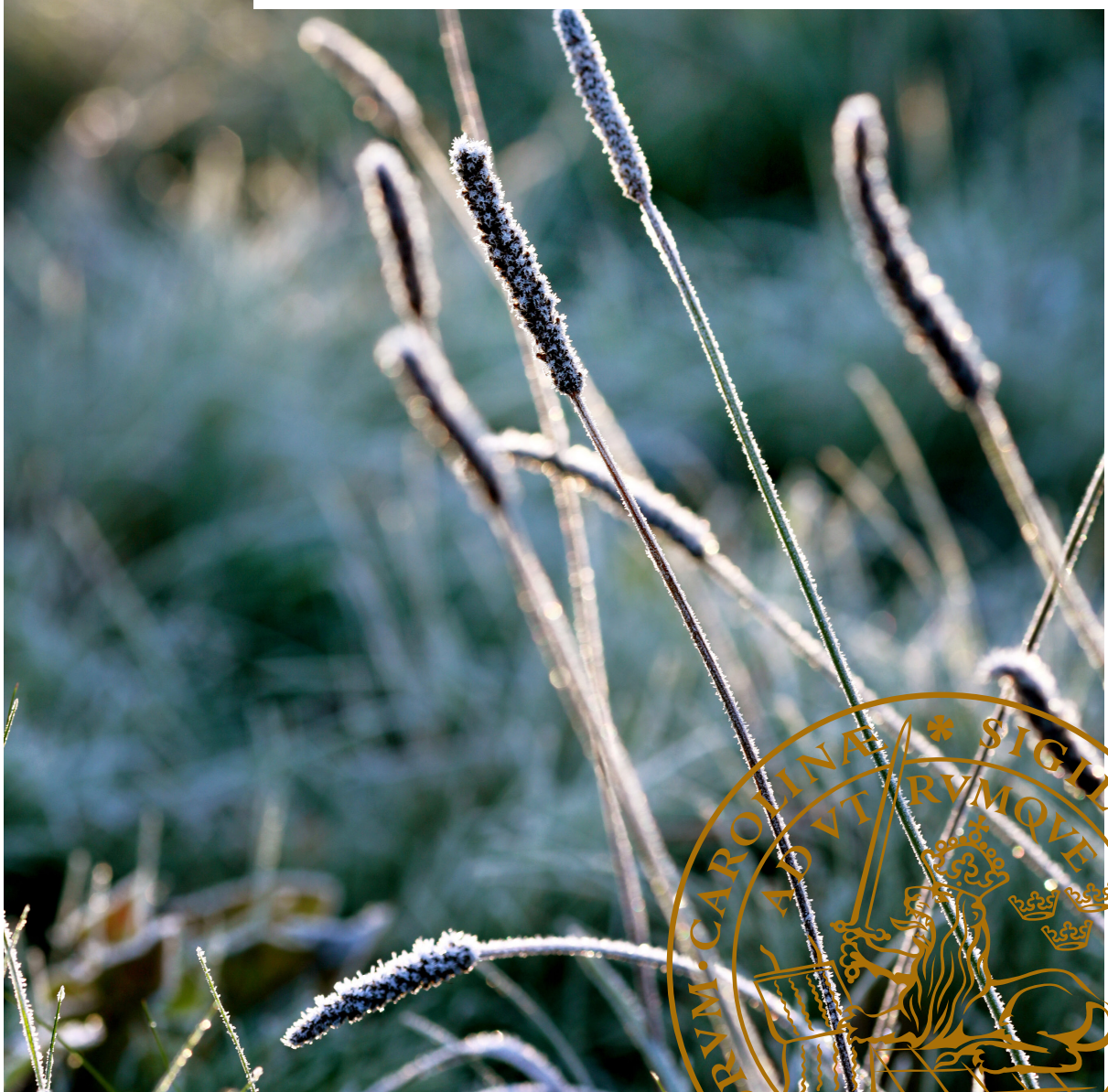

# Bio-based nonionic antimicrobial polymers with isatin or indole functionality

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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**Abstract**

Infections by pathogenic microorganisms remain one of the most serious concerns in several areas, particularly in hygienic applications. Antimicrobials have been used to combat these infections. Using small molecular antimicrobials as additives or coatings is an industrially adopted strategy. However, small antimicrobial agents always suffer from many drawbacks, such as leaching potential, toxicity to the environment, antimicrobial resistance. The development of antimicrobial polymers provides a promising strategy to overcome these issues. Compared to small molecular antimicrobials, antimicrobial polymers have advantages like enhanced antimicrobial efficiency, reduced toxicity, longer lifetime and lower risk to develop antimicrobial resistance.

Most research has been focused on cationic antimicrobial polymers which can disrupt bacterial membranes by electrostatic interactions. The major issues with cationic antimicrobial polymers are their undesirable high-water solubility, toxicity, fouiling potential and poor miscibility with nonionic polymer matrices. To solve these problems, continued exploration of new nonionic antimicrobial polymers is highly valuable and that is where this thesis has been focusing on.

In this work, we have designed and synthesized different nonionic antimicrobial polymers with isatin or indole functionality, including hyperbranched and linear structures. The antimicrobial properties against various bacteria of these polymers were evaluated. In general, these bio-based polymers exhibited significant antimicrobial activity, higher than their corresponding small model compounds. We also found that ether unit may have a synergistic effect to interact with certain bacterial membranes. It was also observed that the N-H moiety of indole in the polymer's structure facilitate the hydrogen bonding with the C=O moiety of polyesters, which improved their miscibility. Finally, we evaluated the hemolytic activity and cytotoxicity of the obtained polymers. As a result, they showed negligible cytotoxic effect to red blood cells and MG-63 osteoblast-like cells, which indicates their potential in biomedical applications.



**Key words:** antimicrobial polymers, nonionic, isatin, indole, miscibility, hemolytic activity, cytotoxicity

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*To my family*

# Popular science summary

According to a WHO report, infectious diseases are one of the most significant causes of mortality worldwide. Mainly, the microbial infections are produced and spread by breathing, touching, drinking, and eating something that are contaminated by the microorganisms. Generally, these infections are defeated or reduced by antimicrobial agents, which has largely improved the state of human beings.

Apart from being used as medicine, antimicrobial agents can also be used as additives in polymer construction or used as coatings for polymers or metals. Using antibiotics or other small molecular antimicrobial agents is an industrially adopted strategy. The problem with small molecular antimicrobial agents is that they can leach out from the materials when used as additives and suffer from poor adhesion when used as coatings. Therefore, researchers have come up with antimicrobial polymers (AMPs), which are also called macromolecular antimicrobial agents, to replace small molecular ones. In comparison with small molecular antimicrobials, AMPs have a few significant advantages: they are more effective due to the local concentrated antimicrobial functionalities; they are not as easy as small molecules to leach out from materials and they are less toxic; they are easier to form films and usually have better adhesion to the surfaces; due to the physical disruption of the microbial cell walls, AMPs are less likely to lead to antimicrobial resistance than small molecular antimicrobials.

What do AMPs look like? It is well-known that most bacterial cell walls are composed of many negatively charged molecules, hence most AMPs are designed with positive charges. Thus they can disrupt bacterial cell walls by straightforward ionic interactions and cause the death of bacteria. However, cationic AMPs have their own problems. Firstly, they are easily soluble in water, and their residues can be toxic to the environment. Secondly, many studies show that the cationic polymers can be harmful to human cells as well. Thirdly, when they are added in nonionic matrix plastics as additives, the properties of the final materials are not homogeneous. Fourthly, given their charged nature, they have the fouling potential by anionic compounds, and their antimicrobial efficacy can be drastically diminished in the presence of organic matter. A promising strategy to tackle these issues is establishing AMPs without charges. However, there are only few studies could be found related to this topic, and their mechanisms of action and structure-property relationships of nonionic AMPs remain unclear. There are many questions to answer: where to find nonionic antimicrobial monomers? without ionic

interactions, do the polymers interact bacterial cell walls efficiently? are they safe to human body? how to apply them in different applications? what structural factors can affect their antimicrobial activity? These questions are where this thesis comes in.

Presented in this thesis is the design, preparation and characterizations of different kinds of nonionic AMPs using isatin and indole building blocks. Without ionic interactions, these obtained nonionic AMPs still exhibited strong and broad antimicrobial activity. We also found some structural factors that can affect the antimicrobial properties of these nonionic polymers. When applied as additives, the indole-based polymer had the ability to form hydrogen bonds with polyester matrices and the resulting polymer blends had homogeneous properties. When applied as coatings, they killed the bacteria very efficiently. Moreover, it was also observed that these obtained polymeric antimicrobials did not have toxic effect to human cells, which could facilitate their biomedical applications.

# List of papers

Paper I **Xiaoya Li**, Sedef İlk, Javier A. Linares-Pastén, Yang Liu, Deepak Bushan Raina, Deniz Demircan and Baozhong Zhang, Synthesis, enzymatic degradation and polymer-miscibility evaluation of nonionic antimicrobial hyperbranched polyesters with indole or isatin functionalities. *Biomacromolecules*, **2021**, 22, 2256-2271.

Paper II **Xiaoya Li**, Xiao Wang, Sathiyaraj Subramaniyan, Yang Liu, Jingyi Rao and Baozhong Zhang, Hyperbranched polyesters based on indole and lignin-derived monomeric aromatic aldehydes as effective nonionic antimicrobial coatings with excellent biocompatibility. *Biomacromolecules*, **2021**, accepted.

Paper III Carlos R. Arza, **Xiaoya Li**, Sedef İlk, Yang Liu, Deniz Demircan and Baozhong Zhang, Nonionic antimicrobial hyperbranched polymers with isatin-based backbone and phenolic terminal units. Manuscript.

Paper IV **Xiaoya Li**, Sedef İlk, Yang Liu, Deniz Demircan and Baozhong Zhang, Synthesis and antimicrobial assay of nonionic indole-based poly(vinyl alcohol) with *N*-substituted alkyl and ether groups. Under review.

# My contribution to the papers

| | |
|---|---|
| Paper I | I synthesized and characterized all the indole- and isatin-based polymers. I performed biodegradation and cytotoxicity experiments together with other coauthors. I wrote the first draft of the paper and made revisions together with other coauthors. |
| Paper II | I designed and synthesized all the indole- and lignin-based monomers and hyperbranched polyesters. I analyzed and interpreted the antimicrobial results together with other coauthors. I wrote the first draft of the manuscript and made revisions together with other coauthors. |
| Paper III | I performed most of the characterizations of polymers regarding molecular and thermal properties. I also performed leaching and contact angle measurements of the obtained polymers. I did data analysis together with other coauthors. I wrote the first draft of the manuscript together with other coauthors. |
| Paper IV | I designed and synthesized all the indole-based polyvinyl alcohol polymers. I performed cytotoxicity test together with other coauthors. I analyzed and interpreted the antimicrobial data together with other coauthors. I wrote the first draft of the manuscript. |

# Abbreviations

| | |
|---|---|
| AAPH | 2,2'-azobis(2-amidinopropane) dihydrochloride |
| AIBN | azobisisobutyronitrile |
| AMPs | antimicrobial polymers |
| ATRP | atom transfer radical polymerization |
| Bt | Bacillus thuringiensis |
| DBTO | dibutyltin oxide |
| DMAP | 4-dimethylaminopyridine |
| DMSO | dimethyl sulfoxide |
| DMF | *N, N*-dimethylformamide |
| DSC | differential scanning calorimetry |
| Ea | Enterobacter aerogenes |
| Ec | Escherichia coli |
| E. coli | Escherichia coli |
| EDC | 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide |
| E. faecalis | Enterococcus faecalis |
| FTIR | Fourier-transform infrared spectroscopy |
| GPC | gel permeation chromatography |
| G(+) | Gram-positive |
| G(-) | Gram-negative |
| HBPs | hyperbranched polymers |
| iPP | isotactic polypropylene |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MW | molecular weight |
| NMR | nuclear magnetic resonance |
| Pa | Pseudomonas aeruginosa |
| P. aeruginosa | Pseudomonas aeruginosa |
| PBS | phosphate buffered saline |
| PBS | poly(butylene succinate) |
| PE | polyethylene |

| | |
|---|---|
| PEG | polyethylene glycol |
| PLA | poly(lactic acid) |
| PHB | polyhydroxybutyrate |
| PCL | polycarprolactone |
| Pm | Proteus microbilis |
| Pv | Proteus vulgaris |
| PVA | poly(vinyl alcohol) |
| RBC | red blood cell |
| $S_N2$ | bimolecular nucleophilic substitution |
| Sa | Staphylococcus aureus |
| S. aureus | Staphylococcus aureus |
| Sm | Streptococcus mutans |
| TGA | thermogravimetric analysis |
| WHO | World Health Organization |

# Table of Contents

# 1 Introduction

Bacteria are often pathogenic and they can cause serious challenges in areas ranging from human health to industrial operations.[1–3] According to a report by the World Health Organization (WHO) in 2004, infectious diseases are one of the major causes of mortality worldwide.[4,5] To fight against bacterial contamination, various disinfectants (*e.g.* hypochlorite, quaternary ammonium, silver ions) are widely used, but their short life time and negative environmental impact have limited their use in certain applications (*e.g.* water sterilization and food preservation).[6] As such, there is a strong demand from society and industry to develop new antimicrobials that are more effective and eco-friendly, and have lower risk to induce antibiotic resistance. However, the development of new antimicrobials is an extremely expensive and lengthy process. Due to the low success rate and profits, most pharmaceutical companies in the world had stopped investigations on the development of new antimicrobials since late 1990s. Therefore, it has become an important task for academia, aiming to develop new concepts and molecular design principles for antimicrobials, as well as to unravel their structure-property relationships.[7]

## 1.1 Bacterial structure

Bacteria are classically divided into Gram-positive and Gram-negative bacteria, which have different cell membrane structures.[8,9] As illustrated in **Figure 1.1**, Gram-positive bacteria such as *S. auras* have a cytoplasmic membrane combined with many peptidoglycan layers, whereas Gram-negative bacteria such as *E. coli* have two distinct membranes, namely inner and outer membrane, with fewer peptidoglycan layers. The outer membrane contains lipopolysaccharide (LPS), which also creates a permeability barrier at the cell surface. Due to the presence of high amounts of electronegative groups in their lipid molecules, the cell membranes of both Gram-positive and Gram-negative bacteria are usually negatively charged.[10,11]

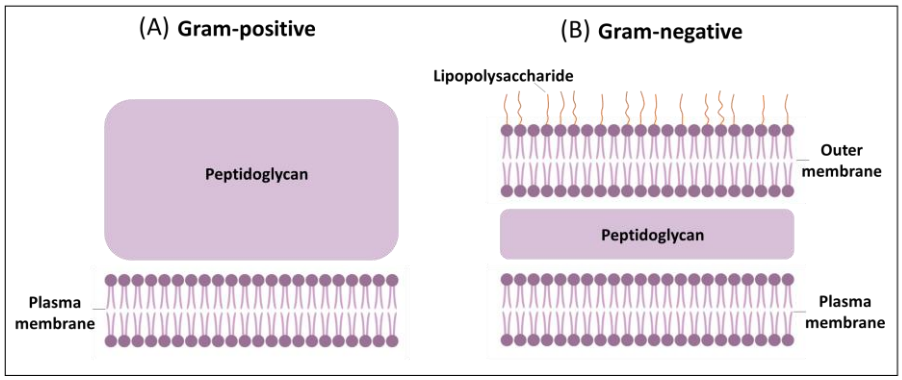



**Figure 1.1.** Structures of bacterial membrane of (A) Gram-positive and (B) Gram-negative bacteria. Created with BioRender.com

# 1.2 Antimicrobial polymers

Antimicrobial polymers (AMPs) have been extensively studied in the past decades (**Figure 1.2**), which are considered as good alternatives to replace the traditional antimicrobials to fabricate antibacterial surfaces in various hygienic applications due to their enhanced efficiency, prolonged lifetime, lower risk to induce antimicrobial resistance, lower leaching potential and reduced environmental toxicity.[12–20]

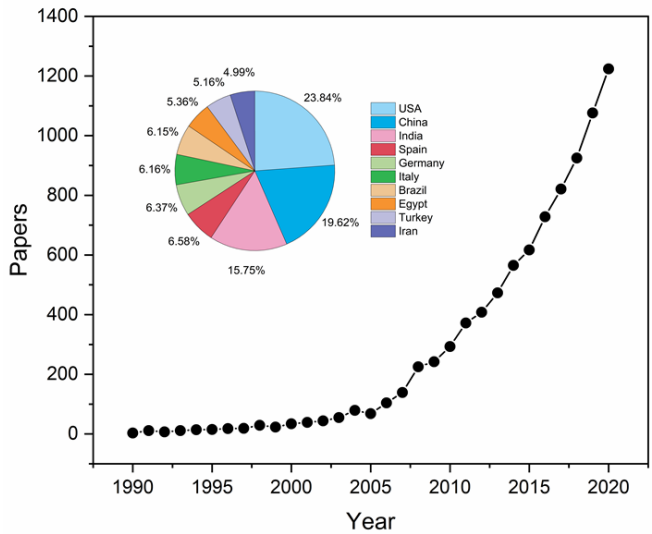



**Figure 1.2.** The number of scientific publications during 1990 and 2020 with the keyword "antimicrobial polymers" according to ISI Web of Science. Inset shows the publication contributions of the top ten states from 1990 to 2020.

Generally, AMPs can be classified into two categories:

(a) Polymers with inherent antimicrobial activity. They exhibit antimicrobial activity because their molecular structures are able to interact with bacteria, such as polymers with quaternary nitrogen atoms, or those mimicking natural peptides.[21–30]

(b) Polymers without inherent antimicrobial activity, but are loaded with external small molecular antimicrobial agents, such as small antibiotics, quaternary ammonium compounds, or metal ions.[25–36]

Regarding the first category, most research has been focused on ionic polymers, which are synthetically flexible and straightforward.[13,43–46] Regarding the second category, small molecular antimicrobial agents are added into non-active polymers. The mechanism of this system depends on the release of the active antimicrobials. The second category of AMPs can only provide short-term activity, may harm to the environment and trigger antimicrobial resistance.[32,47] This thesis will only focus on the first category.

It has also been demonstrated that combining polymers with intrinsic antimicrobial activity and traditional antimicrobial agents could also treat bacterial infections efficiently, while the traditional antimicrobials target specific pathways of bacteria, and cationic polymers target bacterial membrane disruption.[48,49] For example, Gillies et al. synthesized phosphonium-functionalized block copolymer micelles. It was found that the antibiotic tetracycline can be loaded into the micelles, potentially providing orthogonal effect with the cationic polymers.[49]

### 1.2.1 Ionic and nonionic antimicrobial polymers

Most AMPs are designed with positive charges, disrupting the bacterial membranes by electrostatic interactions, which have been revealed with excellent bactericidal activity. However, cationic AMPs may suffer from a few disadvantages: (1) giving the ionic nature, they are usually highly hydrophilic, which is not favorable for constructing moisture-resistant antimicrobial surfaces.[50,51] (2) they may exert toxicity to the aquatic organisms and mammalian cells.[52–55] (3) they usually have poor miscibility with nonionic matrix materials and thus increase the difficulty for manufacturing.[56] (4) cationic AMPs have high fouling potential by anionic compounds in the presence of organic matter, which can dramatically reduce their antimicrobial efficiency.[57,58] Nonionic AMPs have the potential to overcome these issues, and nonionic AMPs bearing chlorine, organotin, carbon-rich, phenol, indole, isatin, astaxanthin, tropolone, aspirin, limonene groups have been reported.[59–68] Many of these functionalities are from naturally occurring molecules, and the resulting polymers are prone to be more biodegradable and biocompatible.[69,70] Due to the lack of charges, nonionic AMPs could interact with the bacterial membrane

through hydrogen-bonding, hydrophobic, dipole, aromatic interactions, etc..[59,62,71,72] However, their general mechanisms of action and structure-property relationships still remain largely unknown.

### 1.2.2 Synthesis of AMPs

AMPs can have diversified structures, such as linear and branched structures (**Figure 1.3**).[13] Such different molecular structures can not only lead to different physical and chemical properties, but also their antimicrobial activities. For instance, branched polymers usually have lower viscosity and higher solubility compared to linear polymers, which can benefit their processing.[19,27,73–78] In addition, the highly concentrated functional groups of highly branched polymers can interact with bacterial membranes simultaneously so such polymers frequently have enhanced antimicrobial potency.[25,72,79]

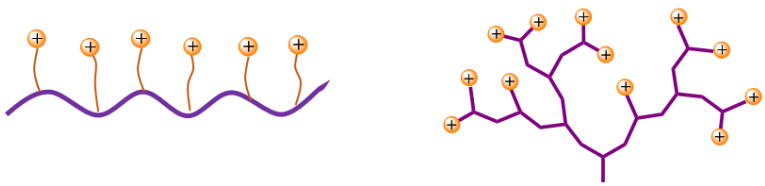


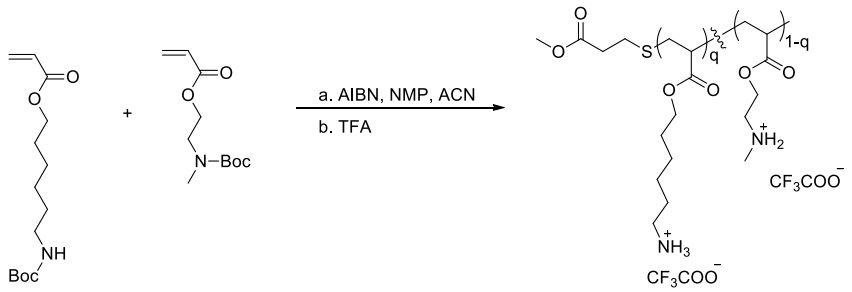
**Figure 1.3.** Examples of linear and branched AMPs.

AMPs can be synthesized by two approaches: polymerization of monomers with antimicrobial activity, and post-polymerization modifications with antimicrobial moieties.

#### 1.2.2.1 Polymerization of monomers with antimicrobial activity

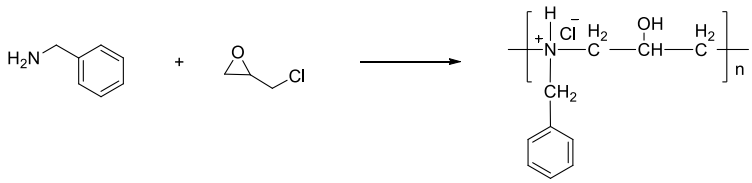
Antimicrobial molecules with reactive functionalities such as hydroxyl, amino, or carboxyl groups can be converted to a variety of polymerizable monomers.[20] Therefore, corresponding AMPs with linear or branched structures can be prepared by different polymerization mechanisms such as chain polymerization or step polymerization.[71,81,82]

There are many cationic monomers and AMPs synthesized by this approach. For example, Punia et al. reported a series of cationic amphiphilic acrylic random copolymers having 2- and 6-carbon spacer arm counits (**Scheme 1.1**), which had superior antibacterial activities with very low hemolytic effect.[83]



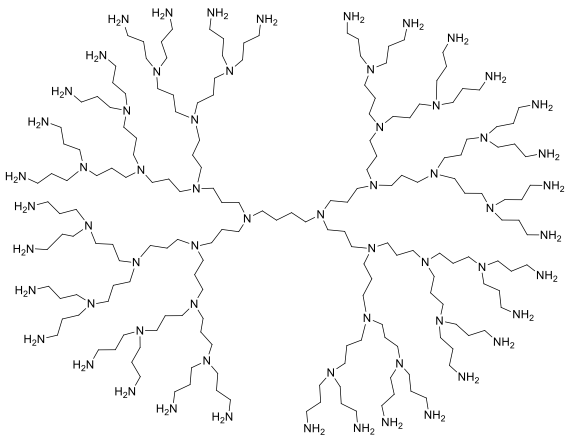

**Scheme 1.1.** Synthesis of acrylic random copolymers with quaternary nitrogens in the side chain.

Apart from incorporating quaternary ammonium groups in the polymer side chain, quaternary nitrogens can also be part of the polymer backbone. Cakmak et al. synthesized a variety of such quaternary ammonium antimicrobials via condensation polymerization of benzyl amine with epichlorhydrin (**Scheme 1.2**), which were proven to be effective against pathogenic bacteria, yeast and fungi.[84]



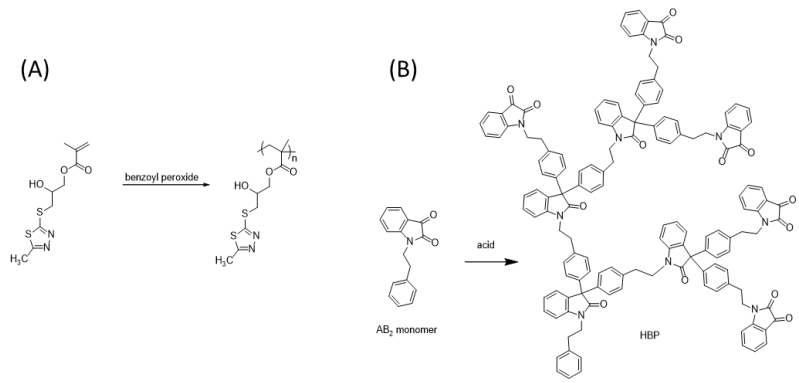

**Scheme 1.2.** Synthesis of a cationic polymer with quaternary nitrogens in the main chain.

Cooper et al. prepared a series of quaternary ammonium functionalized poly(propyleneimine) (PPI) dendrimers (**Scheme 1.3**). Biooluminescence method revealed that their biocidal properties were dependent on their molecular weight, length of hydrophobic chain and counteranion.[19,25]



**Scheme 1.3.** The structure of poly(propyleneimine) (PPI) dendrimers.

Nonionic AMPs were also synthesized by this approach. For example, Moon et al. synthesized an acryl monomer with azole moiety, which was polymerized by free radical polymerization (**Scheme 1.4A**). The obtained polymers exhibited enhanced antimicrobial activity than the monomer.[80] Zhang et al. reported an isatin-based hyperbranched polymer polymerized from an $AB_2$ monomer with isatin moiety (**Scheme 1.4B**). The resulting polymer showed strong antibacterial activity against 9 different pathogenic bacteria and negligible leakage in water.[72]



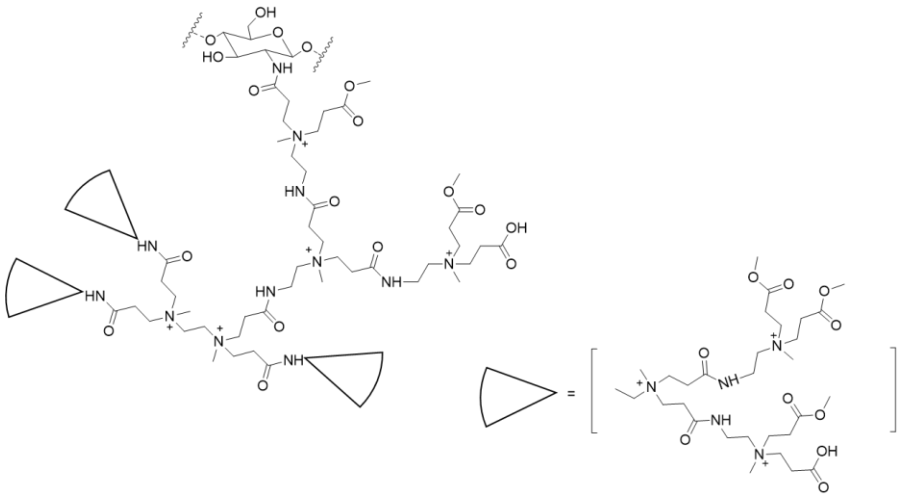

**Scheme 1.4.** Synthesis of nonionic polymers including (A) a linear polymer with azole units and (B) a hyperbranched polymer with isatin units.

The key in this strategy is to find appropriate monomers, and the corresponding polymers are expected to have certain interactions with the bacterial membranes. It should be noted that the corresponding polymers may not always have enhanced antimicrobial effects compared to their corresponding monomers, which depends on the specific mechanism of action of different monomers/polymers.[19,25,79,85,86]

1.2.2.2 Post-polymerization modifications with antimicrobial moieties

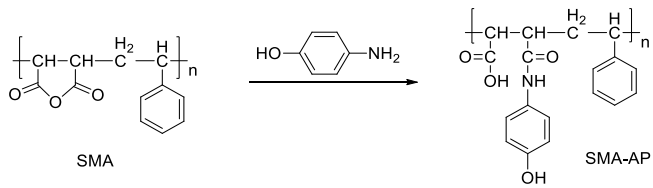
Covalently grafting antimicrobial moieties onto polymers is a convenient strategy to obtain AMPs.[20] Such post-polymerization reactions can take place on specific functional groups (*e.g.* hydroxyl, amino or carboxyl groups) of the matrix polymers.[59]

For example, chitosan was grafted with a cationic hyperbranched dendritic polyamidoamine (PAMAM) (**Scheme 1.5**), which showed excellent antimicrobial activity against *S. aureus* when it was applied onto cotton fabrics.[46]



**Scheme 1.5**. Synthesis of polyamidoamine grafted chitosan.

A copolymer poly(styrene-*alt*-maleic anhydride) (SMA) was used by Lee et al. as the matrix, to which 4-aminophenol (AP) was grafted (**Scheme 1.6**). The resulting nonionic SMA-AP polymer showed strong antibacterial activities against *E. coli* and *S. aureus* according to a flask shake test.[87]




**Scheme 1.6.** Synthesis of 4-aminophenol grafted poly(styrene-alt-maleic anhydride).

For this strategy, the choice of polymer matrix is important, which depends on their availability, cost, thermal, mechanical and chemical properties, as well as the compatibility with other reagents and solvents. A commercially available, inexpensive polymer is usually preferable. A frequently encountered disadvantage of this strategy is the presence of residues of catalysts, reagents or byproducts that are entrapped in the polymer matrix.[88]

## 1.2.3 Factors affecting the antimicrobial activity of AMPs

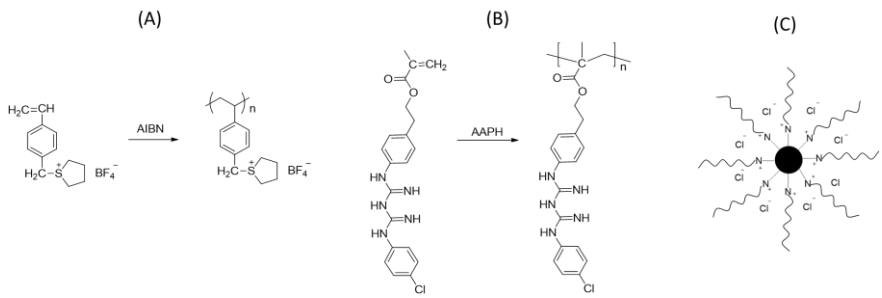
The typical mode of action of cationic AMPs involves: (i) Adsorption of cationic AMPs onto usually negatively charged bacterial membrane. (ii) Diffusion into the membrane. (iii) Binding to the cytoplasmic membrane. (iv) Disruption of the cytoplasmic membrane. (v) Release of electrolytes such as $K^+$ and nucleic materials such as DNA/RNA from the cell. (vi) Death of the cell.[89,90]

Therefore, it is reasonable to think that many factors can affect the antimicrobial properties of cationic AMPs based on each elementary step described above, such as molecular weight (MW), charge density, counterion, and overall hydrophobicity.[14,15,20,91–97]

Many studies showed that there existed an optimal MW range for the cationic AMPs. Increasing MW of polycations could enhance the process i, iii and iv due to the increasing charge density, while could suppress process ii due to the poorer diffusion ability, especially for the Gram-negative bacteria with an additional outer membrane. As such, the overall effect of MW on antimicrobial activity is a combination of two kinds controlling factors.[93,98] It was observed that compounds with MW up to $5 \times 10^4$ Da seem not to have problems with diffusion of cytoplasmic membrane of Gram-positive bacteria such as *S. aureus*; while the diffusion process is more complex for Gram-negative bacteria such as *E. Coli*.[20]

Kanazawa et al. studied the MW dependence of the antimicrobial activity against *S. aureus* of linear polymers with polystyrene backbone and pendent sulfonium salts (**Scheme 1.7A**). They found that the antimicrobial activity increased with increasing MW from $1.1 \times 10^4$ to $4.7 \times 10^4$ Da.[99] Ikeda et al. investigated antimicrobial activity against *S. aureus* of poly(methyl acrylate) with pendant biguanide groups (**Scheme 1.7B**). They found that the activity was increased with the MW lower than $5 \times 10^4$ Da, while decreased with the MW over $1.2 \times 10^5$ Da.[98]

For highly branched structures, MW also played an important role. For example, it was reported that the antimicrobial effect of poly(propyleneimine) dendrimers with quaternary ammonium ions (**Scheme 1.7C**) showed parabolic dependence on MW. Dendritic biocides from generation 3 were the least active, while those from generation 5 showed highest efficiency. Further increase of the polymer size, the activity turned out to decrease due to the poor permeability through the bacteria membrane.[25]

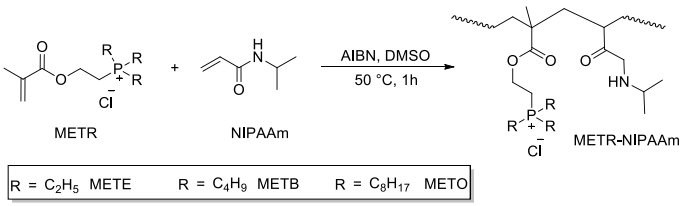



**Scheme 1.7.** Synthesis of cationic polymers including (A) polystyrene with pendent sulfonium salts, (B) polymethyl acrylate with pendant biguanide groups and (C) poly(propyleneimine) dendrimers.

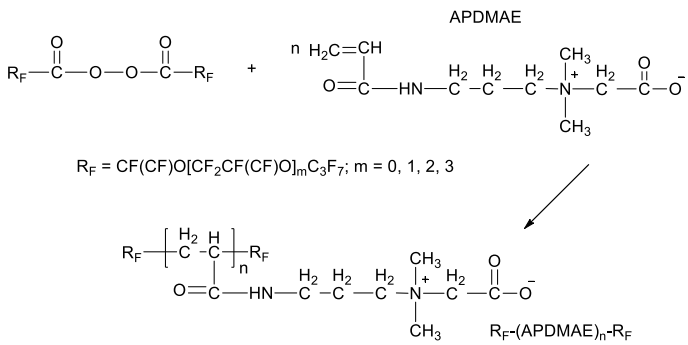
Counterions of cationic AMPs can also affect the antimicrobial activity. For example, enhanced antimicrobial activity was observed for AMPs with phosphonium ions if the counter anions were prone to form a loose ion-pair.[100]

Higher efficiency for bromide than for chloride counterions was observed for the above mentioned polycationic dendrimers (**Scheme 1.7C**).[25]

According to the mode of action, one would expect that cationic AMPs are usually amphiphilic that cationic residues binding to the anionic bacterial membrane by electrostatic interaction whereas their hydrophobic residues insert into the non-polar membrane core by hydrophobic interaction. Therefore, it is easy to understand that the structural hydrophobicity of AMPs can also play an essential role in determining their antimicrobial activity. For example, Nonaka et al. prepared methacyloylethyl trialkyl phosphonium chlorides/N-isopropylacryamide copolymers (METR-NIPAAm) (**Scheme 1.8**). The antimicrobial activity of these polymers against *E. coli* increased with the increased length of the hydrophobic alkyl chains (from $C_2H_5$ to $C_8H_{17}$).[101] Similarly, Sawada et al. found the polymer $R_F$-$(APDMAE)_n$-$R_F$ (**Scheme 1.9**) with longer alkyl chain was more active against both *S. aureus* and *P. aeruginosa* than shorter ones.[102]




**Scheme 1.8.** Synthesis of methacyloylethyl trialkyl phosphonium chlorides/N-isopropylacryamide copolymers (METR-NIPAAm) with various length of hydrophobic alkyl chains.



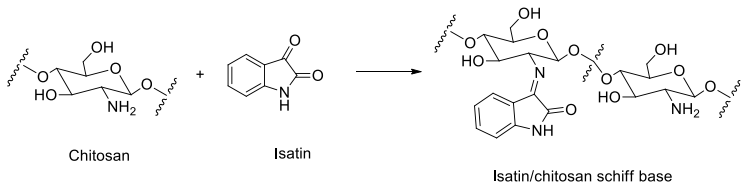

**Scheme 1.9.** Synthesis of fluoroalkylated end-capped betaine polymers.

Other factors such as polymer architecture,[103] end group of low-MW polymers,[104] cationic spacer length (the length of the side chain spacer group to the ammonium cation in the polymer backbone)[105] were also demonstrated to have impact on the antimicrobial activity of polycationic biocides.

## 1.2.4 Isatin and indole as renewable recourses

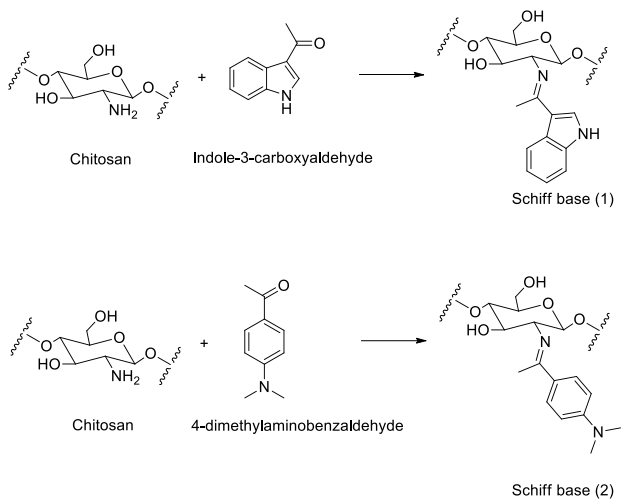
Isatin and indole are biologically active molecules, which widely exist in nature and domestic wastes.[90–94] Many isatin and indole-derived small molecules have shown significant antimicrobial activities.[64,109] Many linear or branched polymers built up from isatin and indole units have been developed.[110–114]

Antimicrobial linear biopolymers such as chitosan with isatin or indole units have been reported. Omer et al. successfully incorporated isatin into chitosan by Schiff base formation, and the resulting biopolymers exhibited superior antibacterial efficiency against one Gram-positive bacterium (*S. aureus*) and three Gram-negative bacteria (*P. aeruginosa*, *Salmonella* and *P. vulgaris*) than pristine chitosan (**Scheme 1.10**).[115]



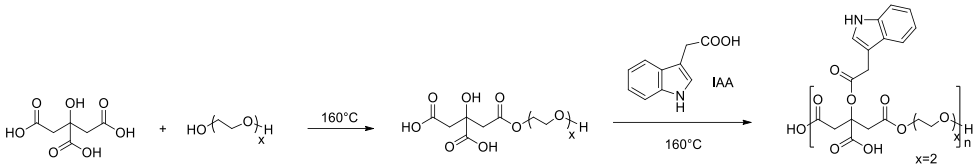

**Scheme 1.10.** Synthesis of isatin grafted chitosan.

Similarly, Hassan et al. synthesized the chitosan derivatives with grafted indole units via Schiff base formation (**Scheme 1.11**). Both agar-well diffusion assay and MIC (minimum inhibitory concentration) tests revealed that the antimicrobial activity of indole-grafted chitosan was remarkably higher than a reference chitosan grafted with 4-dimethylaminobenzaldehyde.[116]



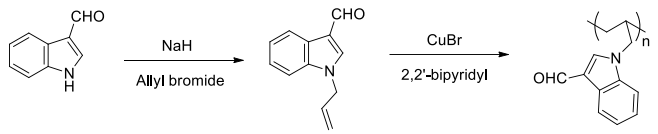

**Scheme 1.11.** Synthesis of indole and 4-dimethylaminobenzaldehyde grafted chitosan.

Indole-based linear synthetic AMPs have also been reported, including poly (N-vinylindole), polymethacrylates, polyacrylates, and polyacetylene derivatives.[117–125] For instance, Chitra et al. synthesized a series of pH-sensitive biopolymeric hydrogels by polycondensation of indole-3-acetic acid, citric acid and diethylene glycol (**Scheme 1.12**), and the resulting hydrogels showed significant antifungal activity against *Aspergilus fumigates, Candida albicans and Rhizopusoryzae*.[121]



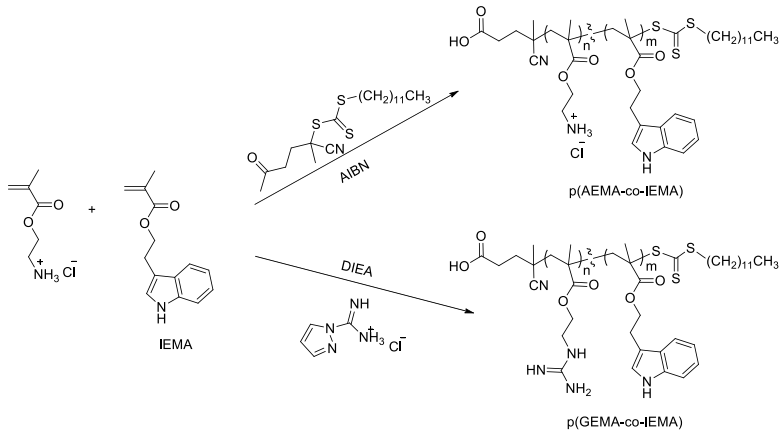

**Scheme 1.12.** Synthesis of indole-3-acetic acid based biological polymers.

Srivastava et al. synthesized a linear vinyl polymer with pendant indole units (**Scheme 1.13**) with narrow molar mass distribution by a living radical polymerization (ATRP) of 1-allylindole-3-carbaldehyde. The resulting polymers showed superior antibacterial activity against *P. mirabilis* and *K. pneumonae*.[120]



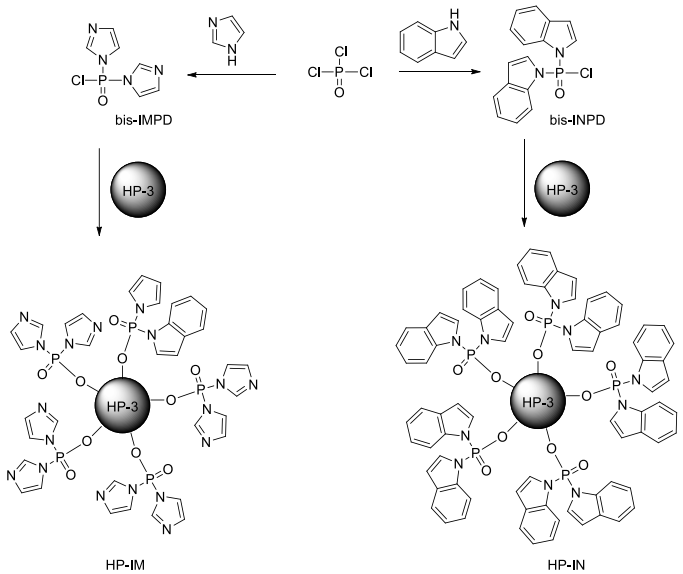

**Scheme 1.13.** Synthesis of a linear vinyl polymer with pendant indole units via ATRP.

Locock et al. reported polymethacrylates with both indole and cationic ammonium or arginine ions (**Scheme 1.14**). It was found that a low level of indole content present in the copolymer structures resulted in enhanced activity against *S. epidermidis* and the methicillin-resistant strain of *S. aureus.* Further mechanistic investigations will be needed to understand the cooperative roles of indole pendant groups in these ionic AMPs.[122]




**Scheme 1.14.** Synthesis of polymethacrylates with both indole and cationic ammonium or arginine ions.

Dendritic or hyperbranched AMPs with isatin or indole units have been also reported. Zhang et al. prepared isatin-based HBPs with broad-spectrum antibacterial activity (**Scheme 1.2**).[72] Karpagam and Guhanathan synthesized hyperbranched polyesters end-capped with bis-indole and imidazole phosphosphoryl chloride (**Scheme 1.15**). According to disc diffusion assay, the HBP with indole functionality showed slightly higher antibacterial activity against Gram-positive bacteria (*S. aureus* and *B. subtilis*) and Gram-negative bacteria (*E. coli* and *K. pneumoniae*) than the HBP with imidazole units.[125]




**Scheme 1.15.** Synthesis of hyperbranched polyesters end-capped with indole and imidazole units.

### 1.2.5 Miscibility of polymer blends

Miscibility is the capability of a mixture that form a single phase over certain range of temperature, pressure, and compositions, which is a key factor for polymer blends.[126] In general, there are three types of polymer blends: completely miscible, partially miscible, and completely immiscible blends.[127] Miscible blends are rare due to the negligible entropy gain upon mixing and the unfavorable enthalpy of mixing without specific attraction forces between the two polymers.[126–129]

Using copolymer as compatibilizer is an effective strategy to obtain miscible polymer blends. For example, Coates et al. synthesized PE/iPP multiblock copolymers that can weld common grades of commercial PE and iPP together.[130] Miscible blends can also be obtained if there are favorable attraction (*e.g.* formation of hydrogen bonds) between the two polymer components.[131–139] The miscibility of polymer blends can be revealed by their thermal behavior, such as glass transition temperature ($T_g$) in DSC heating curves. The single $T_g$ displayed in the heating run usually indicates good miscibility. For example, Lin et al. studied the miscibility of the blend of polylactide (PLA) and a hyperbranched poly(ester amide) (HP) by DSC. As a result, all blends exhibited only one $T_g$, which decreased gradually with the increase of HP content. This could indicate that the obtained HP was miscible with PLA at all tested concentrations, which was likely attributed to the H-bond interactions between the C=O groups (as H-bond accepters) of PLA and NH/OH groups (as H-bond donors) of HP (**Figure 1.4**).[138]

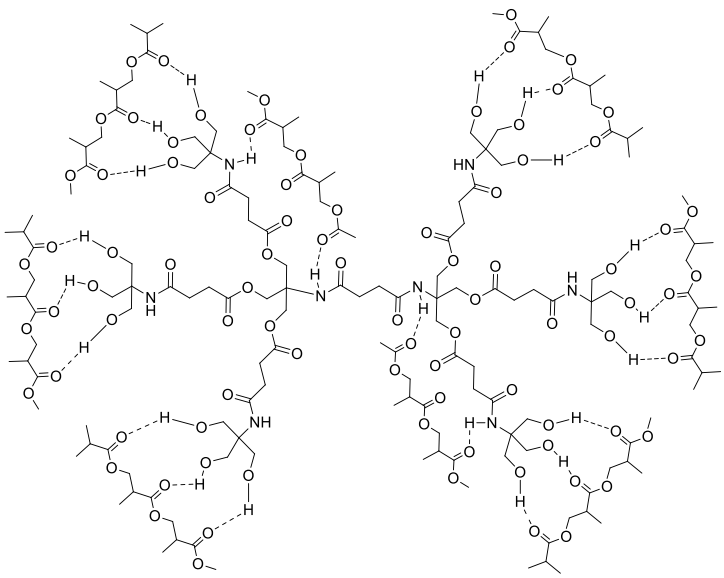


**Figure 1.4.** Illustration of intermolecular H-bonding between PLA and HP.

Most AMPs are ionic polymers, which are usually challenging for blending with nonionic polymer matrices (*e.g.* PLA, PHB, PCL). Using nonionic polymers as antimicrobial additives could improve their miscibility with such matrix polymers.[56]

### 1.2.6 Biocompatibility

On the basis of the mode of mechanisms of cationic AMPs described above, both electrostatic and hydrophobic interactions are essential to disrupt the bacterial membranes. Owing to the structural difference in the cell membranes between mammalian cells and bacteria, cationic AMPs are expected to bind selectively to bacterial membranes by strong electrostatic interaction (**Figure 1.5**). However, it has been reported that excessive hydrophobicity could cause toxicity to the mammalian cells due to the stronger hydrophobic interactions.[95,140–143]

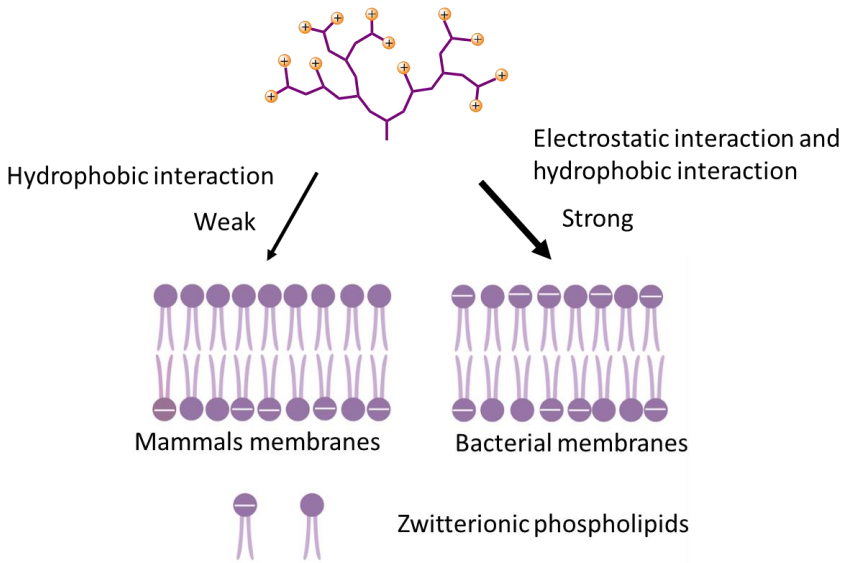



**Figure 1.5.** Illustration of cell selectivity of cationic AMPs. Phospholipid bilayers were created with BioRender.com

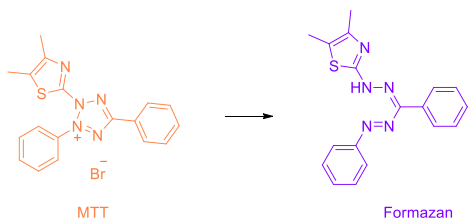
As most studies have revealed, the hydrophilic-hydrophobic balance is a very important factor to consider for designing polymers with desirable selectivities.[83,141,144,145] The minor modifications to the hydrophobic character of cationic amphiphilic polymers were observed to have dramatic effect on the antimicrobial and hemolytic activities.[95]

Kuroda et al. reported that for methacrylate copolymers against *E. coli*, polymers with short alkyl side chains seem to have better selectivity for bacterial over human cells compared to those with longer alkyl side chains.[140] The role of amphiphilic balance on the antimicrobial and hemolytic activity for other polymers such as

poly(glycidyl methacrylate)s, poly(norbornene)s[95] and nylon-3 copolymers[146] were also investigated. Each independent platform required different optimal composition to obtain desirable selectivity.

The MW also has a profound effect on the hemolytic activity of polymers. It has been demonstrated that for most cationic AMPs, lower MW generally resulted in lower hemolytic activity, polymers with high MW usually exhibited remarkably increased hemolytic activity.[147] For example, Mowery et al. found that nylon-3 polymers with low MW (1-4 kDa) expressed the desirable antimicrobial ability and minimal hemolysis, whereas higher MW resulted in significant hemolysis.[146]

Hemolysis is often carried out as a preliminary method to evaluate the cytotoxicity.[148,149] However, the results can be significantly different for the same compound when different methods and buffers are employed due to the vulnerability of the isolated and washed erythrocytes.[150–152] Additional cytotoxic studies with more mammalian cells are necessary for a more accurate assessment of the toxicity of a compound. For this purpose, cytotoxicity assay was developed, which is very important to evaluate AMPs for biomedical applications.[116,153,154] Cytotoxicity is defined as the toxicity caused by the action of chemotherapeutic agents on living cells.[155–157] MTT assay is a common colorimetric method to access the cell viability, which reflects the influence of samples on cell proliferation and cytotoxicity.[158,159] This method is based on the reduction of a tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) with a yellow color to its insoluble formazan, which has a purple color by metabolically viable cells (**Scheme 1.16**).[160–162] The number of the viable cells could be calculated by dissolving the insoluble purple formazan into a colored solution that can be quantified by measuring absorbance at 500-600 nm using a multi-well spectrophotometer.




**Scheme 1.16.** Reduction of MTT to formazan by metabolically viable cells.

Although some antimicrobial agents (*e.g.* phenolic compounds, antimicrobial peptides, quaternary ammonium compounds) exhibited good antimicrobial properties, they showed undesirable cytotoxicity on mammalian cells, which hindered their biomedical applications.[163–165]

## 1.3 Context and scope of this thesis

The focus of this thesis is to design and construct new nonionic AMPs based on isatin or indole building blocks, and to gain a fundamental insight into structure-activity relationship of such nonionic AMPs. This thesis primarily aims to investigate: (1) the effectiveness of such nonionic polymers as antimicrobials. (2) The miscibility of such nonionic AMPs as additives to other polymer matrices. (3) The durability of such nonionic polymers as bactericidal coatings. (4) The structural factors that affect the antimicrobial activity of such nonionic AMPs. (5) The cytotoxicity of such polymers.

To reach these goals, various nonionic polymers bearing isatin and indole units were designed and synthesized. Their chemical structures, thermal properties, miscibility, durability, antimicrobial properties and cytotoxicity were characterized. In paper I, isatin or indole groups were grafted onto a commercially available HBP. The obtained polymers showed superior antibacterial activity against 8 human pathogenic bacteria compared to the corresponding small model compounds, and DSC results revealed that indole-HBP with H-bond donor was miscible with two biodegradable polyesters (PHB and PCL) up to 20 wt%. In paper II, three nonionic hyperbranched polyesters using three $AB_2$ monomers derived from methyl indole-5-carboxylate and lignin-based molecules (4-hydroxybenzaldehyde, vanillin, and syringaldehyde) were synthesized. These HBPs with methoxy groups showed significant bactericidal effect against two Gram-negative bacteria *E. coli* and *P. aeruginosa*, and two Gram-positive bacteria *S. aureus* and *E. faecalis* as coatings. Moreover, no significant difference of the bactericidal activity was observed after 2 cycles of antibacterial experiments. To gain more structure-activity relationship of isatin-based nonionic AMPs, a series of isatin-based HBPs with phenolic groups was synthesized in paper III. Disk diffusion tests revealed that these HBPs showed significant antibacterial activity superior than streptomycin and comparable as gentamicin against 9 different pathogenic bacteria. It was also discovered that the presence of a methoxy or long alkyl group close to the phenolic unit enhanced the activity. To further develop and understand indole-based nonionic AMPs, 6 indole derivatives with different alkyl or ether units were grafted on a synthetic biodegradable polymer poly(vinyl alcohol) (PVA) in paper IV. The obtained 6 indole-based PVAs showed comparable antimicrobial activity as gentamicin against 9 human pathogenic bacteria. It was discovered that the presence of ether substituents could significantly enhance the antibacterial activity against certain tested bacteria, while the presence of linear or cyclic alkyl groups could have negative impact. Finally, the cytotoxicity to a human cell line of these polymers was evaluated. The results revealed that these polymers were non-cytotoxic and some of them even promoted the proliferation of the cells.

# 2 Experimental

The major experimental procedures are described here, and more details are given in the appended papers and/or manuscripts.

## 2.1 Synthesis

In paper I and IV, the synthetic strategy is functionalization of two commercially available biodegradable polymers using isatin/indole derivatives. In paper I, we used a hyperbranched polyester, Boltorn$^{TM}$; In paper IV, we used a linear polymer, PVA. The reaction happens between the hydroxyl group of polymers (Boltorn$^{TM}$ and PVA) and the carboxylic acid group of isatin/indole compounds using EDC and DMAP as catalysts at room temperature. The products are collected by straightforward precipitations. In paper II and III, the synthetic strategy is polymerization of $AB_2$ monomers derived from isatin or indole-based molecules. In paper II, three $AB_2$-type monomers with indole functionality were firstly synthesized, followed by a bulk polycondensation using DBTO catalyst at 165 °C, yielding three HBPs. In paper III, an isatin-based HBP precursor was firstly synthesized via a facial-solvent free polymerization of an isatin-based $AB_2$-monomer, followed by reacting with various phenolic groups (phenol, catechol, guaiacol and hydro-cardanol), yielding other five HBPs.

## 2.2 Characterization

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX400 spectrometer at the proton frequency of 400.13 MHz and a carbon frequency of 100.61 MHz. Gel permeation chromatography (GPC) was carried out with three Shodex columns in series (KF-805, 2804, and 2802.5) and a refractive index (RI) detector (Viscotek Model 250). All measurements were carried out at room temperature at a concentration of 3.0 mg $mL^{-1}$ using chloroform as the eluent, and at an elution rate of 1 mL $min^{-1}$. Calibration was performed with four polystyrene standard samples ($M_n$ = 650 kg $mol^{-1}$ from Water Associates, 96 and 30 kg $mol^{-1}$ from Polymer Laboratories, and 3180 g $mol^{-1}$ from Agilent Technologies). Gel

permeation chromatography (GPC) was carried out with 2xPL-Gel Mix-B LS column and OmniSEC Triple Detectors (refractive index, viscosity, and light scattering). All measurements were carried out at 35 °C at a concentration of 3 mg $mL^{-1}$ using THF as the eluent, and at an elution rate of 1 mL $min^{-1}$. Calibration was performed with polystyrene standard sample ($M_n$ = 96 kg $mol^{-1}$ from Polymer Laboratories). Fourier transform infrared (FTIR) spectra were obtained with an attenuated total reflection (ATR) setup using a Bruker Alpha FTIR spectrometer. Thermogravimetric analysis (TGA) was performed under nitrogen atmosphere with a Thermogravimetric Analyzer (TA Instrument Q500) at a heating rate 10 °C $min^{-1}$. Differential scanning calorimetry (DSC) measurements were performed using a TA Instruments DSC Q2000. The samples were studied with a heating rate of 10 °C $min^{-1}$ under nitrogen with a purge rate of 50 mL $min^{-1}$. The $T_g$ was taken as the midpoint of the endothermic step-change observed during the second heating run; the cool crystallization temperature $T_c$ and melting temperature $T_m$ were taken as those of main exo- and endo-thermal peaks respectively. High resolution mass spectrometry (HRMS) was performed by direct infusion on a Water Xevo-G2 QTOF mass spectrometer using electrospray ionization. Reversed phase liquid chromatography–mass spectrometry (LC-MS) was performed on a XEVO-G2 ESI-QToF mass spectrometer, and Acquity UPLC equipped with a Acquity CSH C18 column (1.7 µm, 2.1 × 100 mm), all from Waters. The mobile phases contained 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), and the gradient profile was 0.0-0.7 min 5% B, 0.7-8.0 min 5-99% B, followed by 99% B for 3 minutes. The column was kept at 60 °C and the flow rate was 0.5 mL $min^{-1}$. Diode-array detection was performed between 190 - 300 nm and the mass spectra between m/z 50-1200 were generated in positive electrospray mode using a capillary voltage of 3 kV, cone voltage of 40 V, source temperature 120 °C, desolvation temperature 500 °C, cone gas of 50 L/h and desolvation gas 800 L/h (both $N_2$). Lock mass correction was performed using leucine enkephalin (according to Waters standard recommendations). A CMB-20A HPLC instrument (Shimadzu), with a UV/Vis detector (SPD-20A) was used. A C18 column Kinetex® 1.7 µm XB-C18 100 Å, LC Column 50 × 2.1 mm was used, with mobile phases A and B, consisted of acetonitrile and 0.1% formic acid solution, respectively. The flow rate was fixed at 0.4 mL/min for 10 minutes. WXRD (wide angle X-ray diffraction) diffraction patterns were recorded with a Stoe Stadi MP X-ray powder diffractometer in transmission mode over 2θ ranges 2–60° with Cu K α radiation. The optical density (OD) values were characterized by a microplate reader (MultiSkan, ND2k). SEM measurements were performed by a field emission-scanning electron microscope (FE-SEM, Hitachi SU8010). UV spectra were recorded by an Ultraviolet-visible Spectrophotometer (UV-vis, HTH HB-7).

## 2.3 Miscibility (paper I)

PHB/HBP polymer blends: a powder of PHB with or without HBP additives (5, 10 and 20 wt%) was dissolved in chloroform/DMF at 100 °C for 5 min in a sealed vessel. The resulting homogeneous solutions were cooled to room temperature and stand for 5 h without any agitation. PCL/HBP polymer blends: a powder of PCL with or without HBP additives (5, 10 and 20 wt%) was dissolved in THF in a sealed vessel. The resulting homogeneous solutions stand for 5 h without any agitation. Afterward, the PHB or PCL solutions were cast at room temperate on a glass Petri dish, and dried at 50 °C under vacuum for 48 hours. The films were kept at room temperature until DSC measurements.

## 2.4 Enzymatic degradation (paper I)

The polymers were soaked in a mixture of phosphate buffer (1000 μL, pH 7.0), water (750 μL), DMSO (200 μL) and PETase (50 μL, 2.10 mg $mL^{-1}$). The reaction mixtures were incubated at 37 °C with shaking at 200 rpm for 72 h. In the meantime, non-enzymatic degradation of polymers was carried out as a negative control under identical conditions. After incubation, the samples were centrifuged at 13 000 *g* for 10 min, and the supernatants were analyzed with LC-MS.

## 2.5 Antimicrobial bioassay

### 2.5.1 Disk diffusion assay (paper I, III and IV)

**Bacterial Culture.** Microorganisms *Escherichia coli* ATCC 25922 (*Ea*), *Staphylococcus aureus* ATCC 25923 (*Sa*), *Proteus mirabilis* ATCC 14153 (*Pm*), *Proteus vulgaris* ATCC13315 (*Pv*), *Pseudomonas aeruginosa* ATCC 27853 (*Pa*), *Enterobacter aerogenes* ATCC13048 (*Ea*), *Bacillus thuringiensis* (*Bt*), *Salmonella typhimurium* SL1344 (*St*) and *Streptococcus mutans* ATCC 25175 (*Sm*) were employed to evaluate the antibacterial properties of the investigated compounds. All bacteria strains were sub-cultured on (Luria Bertoni) LB agar culture at 37 °C for 24 h.

**Disk diffusion assay**. Disk diffusion assay was applied to evaluate the antimicrobial properties. First, the tested solid samples (polymers or small molecular agents) were dissolved in DMF or $CHCl_3$. Microorganisms' susceptibility was adjusted with 0.5 McFarland as a reference standard. The prepared solutions were sterilized under UV light for 5 min before test. Microorganism culture suspension (100 μL, 106 cells per

mL) was swabbed onto a plate within Müller-Hinton agar. Filter disks with a diameter of 6 mm were placed on the Petri plate inoculated with microorganisms, and 20 μL of the prepared sample solutions were loaded on the sterile disks. Afterward, bacteria cultures were incubated at 37 °C for 24 h. Disks containing streptomycin/gentamicin or DMF/$CHCl_3$ (pure solvent) were used as positive or negative controls, respectively. All experiments were performed in triplicate. The results are expressed as the mean diameter of inhibition zone in mm ± standard deviation (mean ± SD). Significant differences between two groups were evaluated as *p* values by t-test using Microsoft Excel software. $p < 0.05$ indicates significant difference, while $p \geq 0.05$ indicates insignificant difference.

### 2.5.2 Antimicrobial tests with polymer coatings (paper II)

**Preparation of monomer- or polymer-coatings**. Silicon wafers (1 cm × 1 cm) were pre-treated with piranha solution (98% sulfuric acid and 30% hydrogen peroxide, 7:3 v/v) for 30 min, then rinsed thoroughly with deionized water and dried with nitrogen flow. Monomer or polymer coatings were prepared by spin-coating (6000 rpm) from 20 μL of DMSO solutions (40 mg/mL) onto the silicon substrates. All coating samples were dried in a vacuum oven overnight at room temperature.

**Antimicrobial tests**. The bactericidal potency of coatings was evaluated by following a contact protocol.[166–168] Bacteria cells (*Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) were grown overnight at 37 °C in PBS medium to a mid-log phase and re-suspended in PBS to $1 \times 10^6$ colony forming units per mL (CFU/mL). 10 μL of inoculum suspension was firstly spread on the uncoated (control), monomer- or HBP-coated silicon wafer, then immediately covered with another piece of control or coated wafer. After incubation at 37 °C for 24 h, the wafer samples were transferred into 400 μL of PBS solution bath and washed vigorously for 10 min. The surviving bacteria were plated on TSA petri dish with 100-fold serial dilutions and incubated at 37 °C for another 24 h. The survival numbers of bacteria were presented as Log (CFU/mL) by counting the number of colonies on each plate. Each experiment was performed at least thrice.

**SEM imaging**. To observe the morphology of bacteria on polymer coatings, **P5c**-coated wafer was used as the representative sample and the uncoated wafer was used as control. The antibacterial test against *E. coli* was carried out as described before. Afterward, the bacteria cells on **P5c**-coating were fixed in the glutaraldehyde solution (pH 7.2, 2.5%) for 2 h at room temperature. The bacterial cells were then dehydrated using gradient ethanol solutions (20, 50, 70, 80, 90, and 100% v/v in water) and dried in a vacuum oven. All samples were coated with gold using Denton Dest II Sputter Coater for 15 s and observed by FE-SEM.

## 2.6 Hemolysis (paper II)

Hemolytic activity was assessed with sheep's red blood cells. Red blood cells were pelletized by centrifuging 1 mL of the blood and washing the pellet four times with PBS (pH = 7.4). A 10 μL of RBC suspension was firstly spread on the uncoated (control), monomer-, or HBP-coated silicon wafer, then immediately covered with another piece of control or coated wafer. After incubation at room temperature for 2 h, the wafer samples were transferred into a 490 μL of PBS or deionized water solution bath and washed vigorously for 10 min. For the uncoated wafers, the positive control was washed with deionized water and the negative control was washed with PBS. 100 μL diluted solution was transferred to a new 96 well plate and the optical density at 540 nm was measured. The hemolysis percentage was calculated by following equation:

$$\text{Hemolysis } \% = \frac{\text{OD}_{540}(\text{Sample}) - \text{OD}_{540}(\text{negative control})}{\text{OD}_{540}(\text{positive control}) - \text{OD}_{540}(\text{negative control})} \times 100\%$$

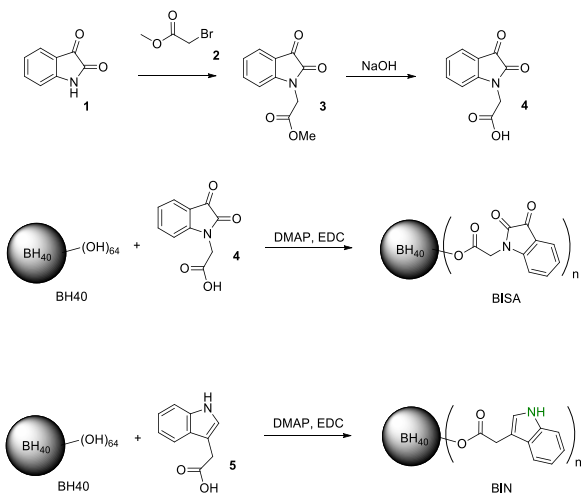
## 2.7 MTT assay (paper I, II, III and IV)

The MG-63 osteoblast-like human cells were employed to evaluate the cytotoxicity of the investigated compounds. The MG-63 osteoblast-like human cells were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% penicillin, and 1% streptomycin in a humidified incubator at 37 ºC. The medium was replaced every 2 days. Cells were trypsinized and centrifuged at 400 $g$ for 4 min to get a concentrated cell pellet when the confluence reached 80%. $1 \times 10^4$ cells/well were seeded on a 96-well plate and cultured for 24 h before adding the materials. Test compounds dissolved in DMSO were added to the cell culture at a final DMSO concentration of 1% (v/v). Fresh culture medium without samples was used as positive control, and each sample was replicated in five wells. After being cultured for 24 h with materials from each group, cell culture medium was discarded and cells were washed with phosphate buffer once. MTT working solution (0.5 mg/mL) was added to the cells and incubated for 2 h at 37 ºC, after which DMSO was added to the reaction products followed by further incubation for 10 min. The solubilized contents were pipetted and transferred into a clear bottom 96-well plate. Absorbance was determined by spectrophotometry at 600 nm wavelength. Plain DMSO was used for blank subtraction.

# 3 Summary of appended papers

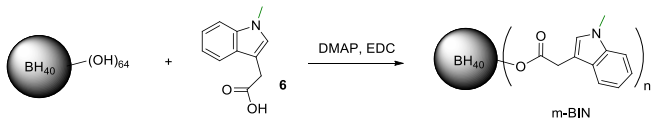
## 3.1 Nonionic antimicrobial hyperbranched polyesters with indole or isatin functionality (Paper I)

The initial idea of paper I was inspired by the previous work from our group, in which two new nonionic hyperbranched polymers based on isatin building blocks were prepared. In spite of these HBPs showing high antibacterial activity and negligible leaching potential, their rigid structures and absence of interacting groups (*e.g.* H-bond donor) limited their miscibility with low-$T_g$ matrix polyesters (*e.g.* PCL, PHB, PBS).[72] Therefore, in this work, we designed and synthesized two nonionic HBPs (BISA and BIN) by reacting carboxylic acid derivatives of isatin or indole (**4** and **5**) with a hydroxyl-terminated commercially available HBP, BH40, in the presence of DMAP and EDC (**Scheme 3.1**). The grafting agent **5** is commercially available, and **4** was obtained from isatin following a 2-step synthetic protocol. The flexible backbone of BH40 was expected to obtain low-$T_g$ HBPs and the NH group of indole was expected to form H-bond with polyester matrix containing C=O group.




**Scheme 3.1.** Synthesis of isatin-based grafting agent **4**, BISA and BIN.

The molecular structures and thermal properties of the obtained HBPs were characterized by GPC, NMR, FTIR, TGA and DSC analyses. Compared to their precursor BH40, BISA and BIN displayed higher $T_g$ (90 and 64 °C, respectively) due to the incorporation of rigid cyclic aryl groups.




**Scheme 3.2.** Synthesis of methyl-modified indole-based HBP, m-BIN.

The miscibility of 5, 10 and 20 wt% HBPs (BISA and BIN) with two different polyesters (PHB and PCL) was evaluated by DSC. In the case of PHB polymer blends (**Figure 3.1**), results suggested that up to 20 wt% indole-based HBP (BIN, **Figure 3.1B**) can form miscible blends with PHB, which was likely due to the favorable hydrogen bonding between N-H moiety of indole in the structure of BIN and the C=O group of polyesters. In contrast, BISA (**Figure 3.1A**) and methyl-modified indole-based HBP (namely m-BIN, **Scheme 3.2**) (**Figure 3.1C**) without active H-donor were immiscible with PHB in the measured composition range. The PCL blends showed the similar trends as PHB blends (**Figure 3.1D-F**).

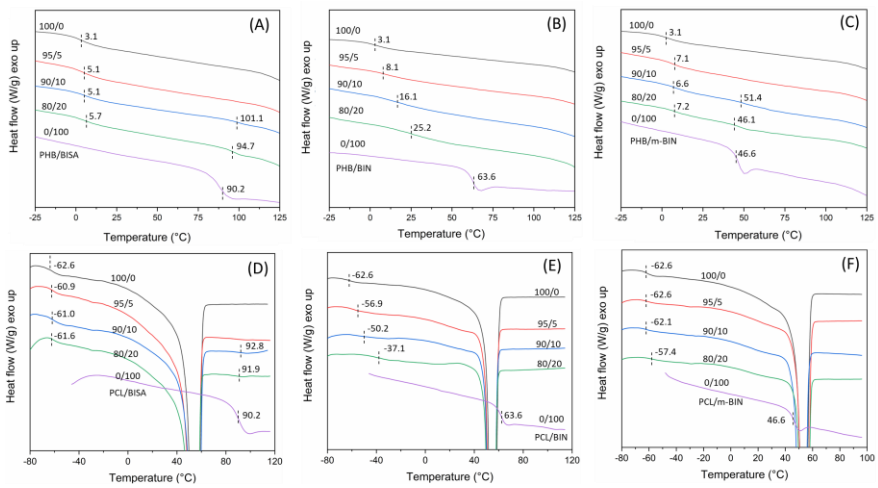



**Figure 3.1.** DSC second heating curves of (A) neat PHB and PHB with 5, 10 and 20 wt% of BISA and neat BISA, (B) neat PHB and neat PHB with 5, 10 and 20 wt% of BIN and neat BIN, (C) neat PHB and neat PHB with 5, 10 and 20 wt% of m-BIN and neat m-BIN, (D) neat PCL and PCL with 5, 10 and 20 wt% of BISA and neat BISA, (E) neat PCL and neat PCL with 5, 10 and 20 wt% of BIN and neat BIN, (F) neat PCL and neat PCL with 5, 10 and 20 wt% of m-BIN and neat m-BIN.

Enzymatic degradation of the obtained HBPs (BISA and BIN) using PETase was preliminarily investigated by following a previously reported method.[112,169] As shown in **Figure 3.2**, various small fragments including oligomers of BIN were found in the products after reaction with PETase for 3 days. However, no small

fragments of BISA were found under the same condition compared with non-enzymatic control experiment (not shown). This result demonstrated that the incorporation of indole into BH40 facilitated its enzymatic degradation with PETase from *Ideonella sakaiensis*, while isatin exerted complex impact.

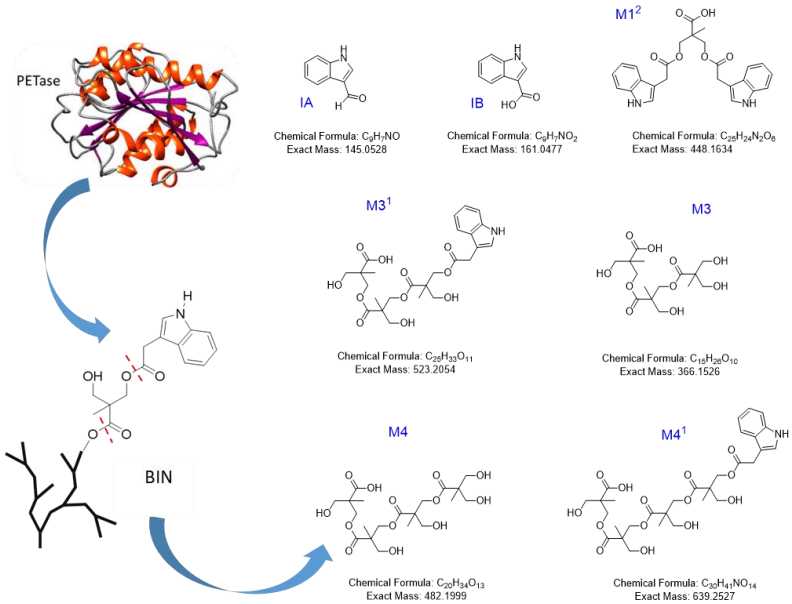



**Figure 3.2.** Illustration of enzymatic reaction of BIN with PETase.

The antimicrobial activity of HBPs and two small model molecules (EISA and EIN, **Figure 3.3**) against 8 pathogenic bacteria was evaluated following a standard disk diffusion assay. As shown in **Figure 3.3**, BISA and BIN with 10 μg per disk loading showed significant inhibition zones (~14-20 mm) against all the tested bacteria, indicating their broad-spectrum antibacterial activity. In comparison with their corresponding small molecules (EISA and EIN) with the same amount of sample loading (10 μg per disk), BISA and BIN exhibited significantly larger inhibition zones against most bacteria except *Pa* for BISA/EISA, and *Pa*, *Sm* for BIN/EIN ($p$ values $\geq 0.05$). The enhanced antimicrobial effect was likely due to the intensified interactions with bacteria by the locally concentrated functional groups (isatin or indole), as other reported AMPs with dendritic structure.[79]

Furthermore, the antimicrobial effects of the resulting HBPs (BISA and BIN) were compared with a commercial antibiotic gentamicin. It was found that BISA and BIN showed either significantly higher or comparable activity as gentamicin against all tested bacteria except two bacteria *Pa* and *Ea* (as confirmed with $p$ values). Specifically, BISA and BIN showed significantly higher efficiency against *Ec*, *Sa*, and *Sm* and comparable efficiency against *Pm* and *Bt*. Moreover, BIN showed significantly higher efficiency and BISA showed comparable efficiency against *Pv*.

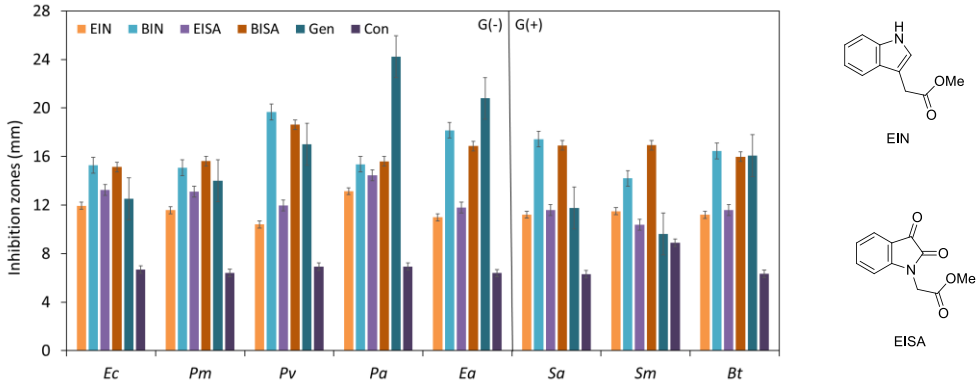



**Figure 3.3.** Inhibition zones of the obtained HBPs (BIN and BISA), small molecular model compounds (EIN, EISA) and gentamicin at the loading level of 10 μg per disk. Gentamicin was used as the positive control experiments (marked as Gen in the figure). DMF was used for the negative control experiments (marked as Con in the figure).

Finally, the cytotoxicity to MG-63 osteoblast-like human cells of HBPs and two small molecules (EISA and EIN, **Figure 3.3**) was evaluated according to a standard MTT assay. As shown in the **Figure 3.4**, none of the obtained polymers (BISA and BIN) showed toxic effect to the tested cells at all concentrations after 24 h incubation. In contrast, their corresponding small molecules (EISA and EIN) showed concentration-dependent toxicity. When the concentration increased up to 500 μg/mL, both EISA and EIN exhibited significant toxic effect. This indicated that the incorporation of isatin or indole functionality to BH40 did not result in cytotoxic effect.

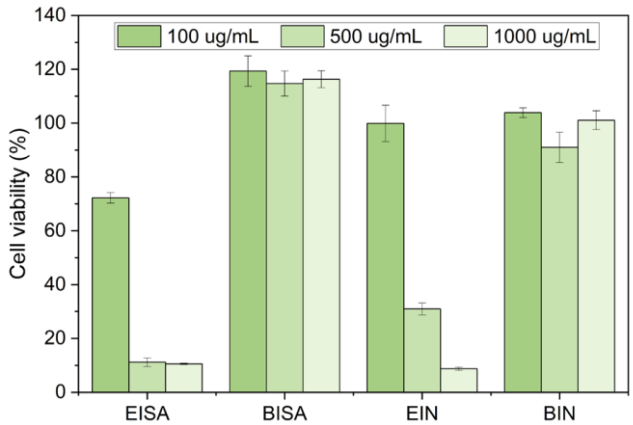
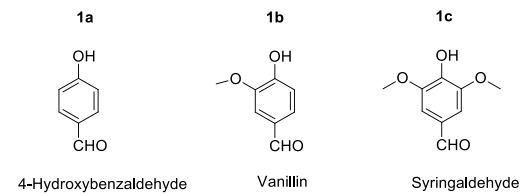



**Figure 3.4.** Cytotoxicity of EISA, BISA, EIN and BIN at three concentrations (100, 500, and 1000 μg/mL). Results are presented as percent viability of treated cells to that of untreated control (100% of cell viability, not shown in the figure).

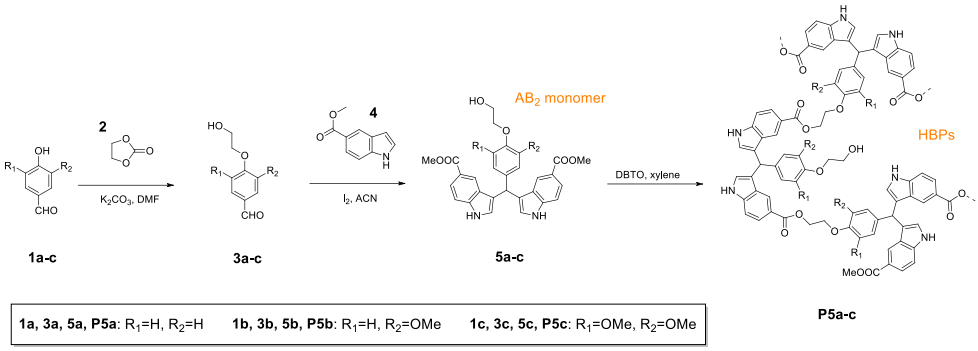
# 3.2 Nonionic hyperbranched polyesters bearing indole and lignin-derived structures as effective antimicrobial coatings (Paper II)

In paper II, instead of grafting indole functionality onto a commercially available HBP (what we have done in paper I), we have designed and synthesized three HBPs from three $AB_2$-type monomers containing bis-indole structure. The monomers were synthesized from methyl indole-5-carboxylate and three lignin-derived compounds **1a-c** (4-hydroxybenzaldehyde, vanillin and syringaldehyde) as seen in the **Scheme 3.3**. The synthesis of monomers and HBPs is shown in **Scheme 3.4**. First, the phenolic groups of **1a-c** were reacted with ethylene carbonate (**2**), yielding the corresponding primary alcohols **3a-c**. Then, **3a-c** were reacted with indole carboxylate (**4**) following an iodine-catalyzed protocol,[170] yielding the corresponding $AB_2$ monomers **5a-c** in satisfying yields (~90%).

Afterward, the obtained monomers **5a-c** were polymerized by a bulk condensation at 165 °C with DBTO catalyst, yielding the HBPs (**P5a-c**).[171,172] A small amount of xylene was used in the system to dissipate heat and remove the condensed methanol.[114] The molar masses of the resulting HBPs were measured by GPC as ~3000-4500 g/mol. The chemical structures and thermal properties were characterized by NMR, FTIR, DSC and TGA analyses. These HBPs showed relatively high $T_g$ values ($T_g$ = 223, 213 and 209 °C for **P5a-c**, respectively) due to their rigid structures and they are also quite thermally stable with high onset degradation temperature ($T_{10}$ > 300 °C).




**Scheme 3.3.** Lignin-derived aromatic aldehydes (**1a-c**).




**Scheme 3.4.** Synthesis of monomers (**5a-c**) and HBPs (**P5a-c**).

The bactericidal activity of monomers **5a-c** and HBPs **P5a-c** as coatings against two Gram-negative bacteria *E. coli* and *P. aeruginosa*, and two Gram-positive bacteria *S. aureus* and *E. faecalis* was evaluated through a conventional contact protocol.[168] After contacting for 24 h, the surviving bacteria on the coatings were plated on TSA petri dish with 100-fold serial dilutions and incubated at 37 °C for another 24 h as shown in **Figure 3.5**.

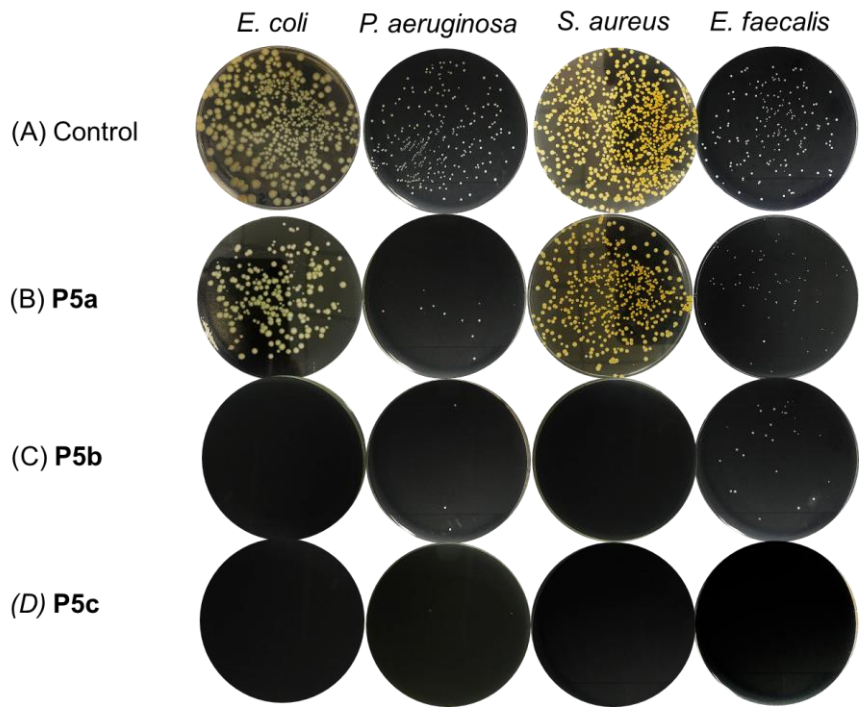



**Figure 3.5.** Petri-plates images of (A) negative control, (B) **P5a**, (C) **P5b** and (D) **P5c** against *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis*.

The surviving colony numbers were counted as presented in **Figure 3.6**. Compared with small molecular monomers, the polymers showed generally higher bactericidal activity against all tested bacteria, which could be attributed to the densely functional groups (*i.e.* indole units) that can enhance their interactions with bacterial membranes. It was also observed that the bactericidal efficiency of polymers was enhanced by the increased number of methoxy groups (**P5a** < **P5b** < **P5c**). Specifically, **P5a** coating exhibited moderate activity of ~2-log reduction in colony counts against *P. aeruginosa*, and low activity of less than 1-log reduction in colony counts against *E. coli*, *S. aureus* and *E. faecalis*. **P5b**-coating exhibited significant activity of at least 6-log reduction in colony counts against *E. coli* and *S. aureus*, and relatively low activity of ~1-log reduction in colony counts against *P. aeruginosa* and *E. faecalis*. **P5c**-coating exhibited significant activity of at least 6-log reduction in colony counts against *E. coli*, *S. aureus* and *E. faecalis*, and moderate activity of ~2-log reduction in colony counts against *P. aeruginosa*. Such observations indicate that the methoxy group could enhance the interactions between polymers and bacterial membranes to some extent, which is consistent with the previously reported cationic AMPs with methoxyethyl side chain.[173]

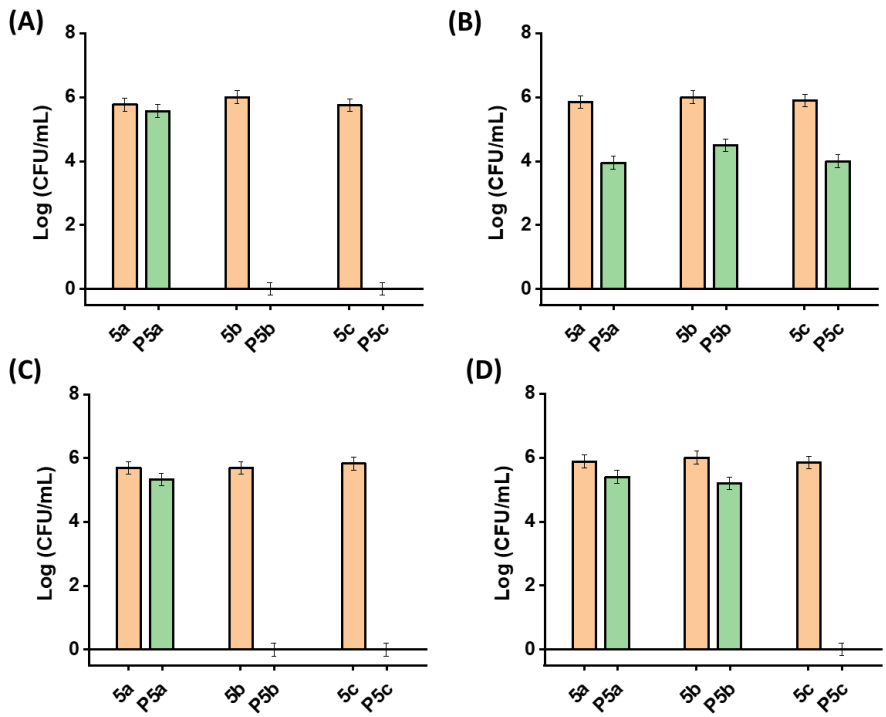



**Figure 3.6.** Colonies of Gram-negative bacteria (A) *E. coli*, (B) *P. aeruginosa* and Gram-positive bacteria (C) *S. aureus*, (D) *E. faecalis* on the surfaces coated with monomers (**5a-c**) or polymers (**P5a-c**). The survival colonies of the negative control (without coating) for all tested bacteria was $1 \times 10^6$ CFU (not shown).

Next, the cells of *E. coli* on the best bactericidal candidate **P5c**-coating was subjected to SEM imaging (**Figure 3.7**). It was found that cells of *E. coli* were partially or completely lysed after 24 h contacting **P5c**-coating, indicating that **P5c** had the ability to disrupt bacterial membranes. This observation suggests a bactericidal mechanism, which is in good agreement with that of other reported cationic AMPs.[143,174]

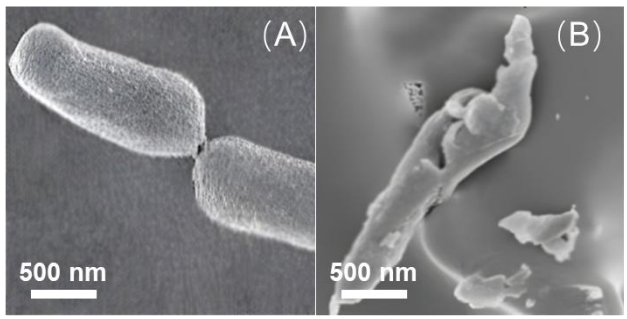



**Figure 3.7.** SEM images of the *E. coli* before (A) and after (B) contacting **P5c**-coating.

To evaluate the durability of the polymer coating, **P5c** coated surface was subjected to antibacterial test against *E. coli* for the second cycle. As shown in **Figure 3.8**, no significant difference of the bactericidal efficiency was observed for the second cycle. This can suggest that the activity of **P5c** coating is durable.

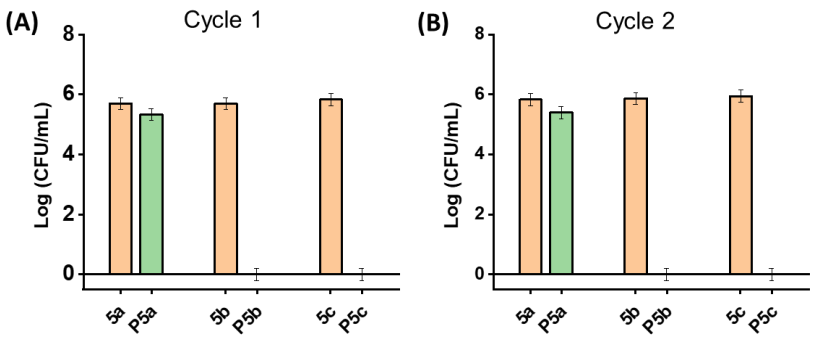



**Figure 3.8.** Colonies of *E. coli* on **P5c**-coating. Including the (A) first and (B) second cycle antimicrobial experiments. The survival colonies of the negative control (without coating) was $1 \times 10^6$ CFU (not shown).

In this work, we also evaluated the hemolytic activity and cytotoxicity to MG-63 osteoblast-like human cells of these indole-based monomers (**5a-c**) and polymers (**P5a-c**). As shown in **Figure 3.9**, the hemolysis of all samples were negligible (less than 0.1%) after 2 h cultivation, which indicates that these monomers and HBPs have good hemocompatibility. The cytotoxicity test was conducted by following a standard MTT assay method and the results are presented in **Figure 3.10**. It was observed that only HBPs with methoxy groups (**P5b** and **P5c**) were non-cytotoxic

to the tested cells according to the ISO 10993-5 standard.[175] This is consistent with the observation for other previously reported polymers with methoxy functionality.[176]

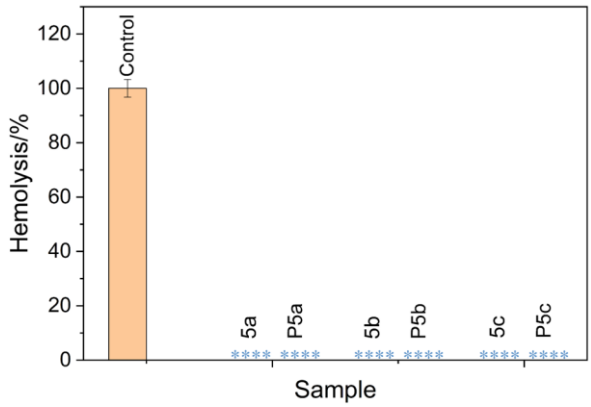



**Figure 3.9.** Hemolysis ratio of monomers **5a-c** and polymers **P5a-c**. **** indicates at least 99.9% survival of red blood cells.

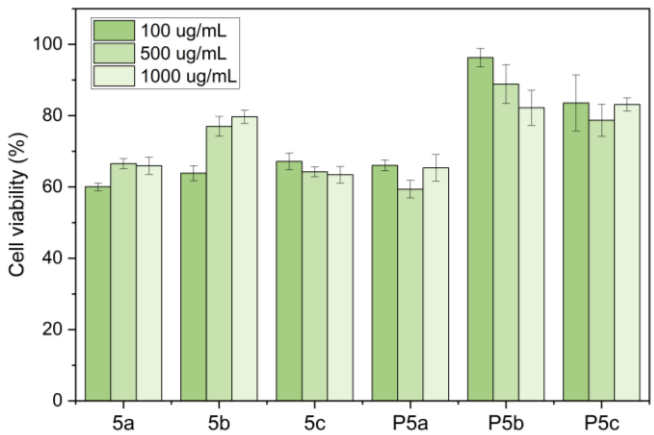
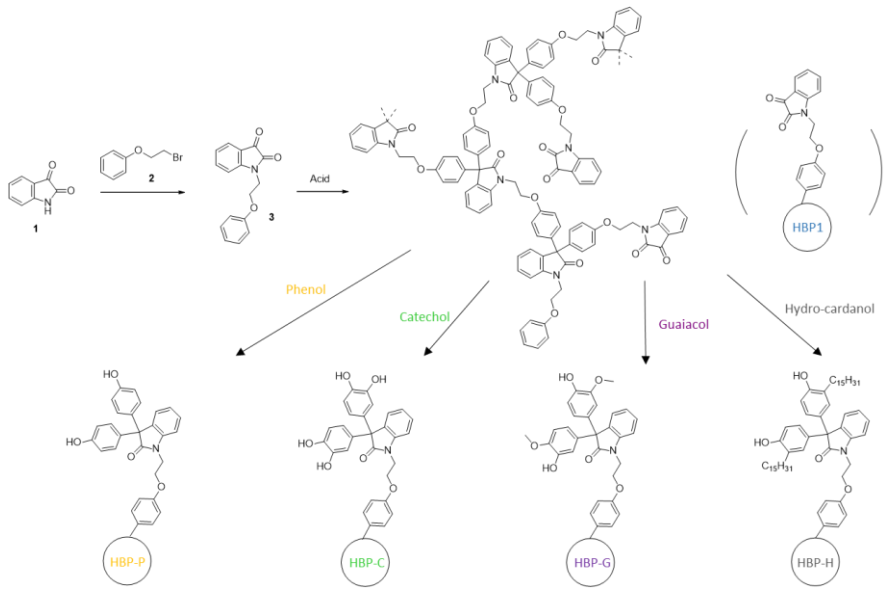



**Figure 3.10.** Cytotoxicity of monomers **5a-c** and polymers **P5a-c** at three concentrations (100, 500 and 1000 μg/mL). Results are presented as relative percent viability of treated cells to that of untreated control (100% of cell viability, not shown in the graph).

# 3.3 Nonionic antimicrobial hyperbranched polymers with isatin-based backbone and phenolic terminal units (Paper III)

Many phenolic compounds are naturally produced with excellent antimicrobial activity, such as phenol, catechol, guaiacol, pyrogallol, and cardanol derivatives (*e.g.* hydro-cardanol, or 3-*n*-pentadecylphenol).[85,177] All these can be potentially used as resources to develop new AMPs. Phenolic polymers with antibacterial effect have been reported,[116,178–180] and it was revealed that the mechanisms of phenolic polymers included disruption of bacterial membranes though hydrogen-bonding interactions between phenol OH and lipid molecules.[62,181,182] In an effort for gaining more knowledge about the structure-activity relationship of isatin-based HBPs, a series of isatin-based phenolic HBPs were prepared in paper III. Firstly, an isatin-based $AB_2$ monomer (**3**) was firstly synthesized in 89% yield, followed by a facile solvent-free polymerization at room temperature overnight to yield an isatin-based precursor **HBP1**. Next, four isatin-HBPs containing phenol moiety (**HBP-P**, **HBP-C**, **HBP-G**, and **HBP-H**) were prepared by grafting four different phenolic molecules onto the obtained precursor **HBP1** (**Scheme 3.5**).




**Scheme 3.5.** Synthesis of $AB_2$ monomer **3**, isatin-based precursor (**HBP1**) and four phenol-containing HBPs, including **HBP-P** (with phenol), **HBP-C** (with catechol), **HBP-G** (with guaiacol), and **HBP-H** (with hydro-cardanol).

The molecular and thermal properties of the obtained HBPs were characterized by GPC, NMR, FTIR, TGA and DSC analyses. According to GPC results, the molecular weight ($M_n$) of **HBP1** is ~16 kDa, higher than that of the previously

reported isatin-based HBP with similar structures ($M_n$~2-3 kDa).[72] This is because of the higher reactivity of the new isatin-based $AB_2$-monomer **3**. The $T_g$ of **HBP-H** (127 °C) was relatively lower than the rest of HBPs ($T_g$ = 188, 242, 234, and 200 °C for **HBP1**, **HBP-P**, **HBP-C**, and **HBP-G** respectively), which was attributed to the effect of its flexible aliphatic chain next to phenol.

The antibacterial effects of these HBPs were evaluated by disk diffusion assay. The tested bacteria include six Gram-negative G(-) bacteria and three Gram-positive G(+) bacteria. As presented from **Figure 3.11**, all samples showed a broad-spectrum antibacterial activity. It was also observed that the polymer **HBP1** showed comparable zone of inhibition as the corresponding monomer **3** ($p$ values ≥ 0.05) against most bacteria. In the case of two G(-) bacteria (*Ec* and *St* respectively), the polymer **HBP1** showed significantly larger zone of inhibition compared to monomer **3**. The enhanced antimicrobial effect was attributed to locally concentrated isatin functional groups in the **HBP1**.

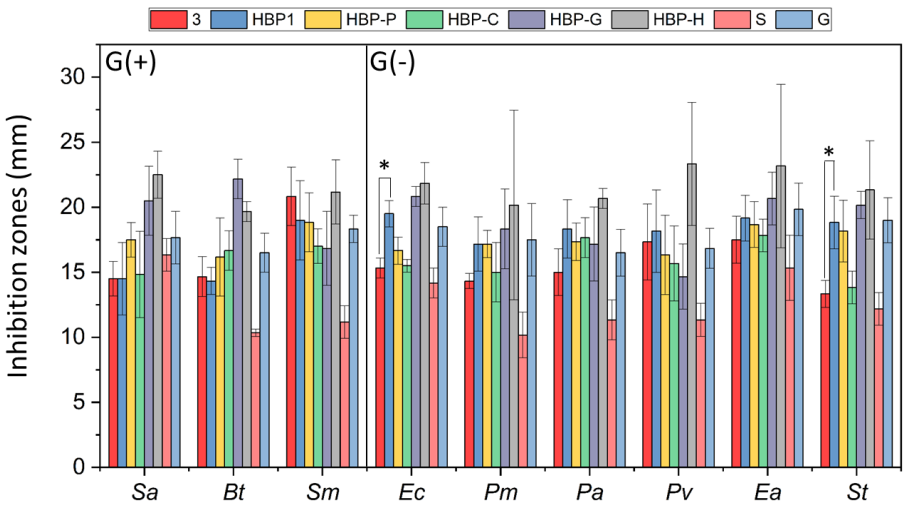



**Figure 3.11.** Inhibition zones of monomer **3**, HBPs (**HBP1**, **HBP-P**, **HBP-C**, **HBP-G** and **HBP-H**) (0.5 µg per disk), streptomycin and gentamicin (25 µg per disk). Streptomycin (S) and gentamicin (G) were used as positive control. The only two cases where the zones of inhibition of **HBP1** had significant difference compared to monomer **3** are marked as * ($p$ values < 0.05).

Next, the antibacterial activity of monomer **3** and the five resulting HBPs was compared to that of two commercialized antibiotics (streptomycin and gentamicin, respectively). In order to facilitate the comparison, the differences between the zone of inhibition of the two antibiotic and samples (monomer **3** and 5 HBPs) were calculated. As seen from **Figure 3.12A**, the monomer **3** and the five HBPs were more effective than streptomycin ($\Delta > 0$, $p$ values < 0.05) against most bacteria. They showed comparable efficiency as streptomycin against certain bacteria ($p$ values ≥ 0.05), which are not shown in the figure (*e.g.* in case of *Ea*). In case of gentamicin (**Figure 3.12B**), the monomer **3** and the five HBPs showed comparable

efficiency against most tested bacteria ($p$ values ≥ 0.05). While for certain bacteria, they showed either higher (Δ > 0) or lower efficiency (Δ < 0). For instance, **HBP-H** was more effective than gentamicin against three bacteria (*Sa*, *Bt*, and *Pa*). **HBP-G** was more effective than gentamicin against *Bt*. For monomer **3** and **HBP-C**, they exhibited lower efficiency than gentamicin against *Ec* and *St*.

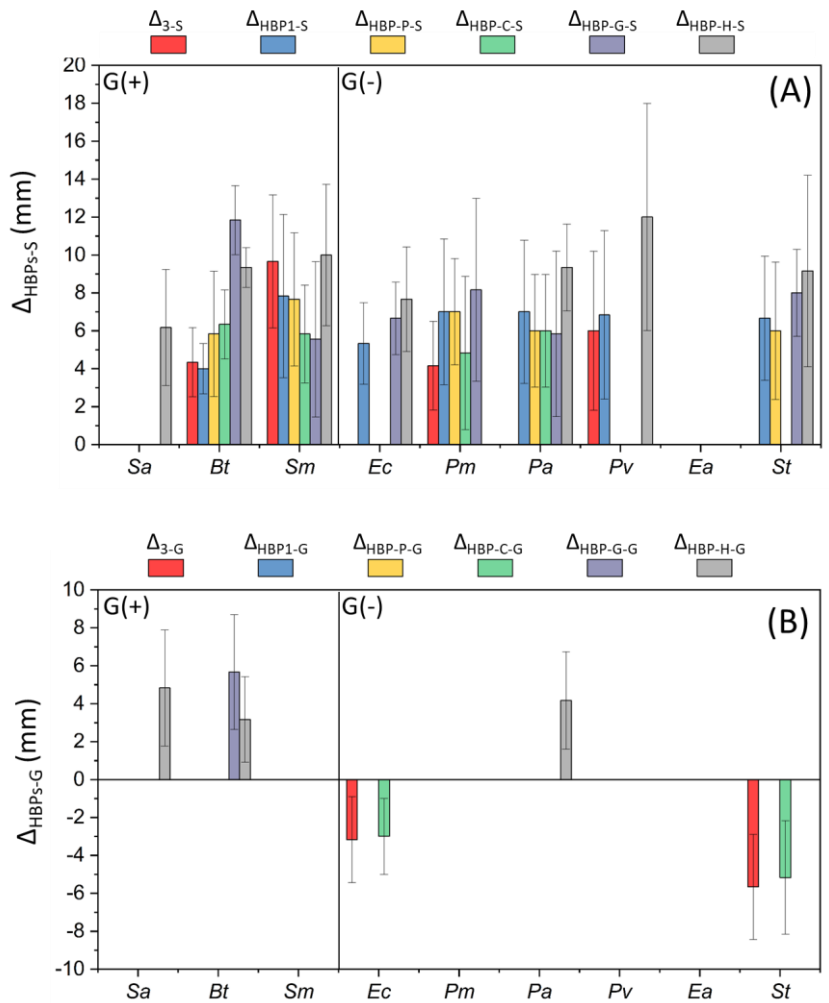



**Figure 3.12.** Comparison of the inhibition zones of monomer **3** and HBPs with (A) streptomycin (marked as S), and (B) gentamicin (marked as G). Those without significant difference are not shown.

Furthermore, differences between the zones of inhibition of HBPs (**HBP-P**, **HBP-C**, **HBP-G** and **HBP-H**) and **HBP1** were calculated (**Figure 3.13**). It was found that these **HBP**s with phenolic functionalities were as effective as their precursor **HBP1** (no significant difference was observed, $p$ values ≥ 0.05) against most bacteria. In some cases, the HBPs **(HBP-P** and **HBP-C**) showed suppressed

efficiency compared to **HBP1**. For example, **HBP-P** showed significantly lower efficiency than **HBP1** against G(-) *Ec*. **HBP-C** showed significantly lower efficiency than **HBP1** against G(-) *Ec* and *St.* On the contrary, the HBPs (**HBP-G** and **HBP-H**) showed enhanced efficiency compared to **HBP1** in a few cases. For instance, **HBP-G** with guaiacol moiety containing a methoxy ether group showed stronger activity than **HBP1** against G(+) *Bt* ($\Delta_{HBP\text{-}G\text{-}HBP1} > 0$, *p* values < 0.05); **HBP-H** with hydro-cardanol moiety containing a long alkyl group exhibited significantly higher efficiency than **HBP1** against G(+) *Sa* and *Bt* ($\Delta_{HBP\text{-}H\text{-}HBP1} > 0$, *p* values < 0.05), indicating the presence of a methoxy or long alkyl group close to the phenolic unit could enhance the interactions between polymer and certain G(+) bacteria.

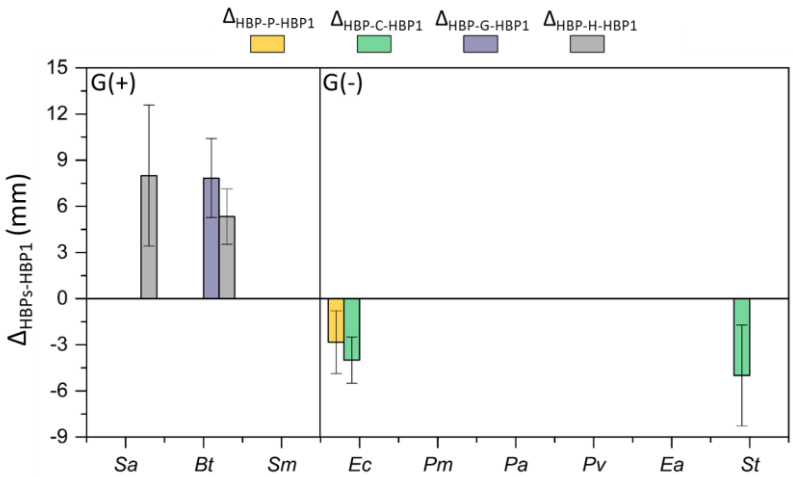



**Figure 3.13.** Comparison of the inhibition zones HBPs (**HBP-P**, **HBP-C**, **HBP-G** and **HBP-H**) with **HBP1**. Those without significant difference are not shown.

Finally, the cytotoxicity to MG-63 osteoblast-like human cells of monomer **3** and the resulting polymers was investigated. As seen in **Figure 3.14**, monomer **3** showed significant toxicity with cell viability less than 50%. In contrast, all polymers showed excellent compatibility with MG-63 osteoblast-like human cells at all concentrations. Interestingly, we observed that **HBP-C** and **HBP-G** promoted the cell proliferation largely with the increase of concentration, which is consistent with the previously reported polymers such as chitosan derivatives.[183,184]

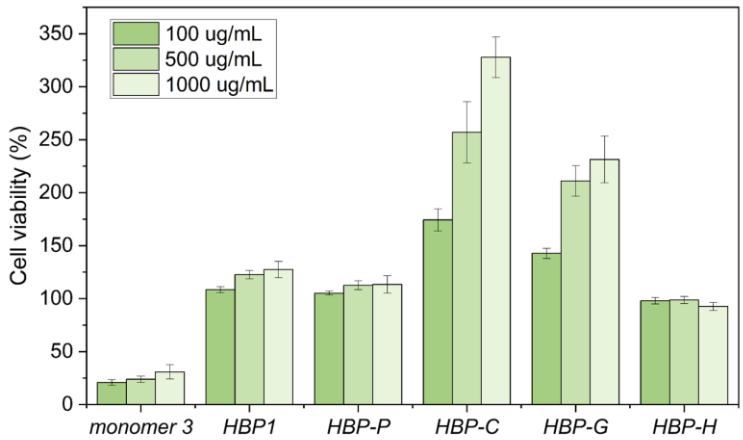
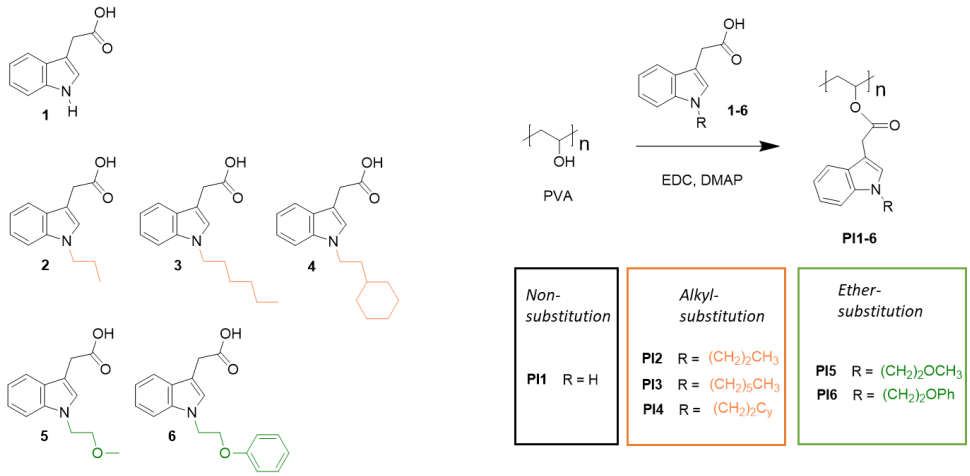



**Figure 3.14.** Cytotoxicity of monomer **3** and HBPs (**HBP1**, **HBP-P**, **HBP-C**, **HBP-G** and **HBP-H**) at three concentrations (100, 500 and 1000 μg/mL). Results are presented as relative percent viability of treated cells compared to that of untreated control (100% of cell viability, not shown in the graph).

## 3.4 Nonionic antimicrobial indole-based poly(vinyl alcohol) with *N*-substituted alkyl and ether groups (Paper IV)

The hydrophobicity of cationic AMPs with various hydrophobic alkyl chains has been widely studied, while the impact of hydrophobic substituents of nonionic AMPs remains unknown. In order to further develop and understand indole-based AMPs, we have synthesized 6 indole-modified PVAs (**PI1-6**) with a series of substitutions (different linear and cyclic aliphatic and/or ether groups) at the indole *N*-position (**Scheme 3.6**) in paper IV. The grafting agent **1** is commercially available and others (**2-5**) were synthesized by straightforward $S_N2$ reactions. The OH conversion ($p_{OH}$) was calculated by using the integrals in the $^1H$ NMR spectra and the values of $p_{OH}$ for **PI1-6** are relatively similar (59-72%). Their molecular weights were measured by GPC, which are ranging from 30 kDa to 70 kDa. In addition, the resulting PVAs showed tunable $T_g$ (39–93 °C) and desirable thermal stability according to DSC and TGA analyses.




**Scheme 3.6.** Structure of six grafting agents (**1-6**) and synthesis of indole-based PVAs (**PI1-6**).

Disk diffusion assay was used to evaluate the antimicrobial properties of the obtained 6 indole-based PVAs (**PI1-6**). The tested bacteria include six Gram-negative G(-) bacteria and three Gram-positive G(+) bacteria. As shown in **Figure 3.15**, compared to the negative control (marked as Con), almost all polymers showed significantly larger inhibition zone. There are only two exceptions that **PI4** showed comparable inhibition zone as the negative control for two G(+) bacteria *Sa* and *Sm* ($p$ values $\geq 0.05$). This observation clearly indicates the obtained PVAs have broad-spectrum antimicrobial activities, regardless of the *N*-substitution.

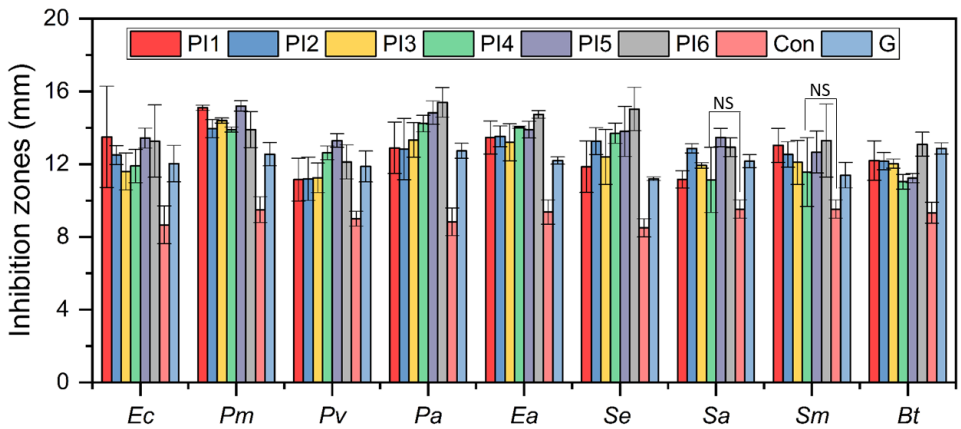



**Figure 3.15.** Inhibition zones of **PI1-6** and gentamicin (10 μg per disk). Pure DMF was used as negative control (marked as Con in the figure). Gentamicin (G) was used as positive control. The only two cases where the zones of inhibition did not show significant difference compared to Con are marked as NS ($p$ values $\geq 0.05$).

Next, in order to compare the antimicrobial activity of these indole-based PVAs with gentamicin, differences between the zones of inhibition of **PI1-6** and

gentamicin (PIx-G. x=1-6) were calculated (**Figure 3.16**). To make discussions easier, only those with significant differences ($p$ values < 0.05) were visible in the figure, and those are not shown in the figure (*e.g.* in cases of *Ec* and *Sm*) mean for these bacteria the effect of the obtained polymers is not significantly different from gentamicin ($p$ values ≥ 0.05). Therefore, we can see that **PI1-6** showed generally comparable antimicrobial effect as gentamicin. In detail, for the six G(-) bacteria, all **PI1-6** showed either significantly higher or comparable (not shown) antimicrobial activity than gentamicin, indicating the effectiveness of such molecular structures against G(-) bacteria. For the three tested G(+) bacteria, the comparison with gentamicin seems more complex. For *Sa*, **PI2** and **PI5** showed significantly higher antimicrobial activity than gentamicin, and **PI1** showed significantly lower antimicrobial activity than gentamicin; For *Sm*, comparable antimicrobial activity as gentamicin were observed for all polymers. For *Bt*, **PI3-5** showed significantly lower antimicrobial activity than gentamicin.

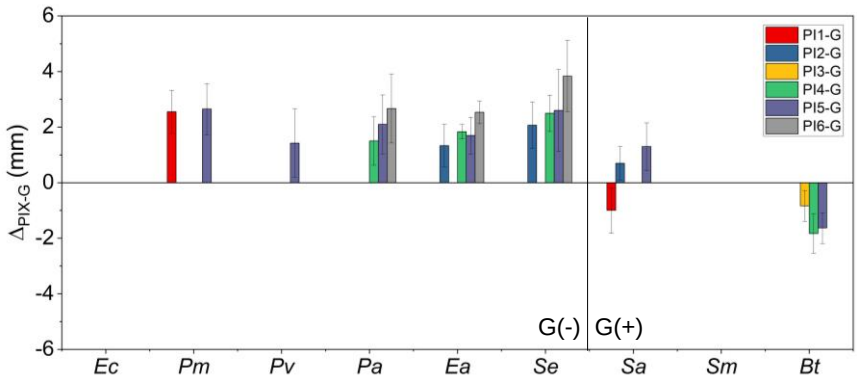



**Figure 3.16.** Comparison of the inhibition zones of **PI1-6** with gentamicin (G). Those without significant difference are not shown.

Finally, we investigated the impact of *N*-substitution on the antimicrobial activity of these indole-grafted PVAs. To achieve this, differences between the zones of inhibition of **PI2-6** and **PI1** were calculated (**Figure 3.17**). Similar to the above discussion, only those with significant difference are shown in the figure, and those not shown mean that their antimicrobial activity is comparable as **PI1**. As a result, most indole-grafted PVAs with *N*-substituted groups (**PI2-6**) showed similar zone of inhibition to **PI1**, which demonstrated that neither the indole *N*-H nor the various *N*-substituted groups had significant influence on their antimicrobial properties. In several cases, the substitution had effect. For example, *N*-substituted propyl groups (*i.e.* in **PI2**) promoted the antimicrobial activity against only one bacterium (*Sa*) and *N*-substituted ether groups (*i.e.* in **PI5-6**) promoted the antimicrobial activity against two bacteria (*Pv* and *Sa* for **PI5**; *Se* and *Sa* for **PI6**). On the contrary, some *N*-substituted alkyl groups showed negative influence in some cases (*i.e. Pm* for **PI3-4)**. These observations suggest that ether groups may have cooperative effect with

indole units for the interactions with membranes of certain bacteria, while the *N*-substitution by linear or cyclic alkyl groups may exert negligible or even negative impact.

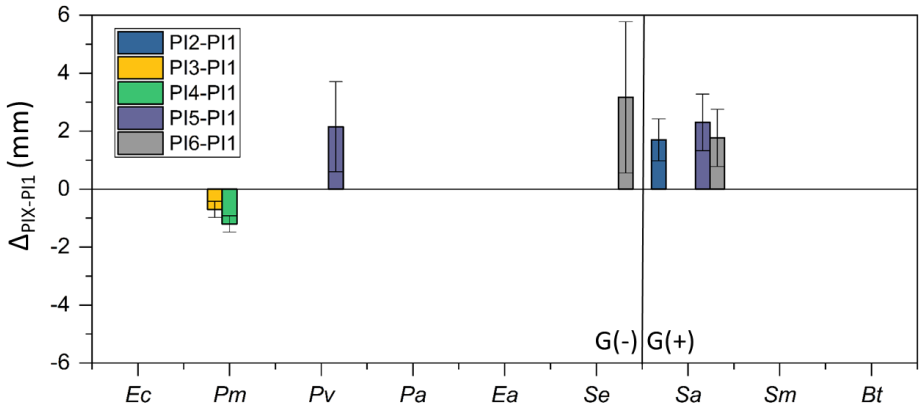



**Figure 3.17.** Comparison of the inhibition zones of **PI2-6** with **PI1**. Those without significant difference are not shown.

Finally, the cytotoxicity to MG-63 osteoblast-like human cells of the resulting polymers **PI1-6** was investigated. As presented in **Figure 3.18**, all polymers showed above 70% cell viability, indicating they are nontoxic to the tested cells according to the ISO 10993-5 standard.[175] It was also observed that **PI1** promoted the cell growth with the increase of concentration, which is in consistent with some previously reported polymers (*e.g.* chitosan- and lignin- derivatives).[183–185]

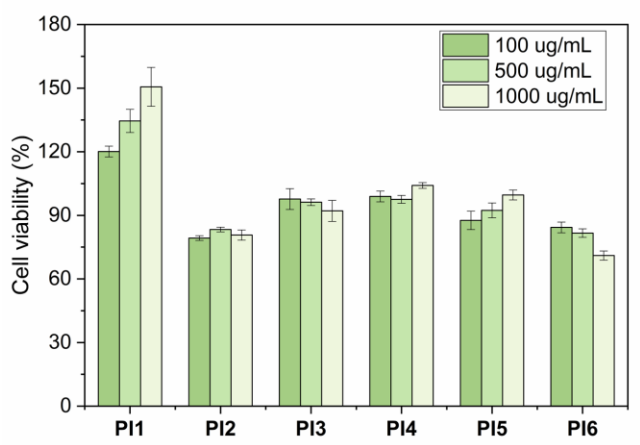



**Figure 3.18.** Cytotoxicity of **PI1-6** at three concentrations (100, 500 and 1000 μg/mL). Results are presented as relative percent viability of treated cells to that of untreated control (100% of cell viability, not shown in the graph).

# 4 Conclusion and outlook

In this study, various new isatin and indole-based nonionic AMPs were designed and prepared. The antimicrobial effectiveness of these nonionic polymers was proved, and their miscibility, durability and cytotoxicity were investigated, providing a preliminary insight on the preparation strategies and structure-property relationships of new nonionic AMPs with isatin or indole functionality. Specifically:

In paper I, isatin and indole were grafted onto a commercially available biodegradable hyperbranched polyester, Boltorn$^{TM}$, yielding two HBPs with isatin or indole units as the end groups. The miscibility study showed that up to 20 wt% of this indole-grafted HBP was miscible with two biodegradable polyesters (PHB and PCL, respectively), which was likely due to the favourable hydrogen bonding interactions between N-H group of indole and the C=O group of polyesters. The disk diffusion assay revealed that the obtained HBPs had significant antimicrobial effect against 8 pathogenic bacteria and negligible cytotoxicity.

In paper II, three $AB_2$-type monomers derived from bio-based indole and lignin resources were firstly synthesized. Then the obtained monomers were polymerized by a conventional bulk condensation, yielding three nonionic hyperbranched polyesters with indole units in the polymer backbone. Afterward the monomers and polymers were coated onto silicon wafers and the antimicrobial activity against 4 pathogenic bacteria of these coatings was evaluated. As a result, these HBP-coatings with methoxy units not only displayed significant and durable bactericidal activity but also excellent biocompatibility.

In paper III, a series of isatin-based HBPs with various phenolic end groups were prepared. It was observed that these HBPs exhibited broad-spectrum and strong antimicrobial effect against 9 different pathogenic bacteria. We also observed that the presence of a methoxy or long alkyl group close to the phenolic unit had an enhanced effect against certain G(+) bacteria.

In paper IV, a series of indole derivatives with different *N*-substitution on indole rings were grafted onto a commercially available biodegradable linear polymer, PVA, yielding a series of PVAs with indole functionalities in the side chain. In general, the obtained nonionic indole-based PVAs exhibited significant antimicrobial activity against 9 different human pathogens (especially for 6 G(-) bacteria) according to disk diffusion assay. Moreover, it was found that the *N*-substitution of indole unit by linear or cyclic ether groups could promote the

antimicrobial effect, while the *N*-substitution by linear or cyclic alkyl groups exerted negligible or even negative impact.

Despite the wide studies and rapid developments of cationic AMPs, nonionic AMPs have been rarely studied. The antimicrobial mechanism and structure-activity relationship of nonionic polymers remain unclear. In the future, it would be interesting to study the mechanisms of nonionic AMPs, including the specific interactions involved that play roles in the bactericidal effect; it would also be very helpful to study more about the structure-activity relationships of nonionic AMPs, including molecular weights, hydrophilic-hydrophobic balance, polymer architectures, and so on. To apply these materials in the biomedical field, it is also important for us to gain more knowledge of the toxic effects of these polymers. Specifically, vitro cytotoxicity assay with more different mammalian cell lines as well as vivo studies.

With more time, I would like to start with the study of interactions between isatin or indole-based nonionic polymers and bacterial membranes by mimicking a system containing polymer molecules and molecular models of bacterial membranes. In order to do this, it might be helpful to learn from some similar studies with antimicrobial peptides. For example, Isabel et al. have evaluated the effect of peptides from *Galleria mellonella* on the model lipid bilayers (that mimic the composition of the *Leishmania* membrane) consisting of dipalmitoylphos-phatidylcholine, dipalmitoyl-phosphatidylethanolamine, dimyristoylphosphati-dylserine, and dimyristoylphos-phatidylglycer. The results showed that these peptides primarily have electrostatic interactions with negatively charged phospholipids, which was expected for cationic AMPs.[186]

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It's so hard to believe that I am about to finish my PhD study. When I recall my first day that I went to school, it was almost 25 years ago. I remember that I cried so desperately after came back from school because I was not able to write Arabic number 2. I kept practicing on the wall with a chalk and refused to have dinner no matter how my parents comforted me. The day I departed to come to Sweden, my mom said: there must be even tougher days ahead you when you decide to study aboard, but I know you will make it just like you worked so hard on learning to write number 2. Now it's about the time to say goodbye to the 4 years' journey full of challenges, joy and happiness, there are so many people that I would like to express my gratitude to.

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