Biomaterials Science

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Journal:	*Biomaterials Science*
Manuscript ID	BM-REV-08-2015-000307.R1
Article Type:	Review Article
Date Submitted by the Author:	08-Sep-2015
Complete List of Authors:	Zhu, Xiaoying; IMRE A*STAR, Loh, Xian Jun; Institute of Materials Research and Engineering (IMRE),

Layer-by-Layer assemblies for antibacterial applications

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Abstract

The adhesion and proliferation of bacteria on various artificial surfaces affects the function of these specific interfaces. To overcome the problems caused by bacteria growth on these surfaces, various antibacterial coatings were developed. In this review, we summarized most of the antibacterial surfaces prepared by the Layer by Layer (LbL) assembly approach and classified these LbL films based on their antibacterial mechanisms. In the first group, the bactericidal LbL assemblies incorporated with various biocides including heavy metals, antibiotics, cationic molecules, antimicrobial peptides and enzymes are able to kill surrounding or contacted bacteria. In the second group, we focused on physical aspects of film surfaces. Bacterial adhesion resistant LbL films were fabricated to adjust the substrate surface properties such as surface free energy (or wettability), roughness, and surface charge which may affect the adhesion of bacteria. Furthermore, to enhance the antibacterial efficiency, multifunctional LbL assemblies combining both bactericidal and adhesion resistant functionalities were discussed. The advantages and limitations of these antibacterial LbL assemblies were summarized and subsequently the future development directions were proposed.

1. Introduction

Bacterium is one of the oldest life forms on our planet, and is extremely adaptable to various conditions, and found virtually everywhere conceivable on Earth.[1] Bacteria will attach to various surfaces and subsequently multiply to form dense aggregations or biofilms with thicknesses ranging from a few micrometers to half a meter.[2] The biofilms found in medical implants, textiles, contact lenses, aquatic flow systems and petroleum pipelines would affect the function of these specific interfaces.[3]

To eliminate the effects caused by bacteria in different fields, there have been great efforts by researchers to develop antibacterial surfaces. The design concept of antibacterial surfaces was to eliminate or prevent the initial adhesion of bacteria, and subsequently the formation of biofilm. In general, the antibacterial surfaces should be able to kill (bactericidal) or prevent the adhesion of bacteria (anti-adhesive). Some antibacterial surfaces may possess both functions.

The Layer-by-Layer (LbL) assembly approach is a versatile coating technology, which was first introduced by Decher et al. in 1992.[4] In general, LbL assembly is carried out by alternately depositing oppositely charged polyelectrolytes onto a charged substrate surface.[5] The substrate surface must either naturally carry charges such as metals, silicones, and glasses or be introduced with charges by treatments such as high energy irradiation,[6] strong oxidation[7] and silanation.[8] The charged substrate surface was firstly dipped into the polyelectrolyte solution with opposite charge. Subsequently, the surface was rinsed by deionised water to remove the loosely adsorbed polyelectrolyte molecules from the substrate surface; the net charge of the substrate's surface was reversed because of the deposition and overcompensation of the polyelectrolyte with opposite charge on top. Analogous procedure with the other polyelectrolyte solution was subsequently carried out, bringing the surface charge back to the starting point. After one cycle, a double polyelectrolyte layer (bilayer) was built up on top of the

substrate. Desired structures and thicknesses of the LbL films can be achieved by adjusting the deposition cycles.

More importantly, the functions of the LbL films were determined by the deposited components. Thus, the selection and synthesis of these charged functional components are the main considerations of the LbL assembly approach. When the driving force of LbL assembly is electrostatic interaction, almost any type of charged species such as organic molecules,[9] metal ions,[10] nanoparticles,[11] biological macromolecules (DNA,[12] proteins[13]) and viruses,[14] are able to build up or be incorporated into LbL films. Furthermore, electrostatic force is not the only the driving force for LbL assembly. Some other interactions such as hydrogen bonding,[15] covalent bonding,[16] biological recognition,[17] etc. can also be used to assemble LbL films to meet different requirements.

Due to the wide variety of fabrication components, substrates and assembly methods, the LbL assembly technology provides a large amount of choices to fabricate antibacterial coatings. A lot of efforts have been made to develop antibacterial surfaces using LbL assembly approaches. However, reviews of the published works regarding antibacterial LbL films are either absent or out of date. Thus, it is crucial to summarize the existing works and then guide the development directions of the antibacterial LbL assembly in future. In this review, we intend to provide a full survey of the LbL films assembled on flat substrates as antibacterial coatings. As shown in Figure 1, the antibacterial LbL films were clarified into three groups according to their functional mechanisms including bactericidal, non-adhesive and multifunctional. The advantages and limitations of the LbL films in different groups will be summarized. Finally, we will propose a few directions for the future development of antibacterial LbL assembly.

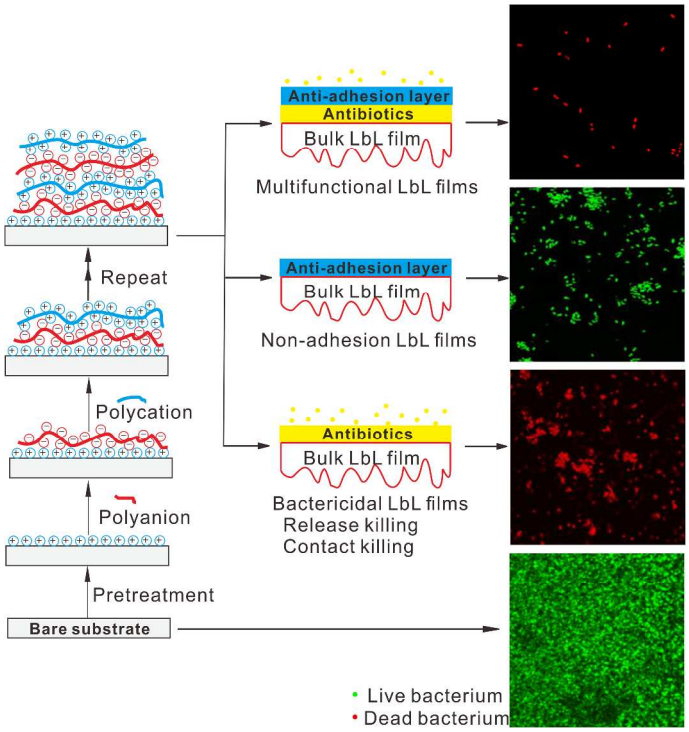

Figure 1. Scheme of LbL assembly and the main antibacterial strategies of coatings.

2. Bactericidal LbL systems

Bacteria can be killed by various bactericidal components based on different mechanisms. Heavy metals such as silver and copper have been widely used as bactericidal agents since antiquity. It is well known that metal species participate in spatially localized or discrete reactions that disrupt enzyme activities, disable membrane function or damage DNA.[18] Although heavy metals showed promising bactericidal effect, their potential toxicity for mammal cells limits their current use. Antibiotics only target bacteria, causing bacterial cell death by inhibiting DNA, RNA, cell wall or protein synthesis.[19] However, the effect of an antibiotic decreases with time because bacteria will develop resistance to the specific antibiotic. Besides antibiotics, cationic molecules and supramolecules also displayed bactericidal properties, which interact with phospholipid components in the cytoplasmic membrane resulting in bacterial cell membrane distortion and protoplast lysis under osmotic stress.[20]

LbL assembly is a versatile method which is able to incorporate all the bactericidal components mentioned above into the multilayer systems. Due to the different interaction strengths between the bactericidal components and the LbL assemblies, the bactericidal components can be either released into the surrounding environment to kill bacteria or firmly stay on the substrate surface to kill bacteria upon contacting. Based on the stability of the incorporated bactericidal agents, we will review release based, contact killing based and mixed killing (release and contact killing) LbL antibacterial systems, respectively.

2.1 Release based bactericidal LbL systems

2.1.1 Bactericidal LbL systems releasing heavy metals

Silver is one of the most widely used bactericidal compounds. Different forms of silver such as ions, metallic nanoparticles and silver halides nanoparticles were incorporated into LbL films and subsequently released to kill bacteria.

Silver ions were deposited on polyacrylonitrile (PAN) nanofibrous mats because of coordination with -CN groups and introduced net positive charge. Subsequently, negatively charged ovalbumin (OVA) was deposited. After a few cycles, the silver/OVA LbL films were assembled on PAN, which showed excellent antibacterial activity against *E. coli* and *S. aureus*.[21] Liposome aggregates loaded with silver ions were embedded into poly(L-lysine)/hyaluronic acid (PLL/HA) multilayer films, which would release silver ions to kill bacteria (*E. coli*) upon temperature increase over the transition temperature of vesicles (34 ºC).[22]

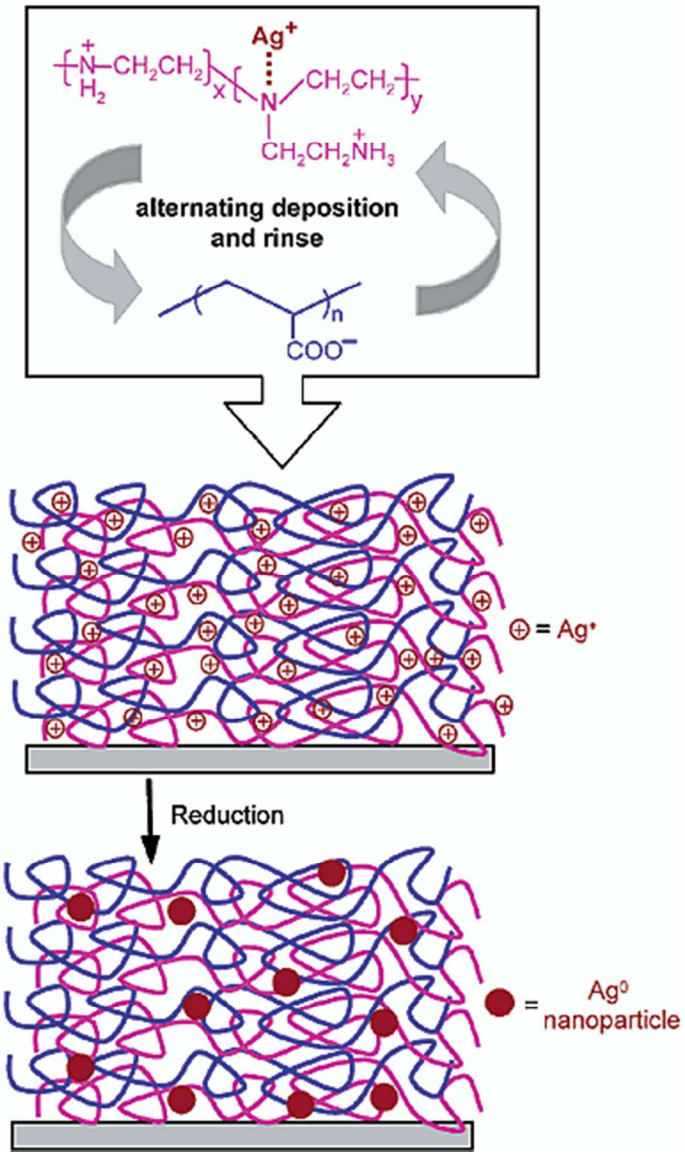

Figure 2. Scheme of LbL adsorption of silver-PEI complex and PAA and reduction of silver ions to silver nanoparticles in the LbL assembly. Reproduced with permission from Ref.[23] Copyright 2002, American Chemical Society.

Silver nanoparticles were synthesized in situ in some LbL systems and released to kill bacteria. For example, as shown in Figure 2, silver – polyethyleneimine (PEI) complexes were prepared and subsequently assembled with PAA to form LbL films, which were further reduced to silver nanoparticles for antimicrobial purpose. The LbL films containing silver nanoparticles showed

promising antibacterial effect against *E. coli*.[23] Silver ions were adsorbed on the cellulose substance (commercial filter paper) which was LbL assembled with titania and chitosan multilayers. The immobilized silver ions were subsequently in situ reduced by UV irradiation to form silver nanoparticles to kill both both *E. coli* and *S. aureus*.[24]

Dopamine (particularly the catechol group) was widely used to modify polyelectrolytes for LbL assembly because of its two functions: first, enhance the adhesion of the LbL film to the substrate; second, reduce the loaded silver ions to silver nanoparticles in the LbL films. Silver ions were immobilized into LbL films fabricated by catechol-modified PEI and catechol-modified hyaluronic acid with enhanced adhesion to the substrate because of the high catechol content which also contributed to the reduction of introduced silver ions in the LbL films to metallic silver nanoparticles providing antibacterial properties.[25] Similarly, to enhance the adhesion of LbL coating to stainless steel, silver nitrate was mixed with a synthetic cationic polymer [poly(3,4-dihydroxy-L-phenylalanine)-co-Poly(2-(methacryloxy)ethyl]trimethylammonium chloride)] to form an aqueous suspension with stable Ag^0 and AgCl nanoparticles. The aqueous P(DOPA)-co-P($DMAEMA^+$)/AgCl/Ag^0 suspension was subsequently LbL assembled with PSS on stainless steel surface. The modified stainless steel showed a high bactericidal activity against *E. coli* bacteria because of the released silver ions from the coating.[26, 27] Dopamine-modified alginate and chitosan were LbL assembled on the surface of titanium alloy to load silver ions and form silver nanoparticles which inhibited the growth of both *E. coli* and *S. aureus*.[28] Thiols/silver nanoparticles/polydopamine were immobilized onto the inner walls of PET bottles *via* LbL assembly technique. Antibacterial activity of the silver nanoparticles effectively prevented the contamination of bacteria to the stored water at room temperature.[29]

Silver ions were loaded into hydrogen-bonded multilayers (PAA/polyacrylamide) and reduced *in situ* into silver nanoparticles as biocidal agent. Silver nanoparticles were leached from the assembled

multilayer thin films and effectively killed both Gram-positive strain (*S. epidermidis*) and Gram-negative strain (*E. coli*) bacteria.[30] Silver ions also were loaded into a synthetic nanogels consisting chitosan and subsequently *in situ* reduced to silver nanoparticles. The nanogels embedded with silver nanoparticles and PSS were LbL assembled on polyether sulfone (PES) membrane surfaces, as shown in Figure 3. The modified PES membrane exhibited long-term antibacterial activities against both *E. coli* and *S. aureus* because of the released silver ions from the LbL coating.[31]

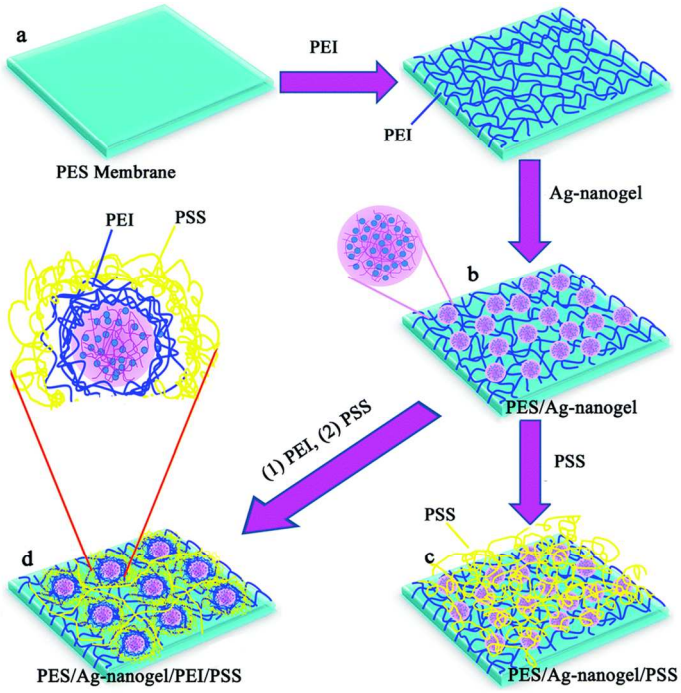

Figure 3. The fabrication processes for the 3D nanogel deposited membranes by surface engineered LBL assembly. (a) Pristine PES membrane, (b) PES/Ag-nanogel, (c) PES/Ag nanogel/PSS, and (d) PES/Ag nanogel/PEI/PSS. Reproduced with permission from Ref.[31] Copyright 2014, Royal Society of Chemistry.

Beside in situ reduction, silver nanoparticles were also directly incorporated into LbL thin films fabricated by PAH and PAA. The silver nanoparticle loaded LbL assemblies indicated bactericidal effect against *S. epidermidis* and supported the growth of the mammalian cells.[32] PAH and PAA polyelectrolyte multilayers were incorporated with silver nanoparticles and subsequently transferred

onto soft materials simulating human skin. The released silver ions form the LbL assemblies caused a significant reduction in colony forming units of *S. epidermidis* and *P. aeruginosa* within 12 h.[33] Silver bromide nanoparticles were also incorporated into covalently cross-linked LbL polymeric assemblies from synthetic methoxysilane polymers as antibacterial agent.[34]

Copper is another commonly used heavy metal in antibacterial applications. Copper ions were coordinated with branched PEI to form a complex which was then assembled with PAA to prepare LbL thin films which could be disassembled under control and release copper ions to kill bacteria.[35]

2.1.2 Bactericidal LbL systems releasing antibiotics

While heavy metals are highly effective bactericidal agents, they were suspected to be toxic to mammalian cells.[36] As an alternative, antibiotics were also embedded in the LbL assemblies and released to kill bacteria. Gentamicin, a positively charged antibiotic and a hydrolytically degradable poly(β-amino ester) were alternatively deposited with the biocompatible polyanionic hyaluronic acid to form LbL thin films. The gentamicin released from the LbL film under control provided promising bactericidal activity against *S. aureus*.[9] Vancomycin and metronidazole were also incorporated in LbL assembled films and released to kill *S. aureus*[37] and *Porphyromonas gingivalis,*[38] respectively. In some multifunctional biomedical coatings, both gentamicin and another therapeutic were incorporated into a complex LbL system and sequentially released under control. The released gentamicin took response for the antibacterial effect of the LbL film.[39, 40] In order to more precisely control the release of antibiotic, gentamicin was LbL assembled with negatively charged prussian blue (PB) nanoparticles. The LbL film was subsequently applied a small anodic electric potential to oxidize the PB nanoparticles from negatively charged to neutral and release gentamicin to kill *S. aureus.*[41]

In some studies, antibiotic releasing from LbL film was determined by surrounding environments. One of several cationic antibiotics (tobromycin, gentamicin, and polymyxin B) was assembled with

tannic acid to construct "self-defense" antibacterial coatings. The loaded antibiotics would be released if only the surrounding environment was acidified by pathogenic bacteria such as *S. epidermidis* or *E. coli*.[42] Similarly, tobramycin was loaded in a cross-linked PAA/chitosan bilayer and released based on pH decrease by biofilm formation.[43] In general, charged antibiotics or charged antibiotic carriers can be easily incorporated into LbL assemblies because of electrostatic interaction and subsequently released to kill bacteria. However, bacteria may develop resistances to antibiotics with time.

Bacteria will also be killed by cationic molecules because of negatively charged bacterial membrane lysis.[44] More important, the potential for bacterial resistance development against cationic molecules is low.[20] For example, positively charged moieties such as quaternary ammonium was incorporated into LbL assembles and released to kill bacteria.[45] But most of the cationic molecules will be firmly immobilized on substrate surface and kill bacteria upon contacting, which will be discussed in Section 2.2.1.

2.1.3 Bactericidal LbL systems releasing peptides

Antimicrobial peptides are part of the innate immune response found among all classes of life. These peptides are highly efficient and act as broad spectrum antibiotics carrying net positive charges. In some studies, antimicrobial peptides were incorporated into LbL systems and released to kill bacteria. However, they were also firmly immobilized in LbL films and working through contact killing, which will be discussed in Section 2.2.2.

Ponericin G1, a positively charged antimicrobial peptide, was assembled with other polyions to form hydrolytically degradable polyelectrolyte multilayer film (as shown in Figure 4) which was able to release the loaded antibiotic and kill *S. aureus*.[46]

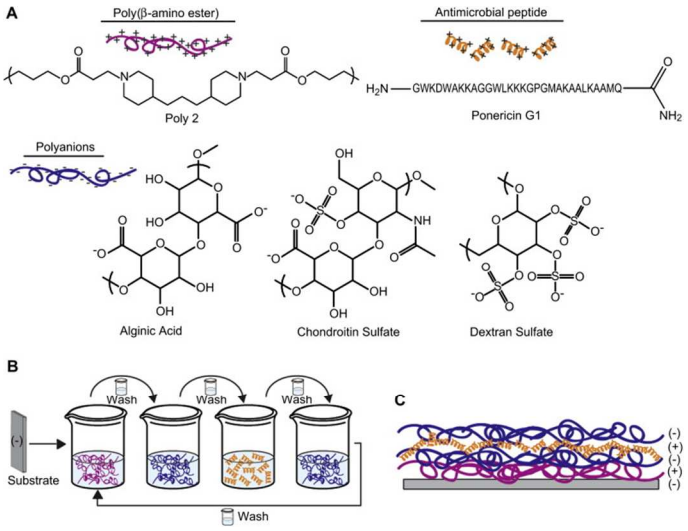

Figure 4. Film components, LbL film assembly, and film architecture. (A) The structure of poly 2, alginic acid, chondroitin sulfate, dextran sulfate, and ponericin G1. (B) The LbL assembly process. (C) A tetralayer films. Reproduced with permission from Ref.[46] Copyright 2009 Elsevier Ltd.

Cateslytin, an endogenous host-defensive antimicrobial peptide, was covalently coupled to hyaluronic acid which was subsequently LbL assembled with chitosan on a planar surface. The hyaluronidase secreted by pathogens would trigger the release of cateslytin from the LbL film to kill Gram-positive *S. aureus*.[47] To provide better antibacterial effect, an antibiotic and a cationic antibacterial peptide were incorporated into LbL assemblies together and released at the same time. Poly(methacrylic acid) (PMAA) multilayer hydrogel coatings were loaded with gentamicin and an cationic antibacterial peptide L5 which were subsequently released in response to pH variations, resulting good antibacterial activity toward *S. epidermidis*.[48]

2.1.4 Bactericidal LbL systems releasing nitric oxide

Nitric oxide is another potent antimicrobial agent produced by the immune system in response to bacterial infection. The bactericidal effect of nitric oxide is contributed by its strong oxidation capability

targeting cell membrane, DNA and proteins of bacteria.[49] Carboxyl-ebselen was covalently bonded to PEI which was subsequently assembled with alginate to form LbL films. Nitric oxide was released from the LbL films with the presence of a reducing agent. The LbL films indicated promising bactericidal effects against a broad spectrum of bacteria.[50]

In general, the bactericidal LbL assemblies based on releasing are highly efficient because the large amounts of biocidal components released from the LbL films will kill surrounding bacteria effectively. However, the release based antibacterial LbL coatings lose their activities as the biocidal substances are released into the surrounding environments. Once the biocidal agent released from the coating is lower than the minimum inhibitory concentration (MIC), the coating will lose its antibacterial function. In addition, the released biocidal substances may bring negative influences to human beings and environments. For example, heavy metals like silver and copper are toxic to mammal cells. Micro pollutants which cannot be removed by traditional water treatment approaches consist of antibiotics leached from various applications. Thus, some research works focused on slow release or non-release antibacterial coatings and tried to avoid these problems.

2.2 Contact killing bactericidal LbL systems

Biocidal components in the non-release based bactericidal LbL assemblies are firmly immobilized and will only kill bacteria upon contacting. In general, most of the biocidal components used in non-release based LbL systems are similar to those used in release based LbL films. The main difference is the stability of the bocidal components immobilized in the LbL assemblies.

2.2.1 LbL systems with positively charged top surfaces

It is well known that cationic molecules in solution are bactericidal. The LbL films which are able to release cationic molecules to kill bacteria have been discussed in the previous section. On the other hand, once the cationic molecules were immobilized on the surfaces of LbL assemblies to provide net positive

charge, bacteria will be killed upon contacting these cationic surfaces. Since the cationic functional groups are stable on the surfaces, the antibacterial effect of contact killing surfaces often lasts longer than release based coatings. Since polycations are one of the building blocks for the formation of LbL films; the deposition amount of polycation in each layer, which can be manipulated by the deposition conditions, is related to the net charge of the film surface. Rubner's group manipulated the assembly and post assembly conditions (e.g., pH) of polyelectrolyte multilayers (PSS/PAH) to expose mobile cationic charge to surface. The PSS/PAH multilayers displaying accessible cationic charge effectively reduced the viability of two strains of Gram positive *S. epidermidis*, and two strains of Gram negative *E. coli*.[44] In another case, multilayers formed with antibacterial cationic polyvinylamine and PAA reduced the growth of *E. coli* because of the positive charge on the surface.[51]

In our group, we recently reported a molecular fabrication approach to precisely control surface ζ potentials of LbL assemblies. The highest isoelectric point of the LbL film prepared from PAA, poly(diallyldimethylammonium chloride) and PEI reached pH 10 showing strong positive charge at physiological pH (7.4). As shown in Figure 5, the cationic LbL film surface attracted more bacteria because of the negative charges of bacterial cell wall but the bacteria viability was not evaluated.[52]

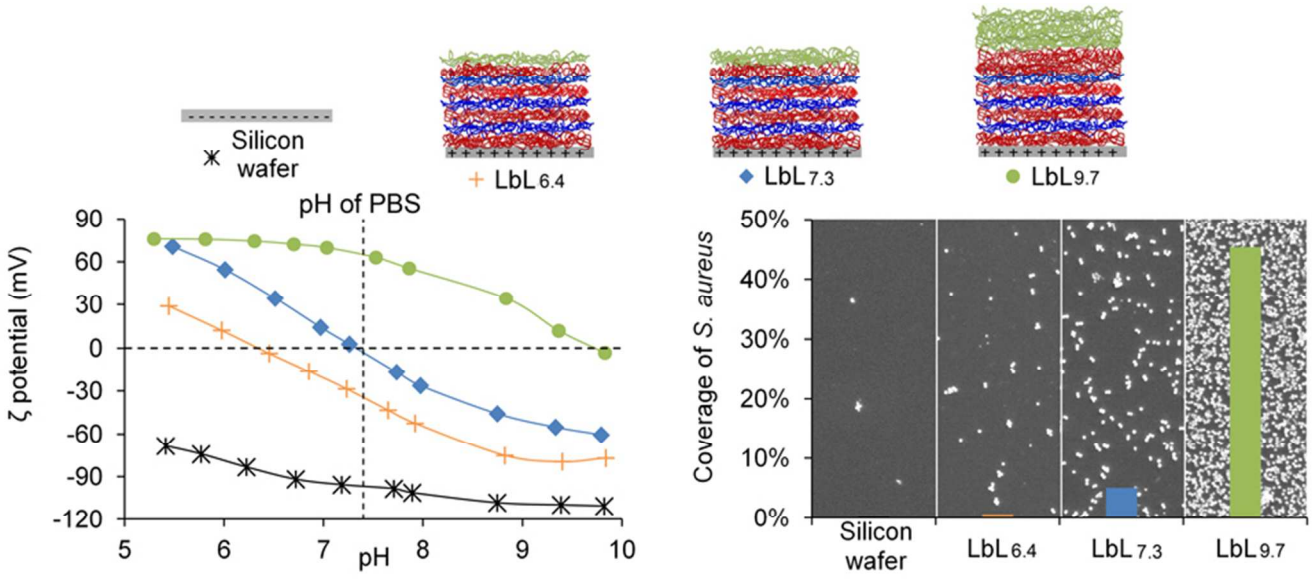

Figure 5. LbL assemblies with different isoelectric points (6.4, 7.3 and 9.7) and the bacteria coverage. Reproduced with permission from Ref. [52] Copyright 2015, American Chemical Society.

Chitosan is a biocompatible cationic polysaccharide that has been reported to be bactericidal because of its positive charge.[44] Chitosan and its derivatives were widely used in LbL assemblies for contact killing purpose. Chitosan and anionic lentinan sulphate were alternatively deposited onto the surfaces of polyurethane *via* LbL assembly, which showed antibacterial activity against *P. aeruginosa*.[53] On cotton samples, LbL films were assembled by chitosan and alginates to suppress the growth of *S. aureus* and *K. pneumonia*.[54] Similarly, chitosan and pentasodium tripolyphosphate (TPP) were assembled on cotton fabrics using LbL assembly method to improve the inhibition against *E. coli* and *S. aureus*.[55] To further enhance the efficiency of contact killing, *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC) with positively charged quaternary ammonium were synthesized and LbL assembled with PAA on plasma-treated poly(ethylene terephthalate) (PET) substrate. The presence of HTACC in the multilayer films improved the antibacterial activity of the modified PET against *E. coli* and *S. aureus*.[56] Sometimes, chitosan serves as a platform that was incorporated with various antibacterial enhancements. Subsequently, the modified chitosan was LbL assembled with a polyanion to achieve better contact killing. For example, negatively charged electrospun cellulose acetate fibrous mats were modified with multilayers of the positively charged intercalated composites including chitosan-organic rectorite (OREC),[57] lysozyme-chitosan- OREC,[58] and quaternized carboxymethyl-chitosan-OREC[59] and the negatively charged sodium alginate *via* LbL assembly approach. The LbL structured fibrous mats enhanced the inhibition against *E. coli* and *S. aureus*. The cationic protein, lysozyme, is another commonly used contact killing agent incorporated into LbL assemblies. LbL films prepared from lysozyme and negatively charged substances such as pectin,[60] poly(L-glutamic acid),[61] and gold

nanoparticles[62] showed promising inhibitory effect against both Gram negative and Gram positive bacteria. Lysozyme with positive charge and DNA with negative charge were conjugated with carbon nanotube and subsequently assembled to form robust LbL films with high Young's Modulus. The LbL film terminated with lysozyme exhibited strong antimicrobial activity, while the film ended with DNA layer on top did not show any effect against *M. lysodeikticus*.[63]

2.2.2 LbL systems with antibacterial peptides on top surfaces

Beside bactericidal enzymes functioned by positive charge, another type of antibacterial enzyme based on quorum sensing disrupting was also introduced into LbL films. For example, the negatively charged quorum sensing disrupting enzyme acylase was deposited with the positively charged PEI on silicone urinary catheters using LbL assembly. The acylase-coated catheters efficiently quenched the QS in the *P. aeruginosa* ATCC 10145, resulting reduced biofilm formation.[64]

Antibacterial peptides that participate in the innate immune defense against microorganisms have not only been incorporated in release based antibacterial LbL films but also stably immobilized in LbL assemblies for contact killing purpose. A cationic antimicrobial peptide, defensin, was incorporated into LbL multilayers built up by positively charged poly(l-lysine) and negatively charged poly(L-glutamic acid). The results indicated the LbL films ended with positively charged poly(l-lysine) showed promising antibacterial effects. The authors attributed the antibacterial effect to the close interaction of bacteria and defensin caused by the attraction of positive charge on the LbL film to bacteria.[65] However, the authors did not consider the bactericidal contribution of positive charge itself.

Nisin, a low molecular weight antibacterial peptide (3500 g/mol), was covalently bound in a LbL system built up by covalent coupling of a synthetic oxidized poly (3,4-dihydroxyphenylalanine) [Pox(mDOPA)] and PAH on stainless steel, as shown in Figure 6. The LbL coating with stably

immobilized nisin showed durable and promising antibacterial effect against *B. subtilis* upon contact killing.[66]

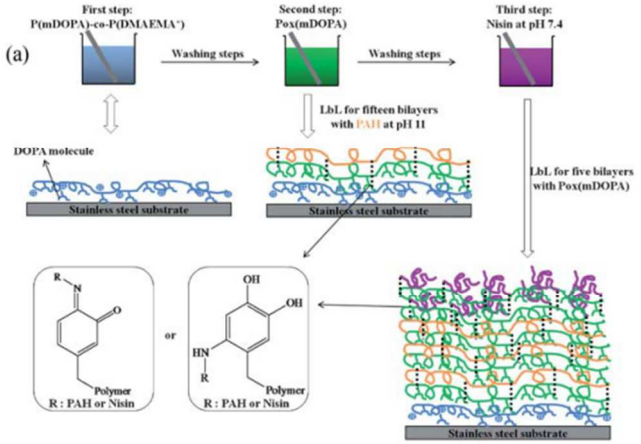

Figure 6. LbL process for imparting long-lasting antibacterial properties to stainless steel. Reproduced with permission from Ref.[66] Copyright 2011, The Royal Society of Chemistry.

2.2.3 LbL systems with antibiotics on top surfaces

Some functional biocides based on contact killing were also incorporated into LbL films. For example, N-halamines contain oxidative halogen which can be directly transferred to the bacterial cell membrane and kill bacteria upon contacting in a very short time without releasing free halogen into the surrounding environment. Two *N*-halamine copolymer precursors, poly(2,2,6,6-tetramethyl-4-piperidyl methacrylate-*co*-acrylic acid potassium salt) and poly(2,2,6,6-tetramethyl-4-piperidyl methacrylate-*co*-trimethyl-2-methacryloxyethylammonium chloride) were synthesized and coated onto cotton fabric *via* LbL assembly approach. After treatment of dilute household bleach, the LbL assembly inactivated both *S. aureus* and *E. coli* within 15 min of contact time.[67] Single walled carbon nanotubes (SWNT) have shown strong antimicrobial activity. SWNT was dispersed into aqueous solution and then incorporated into LbL films assembled with poly(L-lysine) (PLL) and poly(L-glutamic acid) (PGA). The SWNT

containing LbL films effectively inactivated both *E. coli* and *S. epidermidis* on their surfaces after 24 h incubation.[68, 69]

2.2.4 LbL systems with photocatalyses on top surfaces

Photocatalytic substances will generate superoxide and hydroxyl radicals which are able to kill contacted bacteria under the sunlight illumination. Negatively charged photocatalytic 2-anthraquinone sulfonate was LbL assembled with PEI onto the hydrophilic poly(vinyl alcohol-*co*-ethylene) nanofiber composite membranes. The attached bacteria (*E. coli*) on the LbL film surface were killed by the generated radicals under the irradiation of solar light.[70]

The LbL assemblies with the contact killing property have advantages over coatings releasing bactericidal components over time. Firstly, since much lower amount of biocidal agents were released in to the surrounding environment, the contact killing coating will bring less negative influences to the environment than the release based coating. Secondly, the non-release based bactericidal LbL film usually showed a longer life span than the release based coating. However, the bactericidal efficiency of contact killing coatings is a concern for researchers and users.

2.3 Release and contact killing bactericidal LbL systems

Various bactericidal components that can be incorporated into LbL systems have been discussed. At the same time, the stability of the immobilized biocidal agent can be adjusted by the LbL system. Thus, a single bactericidal component sometime can not only kill attached bacteria but also be released to kill surrounding bacteria in one LbL assembly. In some studies, one LbL system contains two bactericidal agents with different incorporation stabilities taking response to both release killing and contact killing.[71]

2.3.1 Release and contact killing LbL systems with single bactericidal components

Starch-stabilized silver nanoparticles were introduced into a biomimetic nanostructured composite prepared from Na(+)-montmorillonite clay nanosheets and poly(diallylmethylammonium chloride) by LbL assembly. The prepared LbL composite showed good growth inhibition of *E. coli*, which was contributed by both the silver eluted from the LbL composite and firmly immobilized silver on the LbL composite surface.[72] Similarly, silver ions were loaded in a LbL assembly fabricated by covalently assembling PEI with terephthalaldehyde and subsequently reduced to silver nanoparticles. The LbL film embedded with silver nanoparticles showed both good release killing and contact killing effects against *E. coli* and *S. aureus*.[73] Copper nanoparticles were synthesized by *in situ* chemical reduction of copper ions chelated by a cotton substrate which was imparted with high negative surface charge in a chemical preconditioning step. The specific LbL electrostatic self-assembly process induced efficient killing of a multidrug resistant bacterial wound pathogen (*A. baumannii*) to the cotton substrate. It may be associated with contact killing, and due to enhanced release of metal ions.[74] A natural antibacterial peptide, gramicidin A, was coordinated with a non-denaturing anionic amphiphilic polysaccharide, carboxymethylpullulan. Subsequently, the negatively charged complex was LbL assembled with cationic poly(L-lysine) to form thin films which were able to inhibit the growth of a Gram positive bacterium, *E. faecalis*. The biocidal activity of the LbL films was contributed by a double mechanism: contact killing on the film surface and release of the peptide into the solution surrounding the film.[75]

2.3.2 Release and contact killing LbL systems with multiple bactericidal components

Two bactericidal components including quaternary ammonium salts and silver nanoparticles were incorporated into LbL films assembled from PAH and PAA, as shown in Figure 7. Due to the weak interaction between silver and the LbL films, silver ions were released showing very high initial

bactericidal efficiency. The stably immobilized quaternary ammonium salts contributed to the retained significant antibacterial activity achieved by contact killing after the depletion of embedded silver.[76]

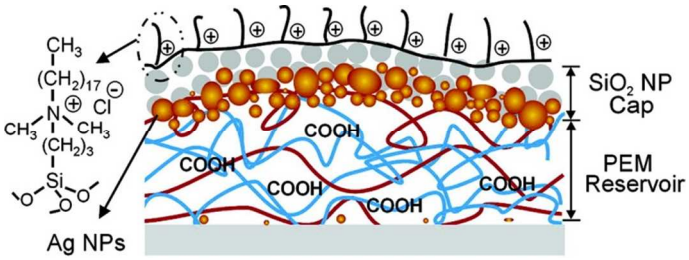

Figure 7. LbL assembly with a reservoir for the loading and release of silver nanoparticles and a nanoparticle surface cap with immobilized quaternary ammonium salt. Reproduced with permission from Ref.[76] Copyright 2006, American Chemical Society.

Chitosan and alginate were alternatively assembled on titanium substrates to form LbL films which were subsequently loaded with minocycline. The incorporated minocycline was sustainably released to kill planktonic and adherent bacteria to inhibit biofilm formation. After minocycline release ceases, positively charged chitosan itself continued its role in the antibacterial performance.[77]

The antibacterial LbL assemblies incorporated with bactericidal agents and working by killing bacteria are still the majority, not only in the research field but also in the market. The advantages of these bactericidal LbL films are obvious such as high efficiency, low cost. By playing with the stability of the immobilized biocidal components, the life span of the bactericidal LbL films can be expanded. However, the main concern of the bactericidal LbL assemblies is the toxicity of the biocidal components. Even in the contact killing mode, the biocidal components will interact with the surrounding environment and bring negative impacts. This has boosted the current wave of interest in research to develop alternative environmental friendly antibacterial approaches.

3. Bacterial adhesion resistant LbL systems

Low or non-adhesion coatings without biocidal agent immobilization are gaining population as an environmentally benign antibacterial solution. In general, the bacterial adhesion is affected by the surface properties of the substrate. The approaches to prepare low-adhesion surfaces are mainly based on tuning the surface properties such as surface free energy (or wettability), roughness, and surface charge of the substrate. LbL assembly is a powerful surface modification approach which was widely used to adjust these surface properties.

3.1 Non-adhesive LbL systems with controlled surface free energy

Hydrophilic surfaces will form a hydration layer to resist the adhesion of bacteria.[78] Thus, hydrophilicity enhancement was achieved by LbL assembly approach on various substrate surfaces. LbL films were assembled from PAH/PAA on polysulfone (PSU) microfiltration membranes to improve the hydrophilicity of the membranes. The modified membranes with LbL films showed good bacterial anti-adhesive properties, which could be attributed to the highly swollen and hydrated multilayers that inhibit the direct contact or close approach of bacteria to the underlying PSU membranes.[79] Poly(ethylene glycol) (PEG) was widely used to improve the substrate surface hydrophiliciy, which was also incorporated into LbL systems for antibacterial purpose. A synthetic Poly(L-glutamic acid) (PGA) grafted PEG was assembled with poly(L-lysine) (PLL) on glass slides. The adhesion of *E. coli* was pronouncedly reduced on the LbL films ending by PLL/PGA-g-PEG bilayers compared to bare substrate.[80] Highly hydrophobic coatings with fouling release property provide another option to get rid of bacterial adhesion. LbL assembly technology was used to prepare highly hydrophobic coatings using fluorinated polyelectrolytes such as nafion[81] or dendritic gold clusters.[82] However, these reported highly hydrophobic LbL assemblies have not been used in antibacterial applications. Purely hydrophilic or purely hydrophobic surfaces are able to reduce bacterial adhesion to some extent; but the amphiphilic

surfaces possessing both hydrophilic and highly hydrophobic domains may provide a better solution to bacterial adhesion. A synthetic polyanion, grafted with amphiphilic perfluoroalkyl polyethylene glycol (fPEG) side chains, was LbL assembled with PEI on silicon wafer. The amphiphilic LbL films effectively prevented the adhesion of the marine bacterium *Pseudomonas* (NCIMB 2021), as shown in Figure 8.[83]

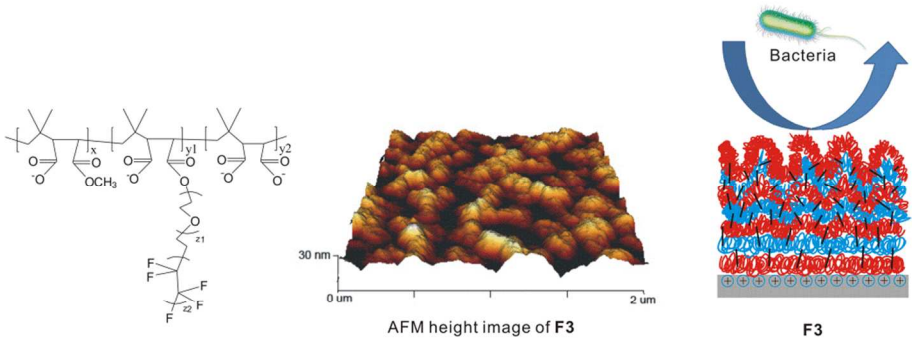

Figure 8. The amphiphilic LbL assembly for bacterial adhesion resistant. Reproduced with permission from Ref.[83] Copyright 2013, American Chemical Society.

3.2 Non-adhesive LbL systems with controlled surface charge and stiffness

Since bacterial cell wall is negatively charged. Surface charge of the substrate may affect the adhesion of bacteria. In our group, three polyelectrolytes including PAA, poly(diallyldimethylammonium chloride) (PDADMAC), and PEI were assembled in different conditions to adjust the surface charge of LbL films. In the bacterial adhesion tests employing *Pseudomonas*, *E. coli*, and *S. aureus*, very limited bacteria were observed on the negatively charged surfaces which were able to repel bacteria bearing negative charge.[52] The stiffness of LbL assemblies will affect the adhesion of bacteria as well. PAH and PAA were used to assemble LbL films at different pH values to achieve

various stiffness of the films. The authors observed that the extent of bacterial attachment was increased with the LbL film stiffness increase.[84] Non-adhesion LbL films are environment friendly antibacterial solutions without biocidal components. However, the antibacterial efficiency and life span of these adhesion resistant LbL films in real applications are still main concerns of researchers and users.

4. **Multifunctional antibacterial LbL systems**

Either bactericidal or non-adhesion LbL films have their own disadvantages or limitations. To optimize the available antibacterial solutions, multifunctional antibacterial coatings possessing both bactericidal and anti-adhesion functions are gaining interest from researchers. LbL assembly is a powerful surface modification tool which can combine these two functions in one multilayer system. In general, the main concern of non-adhesion LbL films is their antibacterial efficiency. To enhance the antibacterial ability of non-adhesion LbL films, the bactericidal function was introduced into the non-adhesion LbL assemblies to fabricate multifunctional antibacterial LbL systems.

4.1 Non-adhesion & release based antibacterial LbL systems

In some of the multifunctional antibacterial LbL assemblies, besides the bacterial adhesion resistant function, the bactericidal function was provided by leaching the immobilized biocidal components into the surrounding environment. LbL assembly was constructed by alternatively depositing of chitosan and heparin on the substrate surface. Silver ions were subsequently loaded into the fabricated LbL films and reduced to silver nanoparticles *in situ*. Even the incorporated heparin contributed to the bacterial adhesion resistance, the immobilized silver nanoparticles greatly enhanced the antibacterial performance of the LbL film because of the additional bactericidal effect.[85]

Heparin, as an anti-adhesive agent, and polyhexamethylene guanidine hydrochloride, as an antibiotic, were LbL assembled on a polyacrylonitrile membrane. The modified membrane with stable LbL film indicated simultaneous easy-cleaning or non-adhesive property and promising bactericidal activity under

physiological condition.[86] PEI was mixed with silver ions to form PEI-Ag^+ complex which was LbL assembled with PAA on a Teflon surface. The deposited LbL film was coated with a thin layer of hydrophobic silane (triethoxy-tridecafluoro-n-octylsilane) *via* vapour deposition during cross-linking and subsequently peeled from the Teflon surface as a free-standing LbL film, as shown in Figure 9. The upper surface of the prepared asymmetric free-standing LbL film was superhydrophobic, which took response of anti-adhesion and self-cleaning properties. In addition, silver ions were released from the LbL film to kill bacteria.[10]

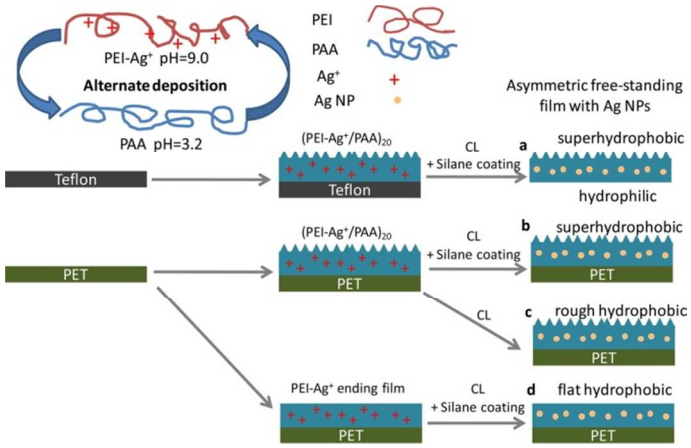

Figure 9. Schematic illustration of the fabrication of free-standing (a) and supported (b, c, d) films. Reproduced with permission from Ref.[10] Copyright 2012, American Chemical Society.

Since the bacterial adhesion resistance of the multifunctional LbL film, much less bacteria will have the chance to attach to the LbL film surface. Thus, lower amount of antibiotic will be enough to kill the attached bacteria. However, the release based LbL film will release huge amount of biocidal components to kill bacteria around the surface. It seems more stably immobilized bactericidal agents with contact killing function (lower or non release of antibiotics) are more suitable for multifunctional LbL films for safe use.

4.2 Non-adhesion & contact killing antibacterial LbL systems

Another group of multifunctional antibacterial LbL assemblies combined both adhesion resistant function and bactericidal function based on contact killing. Hyaluronic acid (HA) and poly(amidoamine) dendrimer (PAMAM) were assembled on a poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (PHB) substrate by LbL assembly method. PHB is a biocompatible polyester which has been extensively studied for biomedical based applications.[87-92] As shown in Figure 10, the improved hydrophilicity of substrate surface by the LbL assemblies took response to the bacterial anti-adhesion activity to *E. coli*. In addition, the positively charged PAMAM outer layer exhibited bactericidal activity against *E. coli*.[93]

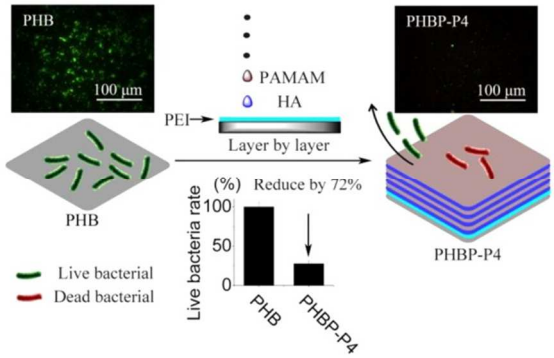

Figure 10. Both the bacterial anti-adhesion activity and the bactericidal activity of the PHB surface coated with the HA/PAMAM multilayers. Reproduced with permission from Ref.[93] Copyright 2015, American Chemical Society.

Heparin as an anti-adhesive agent with negative charge and chitosan as a bactericidal agent with positive charge were alternatively deposited onto aminolyzed poly(ethylene terephthalate) (PET) films to construct anti-adhesive and bactericidal LbL films which were able to reduce the initial adhesion and subsequently the number of viable bacteria (*E. coli*) effectively.[94] Sometimes, heparin and chitosan will be chemically modified or replaced by similar polyelectrolyte to construct LbL assemblies. But the anti-adhesive and killing by positive charge functions of the fabricated LbL films will be the same or enhanced. For example, heparin and N-trimethyl chitosan were alternately deposited on modified

polystyrene films to prepare anti-adhesive and bactericidal multilayer films.[95] A negatively charged water-soluble heparin-mimicking polymer and a positively charged quaternized chitosan (N-trimethyl chitosan) were synthesized and assembled through LbL technology on a poly(ether sulfone) membrane surface. The modified membrane indicated significant improved adhesion resistance against both *E. coli* and *S. aureus,* which was contributed by both the improved hydrophilicity of the membrane surface and the positive charge of the assembled quaternized chitosan.[96] A top-down degradable multifunctional LbL system consisting two types of multilayers was fabricated for antibacterial applications. As shown in Figure 11, the top 10 bilayers were LbL assembled from polyvinylpyrrolidone (PVP) and PAA with cross-linking, which could be degraded and provide fouling release function resulting almost no adhesion of bacteria in 24 h; the bottom 10 bilayers were constructed by LbL deposition of heparin and chitosan, which possessed the contact killing function.[97]

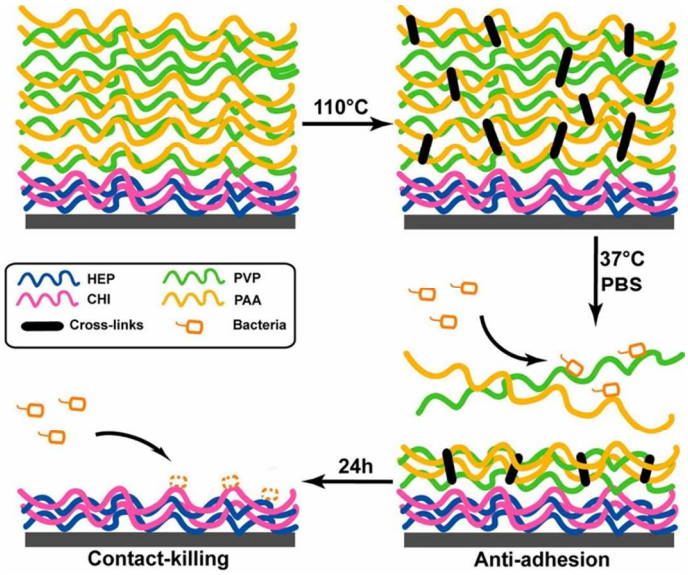

Figure 11. Schematic Representation of Construction, Cross-Linking, Degradation, and Antibacterial Properties of the (HEP/CHI)10–(PVP/PAA)10 Multilayer Film. Reproduced with permission from Ref.[97] Copyright 2013, American Chemical Society.

Adhesion resistant *azido*-functionalized poly(ethylene glycol) methyl ether methacrylate-based polymer chains and bactericidal *alkynyl*-functionalized 2-(methacryloyloxy)ethyl trimethyl ammonium chloride-based polymer chains were LbL assembled *via* alkyne–azide 1,3-dipolar cycloaddition or "click" reaction. The covalently bonded LbL film indicated both resistance to bacterial adhesion and bactericidal effect to marine Gram-negative bacterium, *Pseudomonas. sp.* NCIMB 2021.[98] Even though, the multifunctional LbL films showed promising antibacterial performances, these systems are relatively complicated and difficult to scale up for real applications.

5. Conclusion and future prospects

In this review, we summarized most of the LbL approaches for antibacterial surface preparation. Although the LbL assembly was first reported in 1990s, the applications of LbL method in antibacterial coatings were not many (less than 100 papers). The antibacterial mechanisms of these LbL films were the same as the other antibacterial coatings fabricated by the other methods. In general, the antibacterial coatings should be able to kill bacteria or prevent bacterial adhesion or both. Bactericidal coatings were the most straightforward solution to bacterial adhesion and growth. Thus, a lot of efforts have been made to develop bactericidal LbL films for antibacterial applications. LbL assembly is a versatile method which is able to incorporate various biocides including heavy metals, antibiotics, cationic molecules, antimicrobial peptides and enzymes into the LbL systems. Subsequently, these bactericidal components will be either released to kill surrounding bacteria or stability immobilized on the LbL film surface to kill bacteria upon contacting. Silver was the most commonly used heavy metal for antibacterial LbL assemblies. However, leaching heavy metals into the surrounding environments would bring negative influences. Antibiotics were another group of functional components for antibacterial purpose in LbL systems. The emergence of antibiotic resistant bacteria is the challenge of using antibiotics. Approaches to prepare bactericidal LbL coatings based on cationic molecules gained significant attention. However,

the positive charges of these LbL assemblies may be compensated by some other substances rather than bacteria and lose function. Incorporation of antibacterial peptides and enzymes are emerging approaches which are very interesting. The method of maintaining the activity of these fragile biological molecules in the LbL film preparation and subsequent applications is the main concern.

Surface properties such as surface free energy (or wettability), roughness, and surface charge may affect the adhesion of bacteria. Bacterial adhesion resistant LbL coatings were targeting at tuning the surface properties of the substrate to reduce the bacterial adhesion. However, most of the published papers only addressed one of these factors, for example, hydrophilicity improvement. People may have a deeper understanding of bacterial adhesion behaviour, if more than one surface property can be controlled at the same time in a LbL system. Other issues of bacterial adhesion resistant LbL coatings are the antibacterial efficiency and durability. To enhance the antibacterial efficiency, multifunctional LbL assemblies combining both bactericidal and adhesion resistant functions were developed and showed improved performance. However, the concerns of these multifunctional LbL assemblies are the doubled or tripled complexity of the LbL system and the difficulty to scale up for real applications. In short, we have summarized the strategies or routes to design antibacterial LbL assemblies based on the reported approaches. Most of the studies mentioned in this review indicate promising antibacterial effects. The advantages of different approaches are also clear. However, several of them are coming with limits and defects such as toxicity, durability and efficiency. Future work in fabrication of antibacterial LbL assemblies may focus on overcoming the shortcomings of the existing approaches, for example, controlling multiple surface properties of LbL film to enhance antibacterial efficiency; improving stability of LbL film to expend life span; reducing negative impacts to application environments; modifying the existing fabrication method to scale up manufacturing process.

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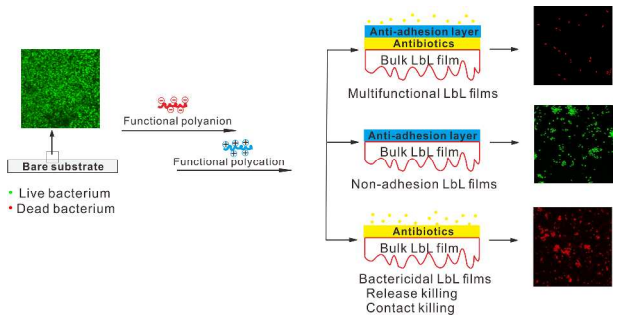
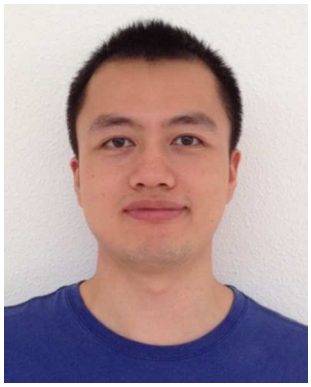
TOC Graphic



This review describes the latest update on the research happenings on the area of layer-by-layer assemblies for antibacterial applications

Biography



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