Chapter 1
Silver Nanoparticle Antimicrobials and Related Materials

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1.1 Antimicrobial Mechanism of Silver and Silver Ion

Silver has a long history of usage in medicine in many cultures. The ancient Greeks, Romans, Egyptians, and many others used silver vessels for water and other liquids storage. It is recorded that Aristotle suggested that Alexander the Great (335 BC) should transport water in silver containers on many campaigns [1]. Pioneers who explored across the Wild West also used silver and copper coins to preserve drinking water from spoilage by bacteria and algae. They also put silver dollars into their milk to retain its freshness [2].

The first documented usage of silver in medical applications can be dated back to around AD 750. However, it was not until late 1800s that scientists proved that silver is an effective germicide. In 1869, Ravelin reported that silver, even in ultra-low concentration, shows antimicrobial activity [3]. Shortly after Ravelin's discovery, von Naegeli found silver ions (9.2×10^{-9} M) from metallic silver are able to kill *Spirogyra* commonly found in water [4]. In 1881, Carl Crede pioneered the treatment of eye infection of neonates by using 2% silver nitrate solution, a technique which has been widely used ever since [2]. In the early 1900s, silver foil was used as a dressing material for burn wound treatment. Silver in different forms, mostly colloidal silver, was extensively used by physicians, and this type of treatment was considered as "high-tech" at that time.

After World War II, novel and powerful antibiotics quickly replaced silver in bacterial infection treatment and the interest in silver as an antimicrobial agent declined sharply. Unfortunately, after decades of use, many bacteria have developed resistance towards one or more antibiotics. This has led to a resurgence in the use of silver in antibacterial applications. For example, in the 1970s, Margraf used a 0.5% silver solution to kill invasive bacteria in burn wounds. Silver sulphadiazine cream

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was also developed to replace diluted silver salt solutions, and is now used extensively in burn wards in America. This technique is considered as a gold standard of topical burn treatment [5]. Other commercial silver antibacterial products can also be found in the food industry, water supply systems, medical care products, clothing, electronic appliances, etc. For example, Agion® is a surface modification technique that turns regular surfaces into antibacterial surfaces by releasing silver ions. Similar techniques have been adopted in various products made by Honeywell®, Dell®, Motorola®, Logitech®, PPG®, the North Face®, Adidas®, etc. Microban® 3G silver is another popular antibacterial solution for polymeric materials that combines antibacterial property of silver with other antibacterial agents.

Although silver has been used as a biocide for hundreds of years, the antimicrobial mechanism of silver has not been fully elucidated. A variety of mechanisms may be involved in the antimicrobial activity of silver against a broad spectrum of organisms. Some of the commonly accepted mechanisms include silver–amino acid interaction, silver–DNA interaction, generation of reactive oxidative species, and direct cell membrane damage.

1.1.1 Interaction with Thiol Groups on Amino Acids

The cytotoxicity of heavy metal ions like Ag^+, Hg^{2+}, Cu^{2+} and Pb^{2+}, in many cases, is due to the interaction between metal ions and thiol groups in proteins or enzymes in microorganisms which leads to the inhibition of their crucial biological functions [6–11]. The thiol group in *L*-cysteine residues can undergo either reversible or irreversible oxidation by chemical agents, including heavy metal ions, causing death of microorganisms (Fig. 1.1) [1].

1.1.1.1 Inhibition of Respiratory Process of Bacteria Cells

Cellular respiration is the process by which cells convert biochemical energy from nutrient into adenosine triphosphate (ATP) and release waste products.

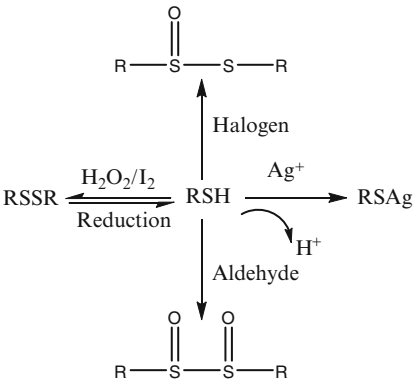

Fig. 1.1 Biocides interaction with thiol group (Reprinted from [1]. With permission from Elsevier)

In eukaryotes, oxidative phosphorylation occurs in the mitochondrial cristae. It involves the respiratory chain which establishes a proton gradient (chemiosmotic potential) across the inner membrane by oxidizing the nicotinamide adenine dinucleotide (NADH) produced from the Krebs cycle. ATP is synthesized by the ATP synthase enzyme, and the chemiosmotic gradient is used to drive the phosphorylation of ADP to form ATP. To complete the respiration process, the electrons are finally transferred to exogenous oxygen and, with the addition of two protons, water is formed. Bard [12] used microelectrode and other techniques to explore the inhibition of respiration of *Escherichia coli* treated by silver ions. The mechanism has been proposed that silver is able to cause cell membranes to be permeable to protons and therefore the proton gradient is lost. To compensate for the loss of the proton gradient, the acceleration of the respiratory process is triggered, attempting to expel more protons to restore the proton gradient. This uncontrolled process generates superoxide or hydroxyl radicals which are extremely harmful to the cells.

1.1.1.2 Enhanced Phosphate Efflux

Rosenberg [13, 14] studied the ability of silver ions to inhibit the intake of phosphate ions and enhanced efflux of phosphate of *Escherichia coli*. In the experiment, *Escherichia coli* was first incubated with either 5 mM succinate or 1 mM glucose to deplete the phosphate ions in cells. Then, the phosphate-depleted cells were washed to remove any extra sugar energy sources. Silver was added 2 min prior to radioactive ^{32}P addition. The ^{32}P uptakes by *Escherichia coli* cells (with silver and without silver) were compared with control (no cells) by radioactivity monitoring. The inhibition of uptake of phosphate by silver ions was observed. Alleviation of such inhibition could be done by adding KBr to decrease effective silver ion concentrations. In another experiment, where $AgNO_3$ was also added to suspension of cells which have already taken up ^{32}P, the enhanced efflux of ^{32}P was observed. Again, addition of KBr can either slow down the efflux of phosphate for the *Escherichia coli* AN710 strain or resume phosphate uptake for the *Escherichia coli* AN1080 strain. Besides the enhanced efflux of phosphate ions, the efflux of accumulated proline, glutamine, mannitol, and succinate was also discovered when *Escherichia coli* suspension was treated with silver ions. The efflux can be stopped and the uptake can be resumed by adding thiol compounds such as dithiothreitol or mercaptoethanol, presumably due to the detoxification reactions between silver ions and thiol compounds.

1.1.2 Interaction with DNA Molecules

DNA is responsible for the reproduction process. Any damage done to it will cause either mutation or death of the organism. The preferential interaction of silver ions with the bases found in DNA rather than the phosphate group is well documented [15–19].

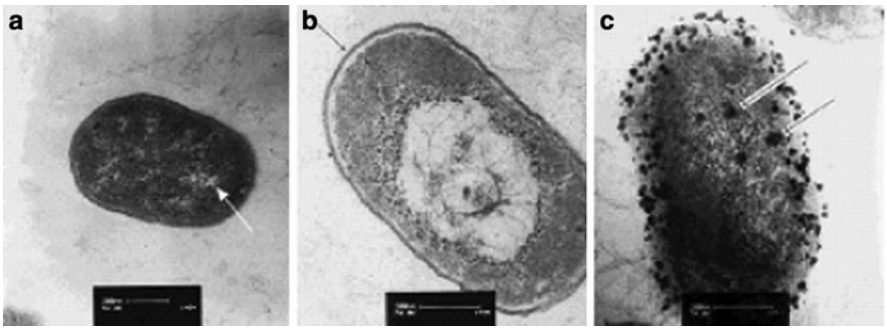

Fig. 1.2 Morphology comparison of untreated and treated *Escherichia coli*: (**a**) Untreated. (**b**) self-defense mechanism tries to protect DNA. (**c**) final stage of a dying *Escherichia coli* cell (Reprinted from [20]. With permission from Wiley)

Feng [20] probed the antibacterial mechanism of Ag^+ by treating *Escherichia coli* and *B. aureus* bacteria with $AgNO_3$ solution. After silver treatment, bacteria samples are subjected to TEM observation. It was found that silver-treated *Escherichia coli* cells showed a large electron-light region (DNA molecules) in the center whereas electron-light areas were evenly distributed in untreated cells (Fig. 1.2a, b). Another phenomenon was also noticed: a large amount of electron-dense granules appeared around the electron-light region but not in untreated bacteria. The researcher proposed that these electron-dense granules were generated by a cell self-defense mechanism as they tried to protect the DNA (electron-light region). When this self-defense mechanism failed to function due to the large amount of silver ions present, the cell was full of electron-dense granules with no electron-light regions at all and the cell wall also completely disappeared, both of which were captured by TEM images (Fig. 1.2c).

The assumption that silver ions may cause increased mutation frequency of microorganism has also been supported in a recent publication [21]. Three different silver materials were used in this research: silver nanopowder, silver-copper nanopowder, and colloidal silver. Both polymerase chain reaction (PCR) and *in vivo Escherichia coli* experiments were carried out and compared. A particular rpsL mutation assay was used in *in vitro* PCR. The rpsL gene encodes a small ribosomal protein S12, which caused resistance to the antibiotic streptomycin after mutation. When the PCR process was finished, the silver-treated genes were used to transform into host bacterial cells MF101. If mutation of rpsL gene occurs, the transformed bacteria will be streptomycin resistant. Therefore, the mutation frequency is calculated from the number of colonies surviving on both ampicillin and streptomycin agar plates divided by the total colonies surviving on ampicillin agar. A substantial increase of mutation frequency was observed: 1.63%, 1.54%, 2.15%, and 0.68% for silver nanopowder, silver–copper nanopowder, colloidal silver, and untreated samples, respectively. These results suggested that silver material treatment can cause significant gene mutation and that this mutation may lead to death of the bacterial cells. A similar observation was made with *in vivo Escherichia coli*

although the mutation frequency was lowered by four orders of magnitude than those in vitro perhaps due to the lower uptake of silver.

1.1.3 Catalytic Generation of Reactive Oxygen Species (ROS)

ROS are normal byproducts of the respiration process. ROS at low level can be controlled by the so-called antioxidant defense such as equilibrium between glutathione and glutathione disulfide. However, excess ROS production may generate free radicals which are extremely deleterious to cells because of the damage to lipids or DNA [22]. It is believed that metals can catalytically promote the generation of ROS with dissolved oxygen in solution [23]. If true, the silver nanomaterials can kill bacteria directly by the catalytic generation of ROS without the presence of silver ions. Hwang [24] found that the antimicrobial activity of carbon-supported silver was observable only in the presence of oxygen. In this case, it was apparent that generation of ROS by silver nanoparticles instead of biocidal silver ions was the predominate mechanism. Kim [25] verified free radicals generated from silver nanoparticles by spin resonance methods. In the same experiments, the biocidal action of both silver nanoparticles and silver ions was stopped with the presence of reductants, which led to the conclusion that the biocidal mechanism against *Staphylococcus aureus* and *Escherichia coli* is the generation of free radicals.

Besides silver nanomaterials, silver ions can also generate excess ROS. Park [26] used superoxide-responsive protein containing bacteria to detect the ability of silver ions to generate ROS. Usually, bacteria have their own ROS sensor systems. For example, *Escherichia coli* has two proteins as ROS sensing systems: SoxR and OxyR. When exposed to superoxide or nitric oxide radicals, the SoxR protein induces a single gene soxS. By monitoring soxS induction in the reporter strains, the ROS level in the solution can be determined. The increased soxS response found after ionic silver treatment suggested that ROS was generated. Another experiment conducted to validate this mechanism was comparing the antibacterial activity of silver ions under both aerobic and anaerobic conditions, since the generation of ROS requires the presence of dissolved oxygen. It was found that silver ions exhibited higher biocidal activity under aerobic conditions (3.3 log bacterial reductions under aerobic condition versus 1.9 log bacterial reductions under anaerobic condition). This result shows that generation of ROS is a plausible biocidal pathway and also suggests that the antibacterial mechanisms of silver ions may be a combination of ROS generation and silver–thiol interactions.

1.1.4 Direct Damage to Cell Membrane

Direct damage to cell membrane caused by silver nanomaterials has been reported recently. Morones and colleagues [27] explored the biocidal mechanism of silver nanoparticles against four kinds of bacteria: *Vibrio cholerae*, *Pseudomonas*

aeruginosa, *Scrub typhus*, and *Escherichia coli*. The accumulation of silver particles on the bacterial membrane surface was observed by using the High Angle Annular Dark Field (HAADF) microscopy technique. The researchers were also able to get electronic microscopy images of the bacterial cross-section. EDS mapping of the silver element showed that silver nanoparticles are able to penetrate the membranes and get enriched inside the cells. The ability of silver particles to get inside the cells is explained by the observation of membrane defects caused by silver nanoparticles. The researchers also compared silver nitrate-treated bacteria with silver nanoparticles-treated bacteria. Silver nitrate-treated bacteria showed both DNA agglomerated regions (electron-light regions) and electron-dense regions, which match with a previous publication [20]. However, this was not seen for silver nanoparticles-treated bacteria, which suggests that silver in different forms may operate differently.

The detailed mechanism of cell membrane penetration and accumulation of silver nanoparticles is not fully understood. One hypothesis is that the accumulation is caused by electrostatic attraction between negatively charged bacterial surfaces and positively charged silver nanoparticles [28]. However, this does not explain why negatively charged silver nanoparticles are able to adhere to and enter the bacteria cells. Another possibility is that the process is initiated by silver–thiol group interaction on the cell surface, and is supported by a recent publication [29].

Another explanation for cell membrane damage is proposed for Gram-negative bacteria such as *Escherichia coli*. Gram-negative bacteria possess an external membrane outside the peptidoglycan layer, which is lacking in Gram-positive bacteria. It was previously found that chelating agent EDTA can cause the depletion of Ca^{2+} and Mg^{2+} ions, resulting in pits and holes in the outer membranes due to the release of LPS (lipopolysaccharide) molecules [30]. Sondi [31] also observed a similar phenomenon by treating *Escherichia coli* bacteria with silver nanoparticles. The SEM images clearly showed that there were many holes in the cell membranes of silver-treated *Escherichia coli* bacteria (Fig. 1.3). The researchers postulated that silver may also generate pits and holes in the cell membrane by causing the release of LPS from cell membranes.

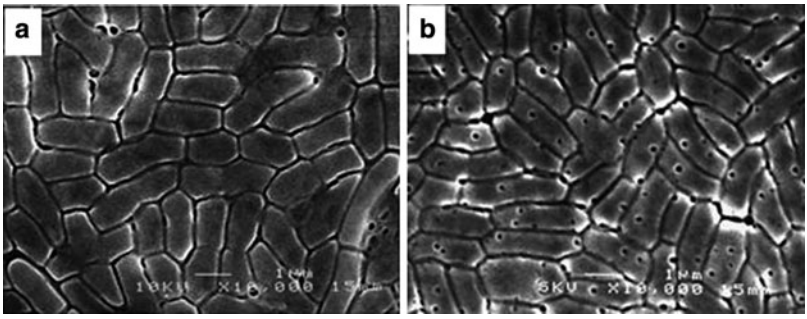

Fig. 1.3 SEM images of (**a**) native *Escherichia coli* bacteria and (**b**) *Escherichia coli* treated with 50 um/mL silver nanoparticles (Reprinted from [31]. With permission from Elsevier)

1.2 Evaluation Methods and Criteria for Silver Antibacterial Materials

Standard evaluation methods and criteria for silver antimicrobial materials are important because quantitative data are necessary for materials screening in terms of antibacterial activities and inter-laboratory comparisons. Most evaluation methods used in publications are adopted from long-standing *in vitro* microbiology protocols for antibiotics.

1.2.1 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) test is an *in vitro* test which determines the lowest concentration of antibiotics needed to inhibit the growth of (but not necessarily kill) bacteria. MIC test is extensively used to evaluate the antibiotic susceptibility of targeted bacteria in microbiology and clinic practice. In theory, it can also be used for antibacterial silver materials and it works practically very well on these samples. Usually, both MIC and minimum biocidal concentration (MBC) are reported for small molecule antibiotics and MBC is usually much higher in value than MIC. However, for silver antibacterial materials, only MIC data are typically reported.

1.2.1.1 Test Protocols

Typically, there are two standard techniques to determine MIC values: agar dilution and broth dilution. In agar dilution test, the antibiotic is mixed with agar powder and made into nutrient agar plates with different concentration of the antibiotic. Then, liquid broth with a predetermined concentration of bacteria is inoculated onto the agar plates. After incubation, bacterial colonies are visually counted. An absence of bacteria colonies means all bacteria are inhibited or killed at that concentration of antibiotics.

The broth dilution protocol uses liquid media with known amounts of bacteria, and different amounts of antibiotic are added to it. After incubation, the turbidity of the media is observed as an indication of bacterial growth. Based on the final media volume (bacterial solution + antibiotic solution), broth dilution can be categorized as microdilution (total volume $\leq$ 500 μL) and macrodilution (total volume $\geq$ 2 mL) [32]. The final results given by the MIC test can be in mg/mL, mg/L, or μg/mL depending on the antibacterial efficiency and bacteria tested. For silver antimicrobial materials, it is difficult to obtain uniformly blended agar plates. Therefore, broth dilution is the commonly used evaluation protocol.

1.2.1.2 Critical Parameters

In order to get reproducible and reliable MIC values for both intra-laboratory and inter-laboratory comparisons, additional parameters must be noted during the test: (a) *Bacteria*: Bacteria tested in the experiments must be isolated in their pure form. Their genus and species level must be identified. Most organisms can be obtained from hospital laboratories, the American Type Culture Collection (ATCC) or other national collections [32, 33]. (b) *Inoculum*: The typical bacterial concentration for MIC is 5×10^5 cfu/mL for final solution (broth dilution) or 1×10^4 cfu per agar plate (agar dilution). It has been documented [34] that more concentrated solutions of drug-resistant strains give increased MIC values due to the generation of enzymes capable of decomposing antibiotics. However, it is still not clear whether increased inoculum will affect the MIC value of silver antibacterial materials. (c) *Determination of bacteria concentration*: The concentration of bacterial media is usually expressed in cfu/mL (colony forming unit/mL). The turbidity of the bacterial solution is proportional to its optical absorption at 600 nm. A calibration curve is usually set up for a specific bioassay reader (or microplate reader) by plotting cfu numbers versus absorption values. The detailed procedure can be found in previous literature [32]. Once the calibration curve is set, the bacterial concentration can be readily calculated from its absorption. If the absorption value is greater than 1, it causes loss of linearity. Therefore, necessary dilution may be needed before obtaining the absorption value.

1.2.1.3 Limitations

The MIC test does not indicate whether tested materials are biocidal or biostatic. Even if the broth or agar plate shows no bacterial growth, there may be viable bacteria present because the tested materials may only inhibit the growth of bacteria (biostatic effect). Bacteria may continue to proliferate when the antibacterial material is removed. To differentiate whether the sample is biostatic or biocidal, aliquots of broth can be withdrawn and spread on a new agar plate with no antibacterial material present. If colonies are observed after incubation, the material is only biostatic at this concentration. Otherwise, the material is biocidal.

1.2.2 Zone of Inhibition (ZOI)

The ZOI test is another *in vitro* test, also widely known as the Kirby-Bauer disk diffusion test [35]. When bacteria are inoculated onto nutritious agar plates, they tend to form continuous spots called colonies. If there is an antibiotic present at a specific area in the agar, colony formation will be inhibited around that area due to

the leaching of the antibiotic, and the zone of inhibition can be visually observed and measured.

1.2.2.1 Test Protocol

The general procedure is as follows: bacteria are first incubated in a liquid broth for a certain period of time (depending on the bacteria); several drops of bacterial broth are streaked onto an agar plate. Then, sample disks are placed over the agar plate and the whole agar plate is incubated. Finally, the net inhibition diameter can be calculated by subtracting the diameter of the sample disk from the diameter of the total zone of inhibition. Kirby and Bauer did a series of experiments comparing their method with the previously mentioned MIC test, and they found that these two tests are highly correlated [35]. Therefore, the net diameter of inhibition zone can be a benchmark for the activity of the specific antibiotic as long as the same type of agar is used during all the experiments (Fig. 1.4).

1.2.2.2 Modification

The ZOI test, though originally developed for water-soluble antibiotics, provides us with a semi-quantitative option to evaluate water-insoluble antibacterial materials when the MIC test cannot be carried out. The ZOI test is especially applicable to antibacterial coatings and films because these types of materials usually perform poorly in the MIC test due to limited availability of biocidal agents.

Due to the simplicity of this method, the FDA has recommended using this technique to evaluate new antibiotics. One disadvantage of the Kirby-Bauer method is the long incubation period, typically 12–16 h. In 1973, Ross [36] shortened this period to 6.5 h without sacrificing the accuracy by spraying cell stains on the agar plates. The live bacteria show a certain color depending on the dye, and the zone of inhibition remains unstained.

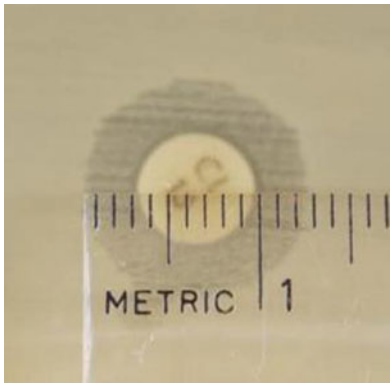

Fig. 1.4 Antibacterial material is placed on a bacteria-inoculated agar plate. A circle of poor bacterial growth surround the material shows the ZOI (Picture credited to Center of Disease Control and Prevention)

1.2.2.3 Limitation

ZOI tests do not necessarily indicate that the bacteria are killed by the antibacterial material. The antibacterial material may just prevent the bacteria growth. Sometimes, clear boundaries are not observed which makes measuring the ZOI diameter difficult. This method is also less quantitative than the MIC protocol.

1.3 Inorganic Silver Composites and Silver Nanomaterials

The increasing demands of better living conditions have prompted scientists to develop and commercialize effective yet affordable antimicrobial materials. Silver is introduced into inorganic substrates in the form of either pure silver nanoparticles or as sparingly soluble silver salts. Silver may either be present on the surface of the material or dispersed in the matrix. The preparations of inorganic silver composites are usually straightforward with little wet chemistry involved. Commonly used inorganic substrates or matrices are titania or silica particles, glasses, ceramics, inorganic fibers, and medical alloys.

1.3.1 Glass

Glass is widely used in windows, sliding doors, and containers which are prone to bacterial adhesion. Thus, antimicrobial modification on glasses is highly desirable. Traditional glasses are made by using melt–quench process, which makes it impossible to fabricate silicate glass/silver composites with a high silver loading. Instead, a sol–gel method is used [37–39]. The first step of this method is polycondensation. Silver salts are mixed with TEOS (tetraethyloxysilane) and triethyoxysilsane, such as $NH_2(CH_2)_3Si(OEt)_3$, in absolute ethanol. After mixing, aqueous ammonia is added under vigorous stirring to cause hydrolysis. The homogenous mixture is then heated and the solvent is removed under reduced pressure to obtain xerogels. The next step involves oxidation of the xerogels in heated quartz tubes under air flow to adjust elemental composition. In the final step, the composites are reduced by reheating under hydrogen flow to further decrease carbon and oxygen content. Glasses made by this method usually show a brown color due to the presence of silver colloids. An improved method has been developed for colorless antimicrobial glasses [40]. First, $AgNO_3$, $Al(NO_3)_3$, water, and ethanol are mixed to obtain a solution A. Second, TEOS and equal amount of ethanol are mixed to form a solution B. Solution A is slowly added to solution B under vigorous stirring. The mixture is then poured into a polystyrene mold to dry and gelate for 7 days. Finally, the gel is milled into a fine powder, put into a mold and baked at 900–1,000°C. Instead of having silver particles, this product is infused with silver ions which are colorless.

As a test, this composite was soaked in *Streptococcus mutans* broth and incubated for 12 h. No surviving bacteria were found in the broth, indicating that the silver ions released from the glass had killed 100% of the bacteria.

1.3.2 Coatings and Films

Surface antimicrobial modification of medical alloy implants is especially important since the adherence and fouling by bacteria on implant surfaces may cause serious complications and sometimes even the death of the patient [41]. Surface modification of surgical materials with silver can greatly reduce this risk. The surface deposition of silver can be achieved by anodic oxidation, electroless plating, CVD (Chemical Vapor Deposition), and other physical or chemical coating techniques. Recently, Duszczyk [42] utilized the PEO (Plasma Electrolytic Oxidation) technique to plate Ag/TiO_2 film onto a commonly used surgical Ti-6Al-7Nb alloy. PEO is an anodic oxidation electrochemical process which uses a voltage higher than the dielectric breakdown point of the metal oxide layer. This technique significantly increases the surface roughness and interconnects the pores in the metal oxide layer. Thus, the metal oxide layers more readily absorb other species. The oxidation process is carried out in a calcium acetate and calcium glycerophosphate solution to create a bioactive porous oxide coating containing calcium and phosphate ions on titanium metal. During the oxidation process, silver nanoparticles present in the electrolyte solution are uniformly incorporated into the oxide coating (Fig. 1.5). Such Ti/Ag disks were incubated with broth medium inoculated with methicillin-resistant *Staphylococcus aureus* at 2×10^5 cfu. After 24 h of incubation, 100% of the bacteria were killed.

Alt [43] developed a double layer coating by using both PVD (Physical Vapor Deposition) and CVD (Chemical Vapor Deposition) techniques. Elemental silver was firstly coated by PVD on a stainless steel implant. This silver coating was covered by a layer of silicon carbide by CVD for better biocompatibility and mechanical durability. The coated implant was able to retain antibacterial activity for at least 28 days in animal experiments, and TEM images showed little silver particle loss after implanted in animal for 28 days (Fig. 1.6).

Besides the aforementioned surface modification of metal, ceramics and glasses are also popular substrates for antibacterial coatings and films. Wang [44] developed a convenient method to co-precipitate Ag/TiO_2 film on the surface of ceramic tiles. A dipping solution was firstly made by mixing an aqueous solution of ammonium hexafluorotitanate with silver nitrate and boric acid. Then, ceramic tiles were soaked in the dipping solution for several hours to form a surface coating. Use of a scanning electron microscope (SEM) and energy dispersion spectrum (EDS) confirmed the formation of the Ag/TiO_2 coating covering the ceramic tile surface. This film is able to leach out silver ions and kill over 99% of *Staphylococcus aureus* and *Escherichia coli* after incubation for 24 h. A variation on this technique has also been employed [45]. Titanium alkoxide was first stabilized by

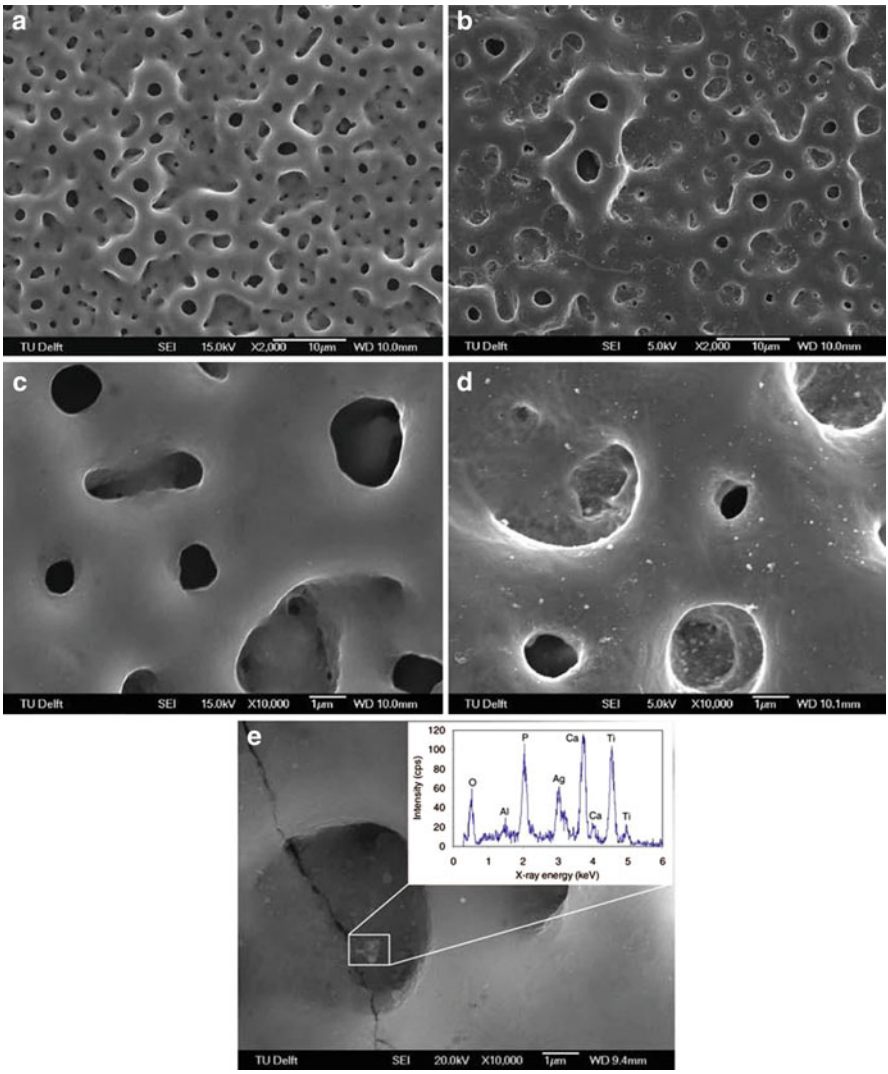

Fig. 1.5 SEM images of titanium oxide (**a**, **c**) and titanium oxide composite coating (**b**, **d**, **e**) produced at 20 A/dm^2 and 5 min oxidation time. EDS analysis indicated presence of Ag particles (Reprinted from [42]. With permission from Elsevier)

pentane-2,4-dione chelation and was then mixed with an $AgNO_3$ aqueous solution to obtain a dipping solution. Different substrates such as glass, aluminum, brass, and stainless steel were soaked in this solution. The coated substrates were finally annealed at 500°C for 1 h before use. Besides ceramics, silver can be coated on float glasses (a type of glass made by floating molten glass on a bed of molten metal) by a more sophisticated technique [46]. Initially, float glasses are pre-coated with silica to prevent diffusion of impurity into the float glasses, followed by a secondary

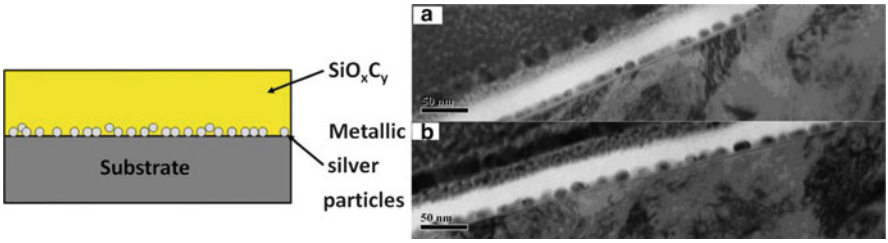

Fig. 1.6 Scheme for double layer coating (*left*) and TEM images of intersection: before implant (**a**) and after 28 days implant (**b**). TEM images show little silver particles loss before and after implant (Reprinted from [43]. With permission from Wiley)

coating of TiO_2 by CVD. Silver film was finally grown in a combustion CVD machine by nebulizing the $AgNO_3$ solution onto the substrate. This method gives the film a photolytic property from TiO_2 to decompose organic contaminants and antibacterial performance from silver. This material is able to kill over 99% of *Staphylococcus aureus*, 69% of *Escherichia coli* and over 99% of *Bacillus subtilis*.

Coating modification of textiles and fibers is another attractive research topic. Fisher [47] developed a silicon-based sol–gel coating solution with both antibacterial and waterproof properties. Triethoxytridecafluorooctyl silane and $AgNO_3$ were mixed to form a sol–gel solution followed by dipping textiles in this solution. Hydrolysis of silane was then initiated. Finally, the textile was dried and tested. It was found that water uptake of the treated textile was considerably reduced due to the highly hydrophobic nature of fluorinated silane. Nearly 100% bacterial inhibition of *Escherichia coli, Candida cariosilignicola*, and *Candida glabrata* were observed even after ten washes.

1.3.3 Pigment Materials

Titania and silica are the two most frequently used pigment materials. Antimicrobial modification of these materials is highly desirable as it may provide a more hygienic environment, especially in hospitals. Another application of such a pigment is in the fabric industry. Conventionally, silver nanoparticles are grafted onto fiber surfaces. However, it is almost impossible to maintain their antibacterial activity after extensive wash cycles. One possible solution is to use a metal oxide, titanium oxide for instance, as a carrier of silver particles so that the silver is less likely to be washed away by detergent. Yuan [48] designed Ag/TiO_2-C composites with a TiO_2 core that gives regular pigment an antimicrobial property. To prepare the Ag/TiO_2-C composite, activated carbon was formed on the surface of TiO_2 by dehydration of sucrose in an autoclave and by soaking TiO_2-C powder in an $AgNO_3$ solution whereupon Ag is precipitated onto the TiO_2-C surface. This composite was tested against *Escherichia coli* and *Staphylococcus aureus* at

10^5 cfu/mL for 24 h. Pure TiO_2 shows moderate antimicrobial activity whereas the Ag/TiO_2-C composite killed 100% of the microorganisms.

Porous silica particles can also be made into antibacterial pigments in a similar fashion. Kwon [49] developed a white pigment based on silica nanoparticles. The porous silica particles were initially synthesized from the hydrolysis reaction of TEOS in the presence of the triblock copolymer Pluronic® as the structure directing agent. The silica particles obtained were a few 100 nm in diameter and morphologically porous to $AgNO_3$ solutions. Then, the silica particles were treated with HCl vapor to generate AgCl. This antibacterial pigment is stable enough to undergo a melt-extruding process in a polypropylene (PP) matrix. In the antibacterial test, the doped PP composite was able to kill 99% *Escherichia coli* after 24 h incubation.

1.3.4 Absorbents/Filter Agents

Zeolites are aluminosilicate minerals with porous surfaces, commonly used as commercial absorbents, water cleaning agents, and filter agents. Adding antibacterial property to zeolites is quite natural and the process is very simple. Sasatsu [50] used commercially available sodium-type zeolites as the host compound. Silver ions were loaded by an ion exchange method that involved soaking Na-zeolite powder in $AgNO_3$ solution for 24 h, then washing with distilled water and drying in air. This zeolite/Ag composite was able to kill *Escherichia coli* in just 5 min. The fast biocidal action is due to the generation of ROS (reactive oxygen species) described earlier.

Another type of porous material is activated carbon. Hu [51] used charcoal obtained from pyrolysis of bamboo as a substrate for silver deposition. To prepare this material, bamboo charcoal was immersed in an $[Ag(NH_3)_2]NO_3$ solution for 1 h and then the silver ion was reduced by slowly adding diluted hydrazine solution under an inert atmosphere. Finally, a filter paper was impregnated with the obtained powder. The powder itself showed low MIC (0.3 μg/mL) towards tested bacteria such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus subtilis*. The filter papers showed clear ZOI for bacteria-inoculated agar plates (Fig. 1.7).

Activated carbon in the form of fiber is also widely used as air filter to remove air pollutants, odor, and airborne microorganisms. An activated carbon fiber filter is also susceptible to bacteria adherence. Bacteria can grow on carbon fibers and thereby become the source of bioaerosols. Incorporation of silver in activated carbon fiber was reported by Hwang [24, 52]. Initially, Pd was introduced as a catalyst onto the activated carbon fiber. Then, an electroless plating technique was employed by immersing the Pd-loaded carbon fiber into a silver salt solution to deposit silver on the carbon fiber (Fig. 1.8). The silver-containing carbon fibers have ZOI of around 11 mm (*Escherichia coli*) and 12–15 mm (*Bacillus subtilis*) compared with none for the control fiber.

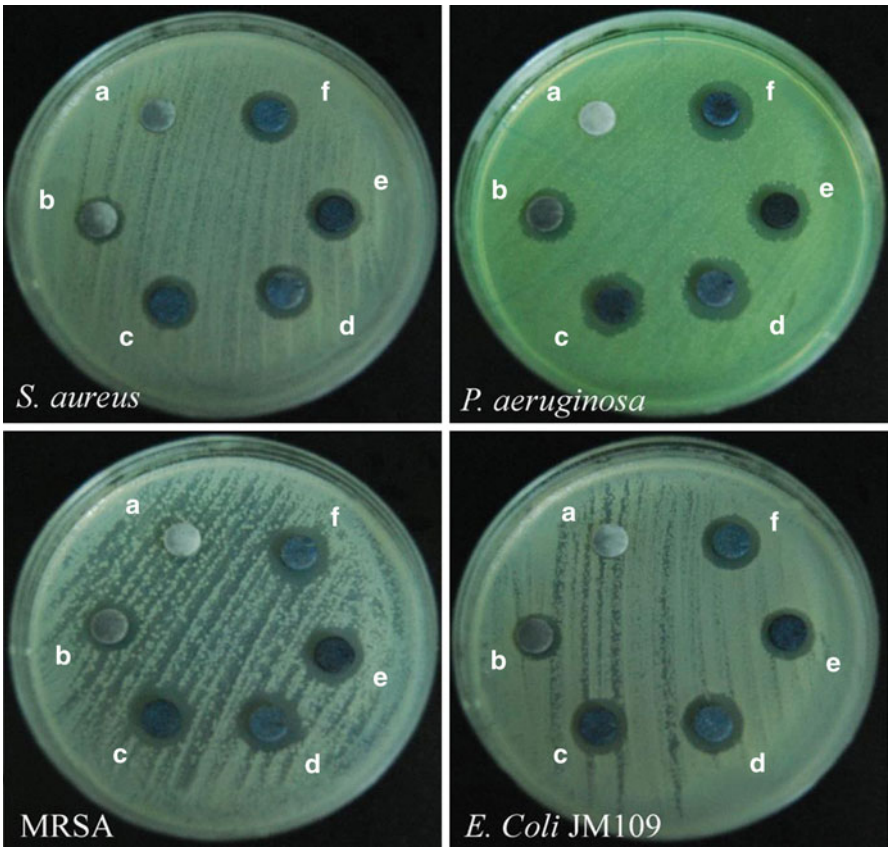

Fig. 1.7 Pictures of filter paper test samples on bacteria inoculated agar plates. Pure bamboo charcoal (**a**) shows no ZOI whereas all bamboo charcoal/Ag samples (**b–f**) show obvious ZOI (Reprinted from [51]. With permission from Elsevier)

1.3.5 Silver Nanomaterials

Contrary to bulk metallic forms or ionic forms, the antimicrobial action of silver in the nano-scale is due to the catalytic generation of reactive oxidative species (ROS) from dissolved oxygen in solution, which can directly attack cell membranes or DNA. The most common synthetic methods for silver nanomaterials are reduction of silver ions by either electrochemical processes or chemical reductants such as hydrazine [53, 54], $NaBH_4$ [55], hyperbranched poly (amidoamine) [56], and formamide [53]. The size of the silver nanomaterial depends on the reductant employed. Strong reductants like $NaBH_4$ usually lead to small silver nanoparticles whereas weak reductants like citrate yield larger nanoparticles with high size dispersion. Factors such as pH and the complexing agent (e.g., ammonia) also contribute to the different morphologies of the obtained silver nanoparticles.

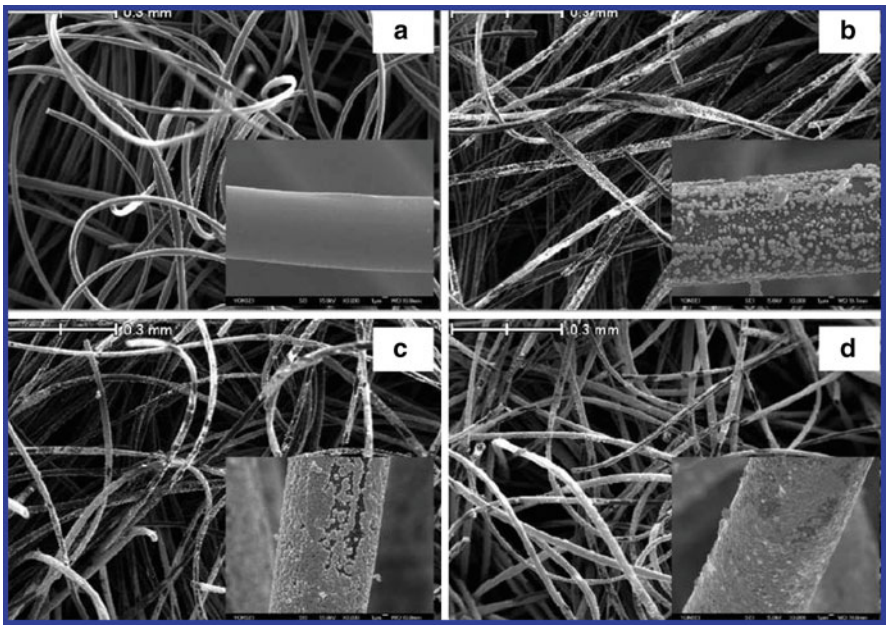

Fig. 1.8 SEM images of (**a**) pure carbon fiber, (**b**) carbon fiber with silver treatment for 10 mins, (**c**) carbon fiber with silver treatment for 20 mins and (**d**) carbon fiber with silver treatment for 30 mins (Reprinted from [52]. With permission from American Chemistry Society)

To avoid using toxic reductants, Zbořil [57] used saccharides as reductants. $Ag(NH_3)_2^+$ complex with glucose, galactose, maltose, or lactose were mixed in solution, and NaOH solution added to initiate the reduction. TEM images of silver colloid nanoparticles synthesized via this method are shown in Fig. 1.9. Besides silver nanoparticles, other silver nanostructures such as silver nanoflakes [58], silver nanofibers [59, 60], silver-coated fibers [61], titania/silver nanocomposites [62], and cross-linked micelles with silver nanoparticle cores [63] have been reported to have antibacterial properties.

Silver nanoparticles synthesized by biological approaches are becoming more and more popular. For more information, please refer to Sect. 2.1.1.3 in Chap. 2.

1.4 Polymeric Silver Composites

Besides inorganic materials, polymers are also encountered daily. Most polymeric materials, such as polyethylene, polypropylene, polystyrene, are not inherently antibacterial. Therefore, the antibacterial modifications of polymeric materials are also important and necessary.

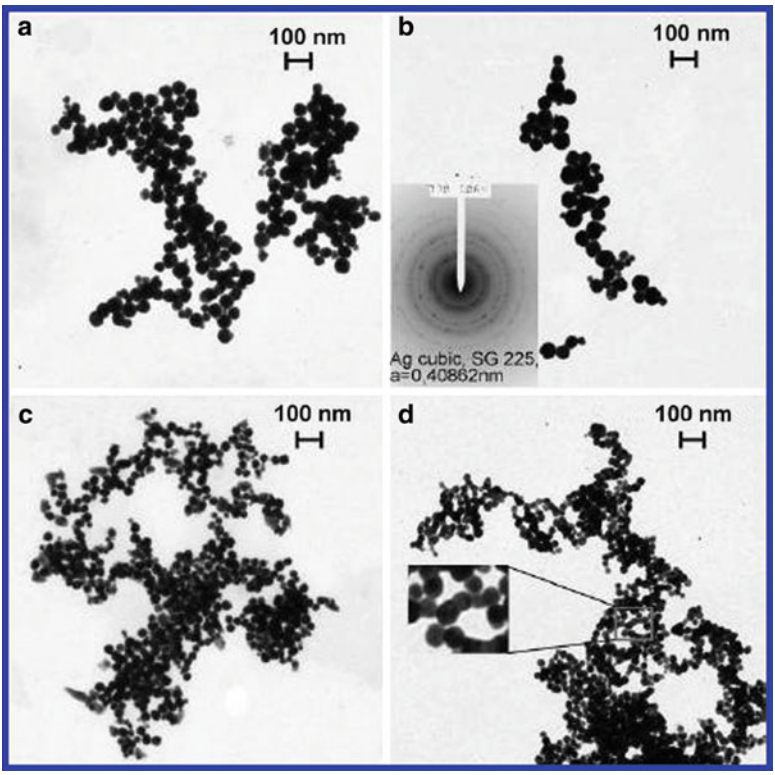

Fig. 1.9 TEM images of silver colloid nanoparticles synthesized via reduction of $Ag(NH_3)_2^+$ by glucose (**a**), galactose (**b**), lactose (**c**), and maltose (**d**) (Reprinted from [57]. With permission from American Chemistry Society)

1.4.1 Coatings and Films

Antibacterial polymeric coatings and films are very useful as surface modifiers that impart antibacterial properties to inherently non-antimicrobial surfaces such as glasses, metals, polymers, wood, and paper. The binding force can be either physical attachment or chemical bonding. Blending antibacterial agents into a polymer coating is one straightforward method. In a recent publication, Khor [64] used Ag/hydroxyapatite (HA) powder and polyetheretherketone (PEEK) to give glass surfaces antimicrobial properties. Ag/HA powder was synthesized by adding H_3PO_4 and $AgNO_3$ solution into a $Ca(OH)_2$ solution, then ammonia solution was used to adjust the pH to 8. Stirred overnight, the Ag/HA powder was separated, washed, dried and mixed with PEEK powder. Later, the powder mixture was sprayed by compressed air at 150°C onto a glass substrate, which exhibited clear ZOI on *Escherichia coli*-inoculated agar plates after coating.

Layer-by-Layer (LbL) self-assembly is another versatile method to prepare functional coatings and films [65, 66]. Basically, electrostatic forces are involved

in the fabrication of LbL films. The glass substrate (negatively charged) is first dipped into a cationic polyelectrolyte solution and dried to form the first layer, then dipped into an anionic polyelectrolyte solution and dried to form the second layer. The two steps are repeated alternately to obtain a multi-layer structure to achieve the desired thickness. Usually, these LbL films are used as drug delivery systems, as water-soluble drugs can be loaded in their positive/negative bilayers and be released when an external stimulus is applied. The commercialization and large-scale preparation are easy due to the simplicity of this technique. For specific antibacterial applications, LbL films can be fabricated into films with multiple antimicrobial actions. First, silver ions or antibiotics (cetrimonium bromide, for example) can be loaded into the LbL films. Second, the positive charged layer can be an antibacterial cationic polymer (see Sect. 1.5.3.2) to offer extra antimicrobial activity. Grunlan [67] made LbL films of polyethylenimine (PEI) and polyacrylic acid (PAA), and loaded silver ions in the PEI polymer layer on polyethylene terephthalate (PET) substrate (Fig. 1.10). After coating, the sample remained transparent. The antibacterial activity of the PET-coated disks was evaluated by the ZOI test for *Staphylococcus aureus* or *Escherichia coli* (Fig. 1.11). The LbL-treated PET disks with different numbers of layers showed different ZOI radii compared to no ZOI for untreated PET.

Theoretically, the LbL technique can be used on any surface. However, in practice, problems often arise when the target substrate is a highly hydrophobic polymer such as polytetrafluoroethylene (PTFE), polyethylene (PE), and polypropylene (PP). Traditionally, a harsh and complicated "priming" such as plasma or oxidation is required prior to applying LbL. The priming process introduces reactive, hydrophilic hydroxyl groups on these polymeric surfaces. A recent paper proposed a novel strategy to address this issue. Messersmith [68] used PEI and hyaluronic acid (HA) (Fig. 1.12) molecules modified with dopamine moieties to mimic the protein structure of natural mussel adhesive. After the modification, the researcher was able to coat LbL film onto a PTFE substrate, which is considered to be the most challenging surface due to its extremely anti-adhesive nature. After three cycles of coating, fluorine composition on the PTFE surface sharply decreased from 69% to 1.6% and the contact angle of water dropped remarkably from 106° to 19.7°, both of which proved that LbL assembly was successful on the PTFE substrate. To grant this LbL coating of PTFE with antibacterial properties, silver nanoparticles were formed in situ inside the LbL coating from reduction of $AgNO_3$. Although only moderate biocidal efficiency was observed (approx. 18% of the total bacteria was killed after 4 h of incubation) from an antibacterial test on *Escherichia coli*, this work proposed a useful solution for antibacterial coating on surfaces like PTFE.

By combining the bacterial killing power of silver nanoparticles and the quaternary ammonium functionality, a dual-functional antibacterial coating was reported by Rubner [69]. The polystyrene substrate was first coated with ten bilayers of poly (allylamine hydrochloride) (PAH) and PAA. An additional ten bilayers of PAH and SiO_2 nanoparticles (~20 nm in diameter) were then deposited onto it. Later, ammonium groups were covalently bonded to the silica nanoparticles by reaction

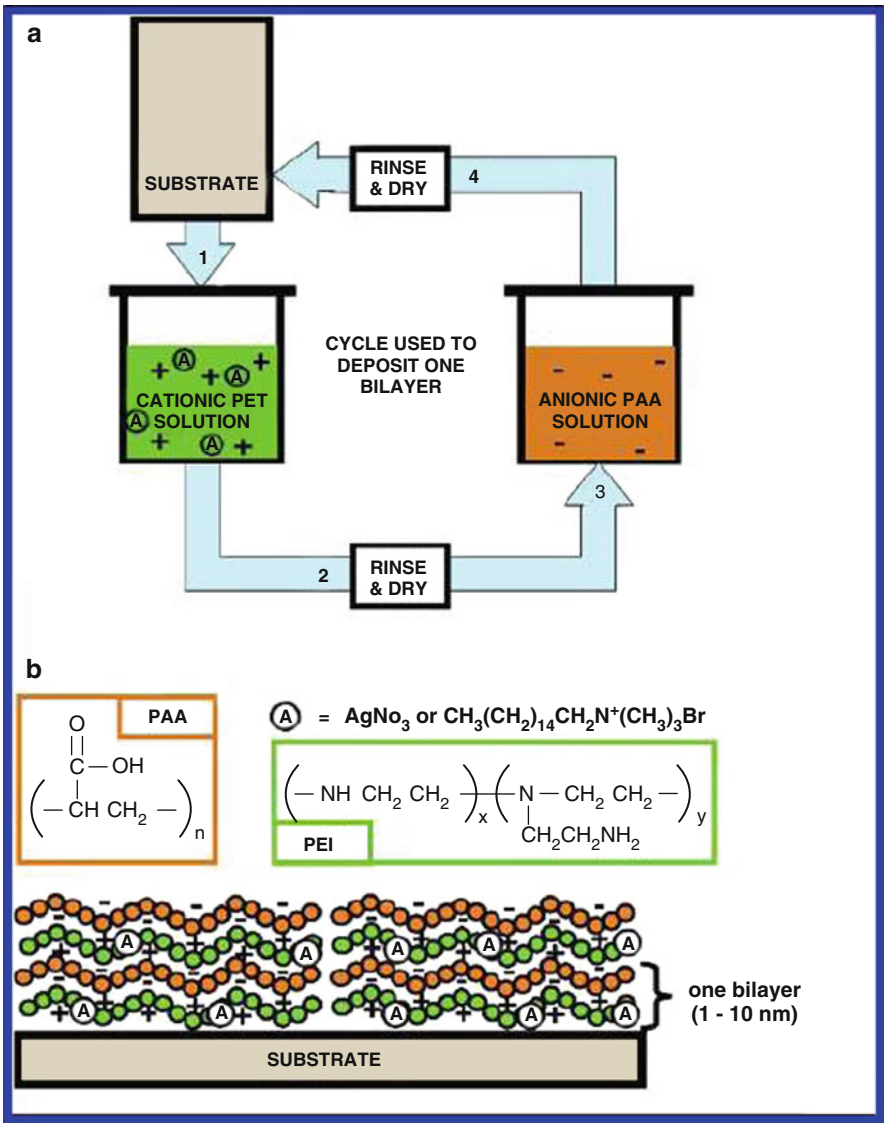

Fig. 1.10 Scheme of LbL self-assembly coating (Reprinted from [67]. With permission from American Chemistry Society)

with Si-OH groups. Finally, the coated substrate was dipped in silver acetate solution and the silver ions were subsequently reduced. The coating now had quaternary ammonium groups on the top surface to kill any bacteria on contact and silver nanoparticles beneath the protective silica particles layer to leach out biocidal silver ions (Fig. 1.13).

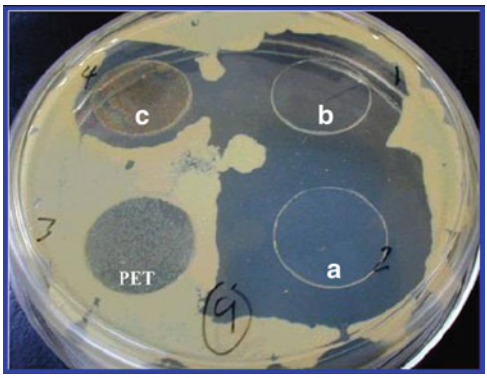

Fig. 1.11 ZOI test: PET with no coating (PET), PET with different numbers of bilayers (**a**–**c**) (Reprinted from [67]. With permission from American Chemistry Society)

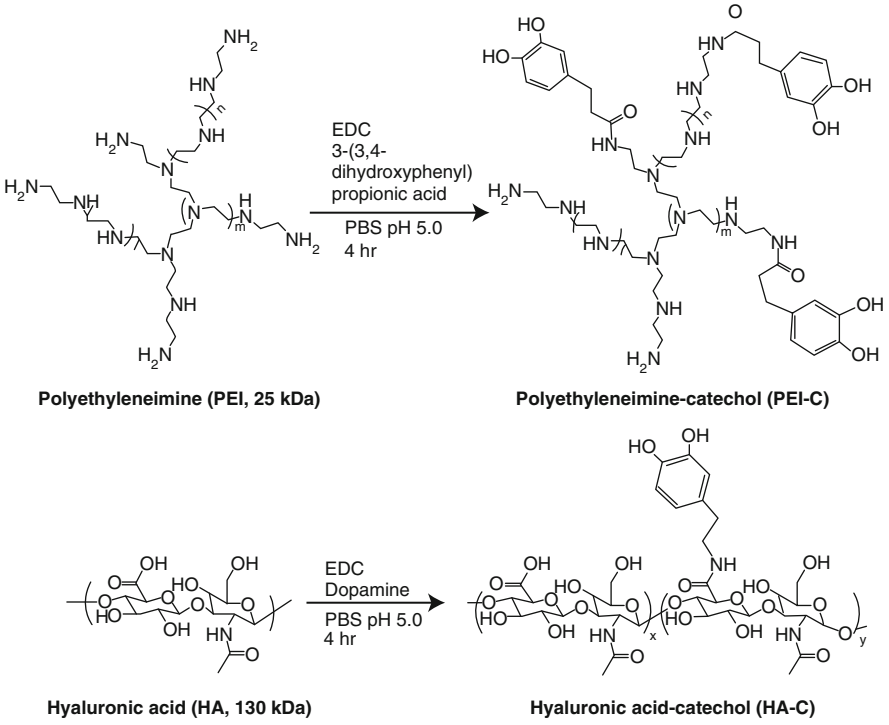

Fig. 1.12 Synthesis of dopamine-containing polymers used in surface independent layer-by-layer assembly (siLbL) (Reprinted from [68]. With permission from Wiley)

Another coating option is electrochemical anodization, which is especially suitable for metal surfaces. This method works similarly to electroplating. Pyrrole monomer is often used in this technique because it easily forms anodized films on metal and it is biocompatible with mammal cells [70]. Jérŏme [71] used this technique and successfully coated stainless steel and carbon fiber specimens with

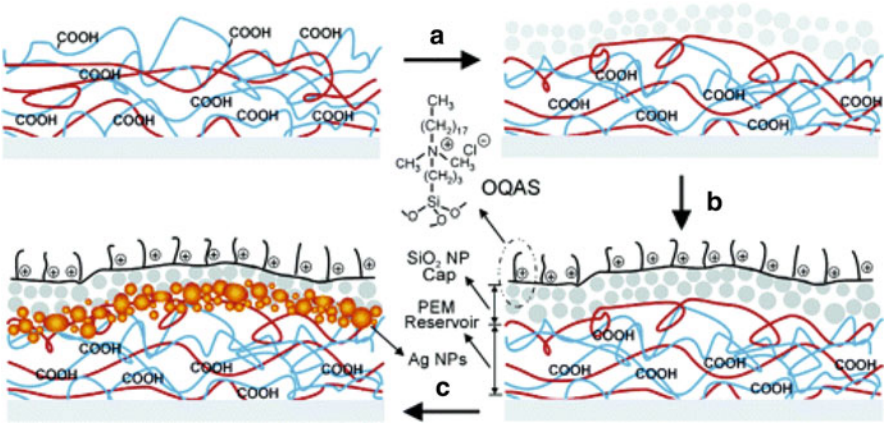

Fig. 1.13 Scheme showing the design of a two-level dual-functional antibacterial coating with both quaternary ammonium salts and silver. The coating process begins with LbL deposition of a reservoir made of bilayers of PAH and PAA. (**a**) A cap region made of bilayers of PAH and SiO_2 nanoparticles added to the top. (**b**) The SiO_2 nanoparticle cap modified with a quaternary ammonium silane, OQAS. (**c**) Ag^+ loaded inside the coating using the available unreacted carboxylic acid groups in the LbL multilayers (Reprinted from [69]. With permission from American Chemistry Society)

PAMPS [poly(2-acrylamido-2-methyl-1-propanesulfonic acid)] and PHMA [poly (2-hydroxy-4-N-methacrylamidobenzoic acid)]-doped polypyrrole film. The films themselves are not antibacterial but silver ions can be anchored onto either PAMPS or PHMA by chelating to the polymer chains, giving these films antibacterial activity. Both *Escherichia coli* and *Staphylococcus aureus* were used to evaluate antibacterial activity of coated stainless steel and carbon fibers. Viable bacteria were significantly reduced when incubated with these samples. The advantages of this technique are: (1) simplicity in controlling the film thickness by altering the current, voltage and time, and (2) generation of robust film with high resistance to peeling force [71].

One interesting type of a polymer coating bearing silver nanoparticles was reported by John [72]. The monomer described in this publication was derived from unsaturated natural fatty acids. The unsaturated fatty acids readily undergo self-cross-linking reaction of the double bonds caused by external oxidants like oxygen (drying oil effect). The author utilized silver ions to oxidize the double bonds to form a cross-linked polymer film. This type of polymer film can kill bacteria on contact.

The design of an antibacterial coating with a stronger binding force is always desirable and can be achieved by chemical bonding between the coating material and the substrate. The Sen group has done extensive research on antibacterial materials. One of their papers proposed an antibacterial modification of various substrates by well-established silane chemistry [73]. This technique involved the irreversible reaction methoxysilane groups with free hydroxyl groups or alkoxysilane groups

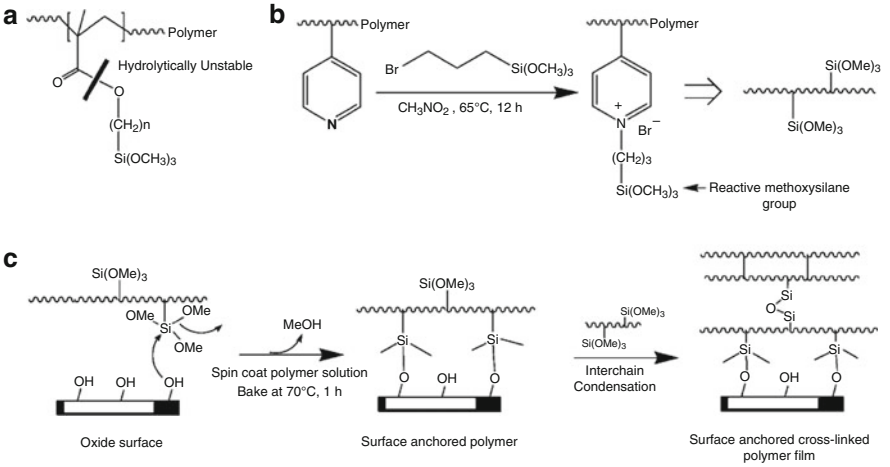

Fig. 1.14 Synthetic route to polymer coating and chemical reaction of polymer coating with OH groups on the substrates (Reprinted from [73]. With permission from American Chemistry Society)

to form strong Si-O-Si bonds. The polymer used in this method was poly (4-vinylpyridine). Most of the nitrogen atoms were quarternized by alkyl bromide to render the polymer antimicrobial, while some of the tertiary nitrogen atoms were quarternized by 1-bromopropylytrimethoxy silane to provide reactive anchor groups (Fig. 1.14). The polymer was then spin-coated onto solid surfaces or dip-coated onto fabrics. The antibacterial activity was further enhanced by adding $AgNO_3$ in polymer solution to form precipitated AgBr nanoparticles that were incorporated into polymer films. This method provides a practical and facile surface modification option for virtually all substrates due to the universal presence of OH groups on glass, metals, ceramics, etc.

1.4.2 Hydrogels

Polymeric hydrogels are cross-linked three-dimensional polymer networks synthesized from hydrophilic monomers [74]. Generally, monomers used in hydrogel synthesis are: acrylic acid, acrylamide, N-isopropyl acrylamide, 2-hydroxyethyl methacrylate (HEMA), methacrylic acid, dimethylethylamino acrylate, etc. Hydrogels are ideal wound-dressing materials for multiple reasons. First, the water in the hydrogel keeps the wound hydrated and prevents the formation of a scar that is undesirable for cosmetic reasons. Second, the low abrasion property of hydrogels keeps the patient comfortable. Finally, the nutrients in the hydrogel can promote the healing process. Commercial products such as HDR® and AQUAGEL® have been available on the market for years. Due to the high susceptibility of

wounds to infection, antibacterial modification of hydrogels is not only academically important but also practically urgent. The general strategies involved are releasing antimicrobial agents such as antibiotics or silver ions, using an inherently antibacterial polymer network, or a combination of the two.

Raju [75] used a semi-IPN hydrogel to obtain an antibacterial material. Acrylamide monomer, a cross-linker, ammonia persulfate (free radical polymerization initiator) and polyvinyl acetate (PVA) were mixed in water and the polymerization was carried out at 35°C for 8 h. Ionic silver solution was absorbed by dry gels, and silver ions were further reduced by $NaBH_4$ yielding hydrogel–silver nanoparticle composites. The resulting solution of the composite was dropped onto bacteria-inoculated agar plates. Pure hydrogel showed no antibacterial activity whereas hydrogel–silver composites showed significant ZOI. Interestingly, the hydrogel–silver nanoparticle composite has a higher antibacterial activity against *Escherichia coli* than the hydrogel–silver ion composite which is obtained without reduction.

Another hydrogel–silver composite for wound-dressing application was reported by Aggor [76]. The acrylamide-based hydrogel used N,N′-methylenebisacrylamide as cross-linker. Silver ions were directly incorporated into the hydrogel by mixing with $AgNO_3$ reaction mixture and then reduced to silver particles after the hydrogel was formed. Although no antibacterial test was done in this paper, antibacterial properties can be expected from this material.

1.4.3 Fabrics and Textiles

The development of antibacterial fabrics and textiles is receiving more and more attention for obvious reasons. First, antibacterial clothing minimizes cross-contaminations and risks for doctors, nurses, and patients. Second, antibacterial combat garments can increase soldiers' survivability in biological warfare. Other types of clothing, such as athletic garments, can be made to possess antibacterial activity. The most straightforward way to impart antibacterial activity to fabrics is by physical deposition of biocidal silver [77–80]. Baglioni [81] reported a simple way to modify some common fabrics like cotton, wool, and polyester. Silver nanosol was synthesized by reduction of Ag^+/PAA solution by either $NaBH_4$ or UV irradiation. Wool, cotton, and polyester textiles were soaked in the solution, then squeezed, rinsed, and dried at elevated temperatures. ZOI test was used to evaluate the antibacterial activity (Fig. 1.15). Obvious ZOI were found for treated cotton sheets on different bacteria-inoculated agar plates.

Previously, the LbL method was used to obtain antibacterial coatings (see Sect. 1.4.1). It turns out to be useful in fabric and textile modifications as well [82]. To start with, silver nanoparticles are reduced by UV treatment with polymethacrylic acid (PMA) as a capping agent. Then, poly(diallyldimethylammonium chloride) (PDADMAC) and Ag/PMA are used as positively and negatively charged polymers for LbL self-assembly. By alternately dipping nylon or silk fibers into the two polymer solutions, antibacterial fibers with 10 or 20 layers

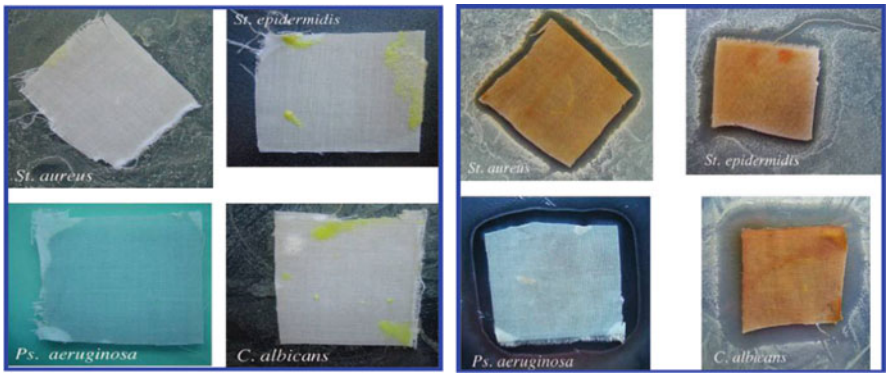

Fig. 1.15 Untreated cotton samples show intense bacterial growth (*left*) and treated cotton samples show ZOI against multiple bacteria (*right*) (Reprinted from [81]. With permission from American Chemistry Society)

of polymer were obtained. Antibacterial efficiencies of both fibers were compared by using *Staphylococcus aureus*. Generally, with the same number of polymer layers, silk showed a better antibacterial activity than nylon, attributed to a higher surface charge of the silk fibers, which facilitates the LbL process.

One challenge in antibacterial fabric and textile fabrication is to maintain antibacterial activity after multiple washing cycles. Silver ions are water soluble and may get depleted after laundry; mechanical abrasion and the usage of detergents during cleaning are also detrimental to the integrity of these materials. Previous reports utilized capillary forces to incorporate silver ions, which are weak in nature and may suffer significant loss of silver species after washing. To cope with this issue, stronger binding of silver onto fabrics was proposed and tested [83]. TiO_2, silver nanoparticles and water-based polyurethane were mixed together and then allowed to soak into polyester fibers. When 90% uptake was achieved, the treated fiber was dried, baked and cleaned with soap. During a water-borne bacteria test, an interesting synergetic effect of TiO_2 and silver nanoparticles was discovered. Samples pretreated with UV irradiation before antibacterial test showed enhanced antibacterial activity because of the activation of TiO_2 particles by UV light. Samples without UV pretreatment showed only 70% biocidal efficiency comparing with the 84–100% for UV-pretreated samples. The researchers ran antibacterial tests after multiple wash cycles. The results looked promising as polyester samples were able to maintain up to approx. 86% biocidal efficiency against *Escherichia coli* and *Staphylococcus aureus* after 3,000 wash cycles.

A similar strategy was also proposed in another publication [84]. First, SiO_2/Ag nanoparticles were made by absorbing silver ions from solution onto SiO_2 nanoparticles. The SiO_2/Ag particles, PET, and coupling agents were mixed, heated, and extruded to get PET bulk material. Finally, bulk PET was subject to melt-spinning to form its corresponding fiber. The resultant PET fiber showed high antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Also, the

PET sample with 5% antibacterial agent load can retain 80% efficiency after 30 wash cycles.

1.4.4 Bulk Materials

Bulk polymeric materials blended with silver can be considered as the counterpart of inorganic silver composite in glasses. For example, Nia [85] blended Ag/TiO_2 composite powder with polypropylene (PP) powder and melt-molded it into an antibacterial plastic. Plastic sheets were tested against *Staphylococcus* and over 99% kill was observed compared with pure PP sheets. Another recent example is using the sol–gel technique to synthesize PBAT polymer with SiO_2/Ag [86]. In addition, silver-doped polyurethane catheters are used as improved medical devices to inhibit bacterial infection [87].

One important application of bulk polymeric silver composites is in dental care materials. It was found that more bacteria or plaques tend to accumulate on conventional restorative resin [88]. Efforts have been made to address this problem by loading chlorhexidine into resin material [89]. However, the antibacterial activity is lost once the drug is depleted and loading of the drug compromises material integrity. Silver-containing dental materials with non-leaching properties were developed to overcome this problem. Preparation of antibacterial resin with silver nanoparticles was described in a recent paper [90]. Silver nanoparticles were synthesized as described in Sect. 1.3.5. Then, these nanoparticles were doped into liquid acrylate monomer and the mixture was cured into the desired shape resin. The material killed 100% *Escherichia coli* bacteria in solution. Similar work was reported by Asuta [91] using commercial silver-incorporated Novaron® and Amenitop®. Acrylate derivatives were used in this system and SiO_2 was added to form a paste, which was later cured under UV light to give a resin disk. In the MIC test, the silver-loaded resin showed no biocidal effect. However, in the ZOI test, the same sample killed *Streptococcus mutans* on contact. The reason for these conflicting results is that there are no silver ions leached into the liquid media, but the resin can kill bacteria on contact by catalytic generation of ROS with silver. The author also tested the silver ion concentration in water after 6 months exposure to water and no silver ions were detected.

1.5 Challenges and Future Prospects

1.5.1 Bacterial Resistance to Silver

Since silver-based antimicrobial materials are now widely used, it is almost inevitable that bacteria will develop a certain resistance to silver. The first report addressing this issue dates back to 1975 [92]. Research showed that the resistance

can be intrinsic. Intrinsic silver resistance of bacteria is defined as the phenotype demonstrated by micro-organisms before the use of an antimicrobial agent. Intrinsic silver-resistant bacteria are usually found in the areas where there is frequent silver exposure such as burn care wards in hospitals, hospital water distribution systems, and soils near silver mines. This resistance can also be acquired by genetic mutation or caused by the acquisition of silver-resistant plasmid or transposon [93].

1.5.1.1 Mechanism of Silver Resistance

Bacterial resistance to silver and other heavy metal ions is generally from genes encoded in either plasmids [94, 95] or chromosomes [96, 97]. Thus far, the silver resistance determinant studied in most detail is plasmid pMG101, which confers bacteria resistances to Ag^+ and Hg^{2+}, as well as to several antibiotics [92]. When this plasmid was transferred by conjugation with *Escherichia coli*, the mutant *Escherichia coli* was able to survive in 0.6 mM silver ions in broth media, a 600% greater tolerance compared to silver-susceptible *Escherichia coli*. Sequencing pMG101 revealed that it contains nine genes, and the functions of eight named genes and their protein products are considered responsible for silver resistance due to the fact that they are the homologues of proteins known for other metal resistance. The first protein product of pMG101 gene is SilCBA, one member of the potential-driven cation/proton efflux pump. It contains three polypeptides. SilA, SilB, and SilC [98]. SilA is a large inner membrane cation pump, SilC is the outer membrane protein, and SilB is the protein which connects SilA and SilC (Fig. 1.16). SilA and SilB together work as an antiporter that carries silver ions out of the cell

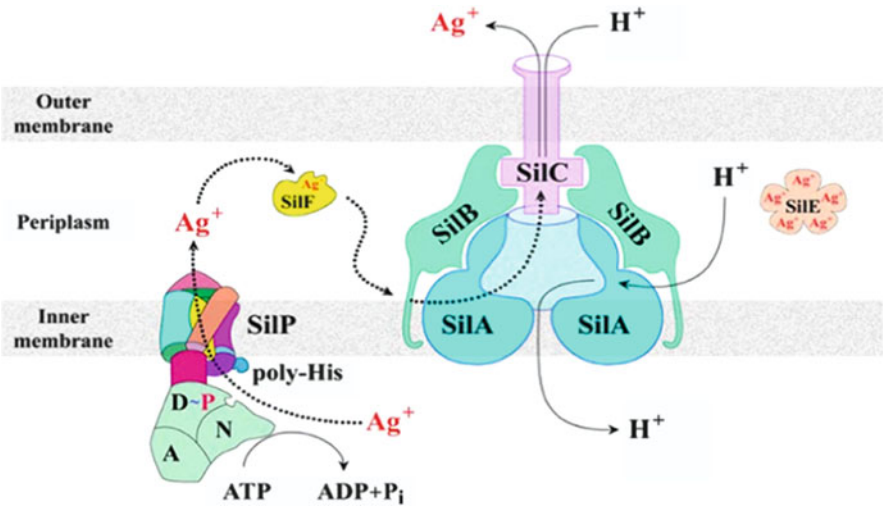

Fig. 1.16 Plasmid silver resistance mechanism (Reprinted from [100]. With permission from Springer)

while SilC is an ATPase that transports silver ions from the cytoplasm to the periplasm. Besides the SilCBA cation/proton pump, another set of pumps also exists: SilP and SilF, both of which are also the protein products of pMG101 gene. SilP is a P-type ATPase, which is a member of large family of cation pumps [96, 97]. SilF is a chaperone protein which picks up silver ions released from SilP and drops off to SilCBA. SilP, SilF, and SilCBA, when working together, complete a full cycle of silver ion efflux and protect bacteria from the deleterious effect of silver. In addition, SilE, another protein product, can also help to increase silver resistance by binding to five silver ions and preventing the silver ions entrance to cytoplasm (Fig. 1.16) [99, 100].

1.5.2 Material Fouling and Biofilm Formation

Fouling is defined as accumulation of undesired materials on a solid surface, mostly in aquatic environments. The most common fouling situation is the adhesion of marine species to ship hulls, which enhances drag, and lowers fuel economy. Similar to fouling on ship hulls, biomaterial surface is subject to protein absorption, which is considered to be the initial step of biofouling. In addition, many proteins such as fibronectin [101–105], fibrinogen [101, 106–108], vitronectin [109, 110], thrombospondin [111], and Von Willebrand factor [112] can enable the specific binding of *Staphylococcus aureus* to biomaterial surfaces. It has been reported that this type of fouling can result in many serious consequences such as heart valve failure [113].

To avoid biofilm formation is another challenge for antimicrobial materials. Defined as microorganism built-up adhering to either biological or non-biological surfaces [114], biofilm is an intrinsic defense mechanism of bacteria against antibiotics and human immune systems. NIH estimates that 75% of bacterial infection is biofilm based [115]. Bacteria within biofilms are 1,000 times or more resistant to antibiotics and inherently inert to host immune response [115]. In addition, exchanges of genes responsible for drug resistance across biofilms have resulted in many bacteria possessing multidrug resistance [116, 117].

1.5.3 Possible Solutions

To cope with ever increasing bacteria resistance to antibiotics, new antimicrobial strategies and new types of antibiotics that do not induce bacterial resistance must be developed. New synthetic materials that can avoid fouling or prevent biofilm formation will also significantly increase the antimicrobial effectiveness of silver and other antibiotics.

1.5.3.1 Cationic Antibacterial Peptides

Naturally occurring antimicrobial peptides focus on the fundamental structural difference between microorganisms and normal cells: the cell membrane. Bacterial membranes are usually constructed in such a way that the outermost layer is extensively occupied by negatively charged lipid head groups. In contrast, the outer layers of cell membranes of plants or animals are usually neutral with most negatively charged lipids pointed into the cytoplasm [118]. Despite the diversity of antimicrobial peptides present in nature, most function in a similar fashion. Peptides can adopt configurations in which the hydrophobic chains and cationic amino acids spatially organize into separate domains [119]. The Shai–Matsuzaki–Huang (SMH) model explains how antimicrobial peptides interact with microorganisms using their hydrophobic chains and cationic amino acids [118, 120, 121]. This model (Fig. 1.17) suggests that these peptides kill the microorganisms through the following steps. First, peptides are absorbed onto bacteria surfaces due to electrostatic forces. Second, lipid membranes are disrupted and membrane structures are altered. Sometimes, this process involves the entrance of peptides into the bacterial interior. Finally, bacteria are killed due to the leakage of internal contents. This model is indirectly supported by the following experimental observations: (1) cholesterol can lower peptide antibacterial activity by

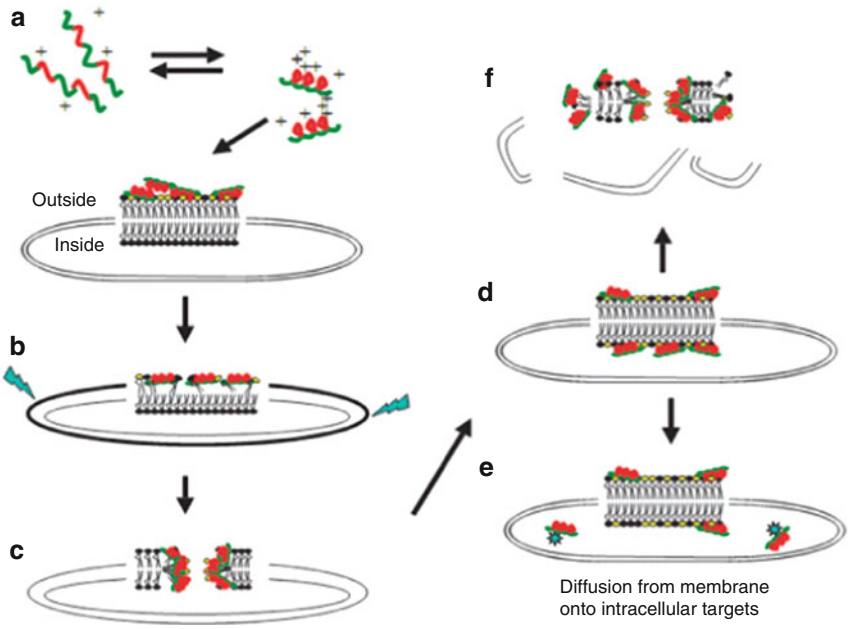

Fig. 1.17 The SMH model. (**a**) Peptides absorption on cell membranes. (**b**) "Thinning" process of cell membranes. (**c**) Disassociation of cell membrane creating "worm-hole." (**d**) Transportation to inner lipid layer. (**e**) Diffusion into cytoplasm. (**f**) Fragmentation of cell membranes (Reprinted from [119]. With permission from Nature)

providing extra stabilization of membranes, and (2) increased ionic strength in solution weakens the antibacterial activity of peptides by shielding the electrostatic forces between cationic peptides and negatively charged membranes.

The detailed mechanism on how peptides kill these microorganisms is not clear. Suggested mechanisms include: disintegration of cell membranes which causes leakage of internal contents [120], disturbance of cell membranes by altering the distribution of lipids between leaflets of bilayers [118], damage to vital intracellular targets after insertion of peptides [122], and degradation of the cell wall by introduction of hydrolases [123].

The major motivation for the study of antimicrobial peptides is that their biocidal activity can even be extended to fungi and viruses. In addition, although these peptides are not as efficient as traditional antibiotics toward susceptible microbes, the peptides are able to kill drug-resistant pathogens at similar concentrations with much faster rates. Unlike antibiotics, acquisition of resistance by bacterial strains is also less likely to happen due to their unique antibacterial mechanism. Since the target site of antibacterial peptides is the cell membrane, to develop resistance towards peptides requires changes of membrane structure and alteration of composition and/or reorganization of lipids, which is very difficult for most microorganisms [119].

1.5.3.2 Synthetic Polymer Mimics of Natural Antimicrobial Peptides

Despite the attractive antibacterial properties of naturally occurring peptides, their extensive use is precluded by the high cost ($50–400/g) compared to conventional antibiotics (approx. $1/g) [124]. In addition, their susceptibility towards enzymatic degradation is a concern. Therefore, the design of synthetic polymer mimics is of great interest.

Polymers with quaternary ammonium and phosphonium groups are structurally designed to mimic the antibacterial peptides with positively charged blocks and hydrophobic alkyl chains, and have the advantage of low cost, ease of synthesis, and resistance to enzymatic degradation (Fig. 1.18). Generally, cationic quaternary polymers share the same antimicrobial mechanism with their peptide counterparts. First, electrostatic attraction between the cationic groups and negatively charged bacterial membrane brings them together. Second, the long hydrophobic alkyl chains are able to penetrate into the bacterial membrane through hydrophobic interactions. Finally, the polymer induces membrane disruption and leakage of cell contents.

In a recent publication [125], biodegradable quaternary ammonium polymers were synthesized as possible next generation drug to replace conventional antibiotics. An *in vitro* test showed that these polymers are able to kill

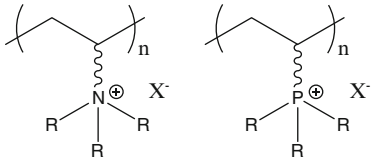

Fig. 1.18 General chemical structures of quaternary ammonium and phosphonium polymers

methicillin-resistant *Staphylococcus aureus*. Short-term in vivo toxicity studies on animal kidney and liver showed no functional abnormality of these vital organs.

Another desirable aspect of quaternary ammonium or phosphonium polymers is that silver halide particles can be introduced inside the polymer matrix if halide counter ions are used during the polymer synthesis. By combining the antimicrobial properties of both the quaternary-functionalized polymers and silver halides, the synthesized "dual action" antibacterial materials further increase the antibacterial performance. In a recent publication [126], Sen partially quarternized poly (4-vinylpyridine) with n-hexyl bromide. Then, soluble silver salts were added into the polymer solution to prepare a polymer–silver bromide composite. Finally, this polymer–silver bromide composite was coated onto glass slides by film casting. This film was subjected to multiple antimicrobial tests. ZOI tests showed bacteria-free areas around the coated slide. The size of ZOI was found to be dependent on the size of silver bromide particles: samples with small silver halide particles yielding larger ZOI and vice versa. MIC tests on both the polymer and polymer–silver bromide composite were also carried out, in which the composites showed much lower MIC values compared to pure polymer coating simply because of the additional antibacterial silver ions in the composite. Finally, the anti-biofilm property was tested using *Pseudomonas aeruginosa*. The pure polymer surface was susceptible to biofilm formation and prone to lose its antibacterial ability, whereas the polymer–silver bromide composite maintained the antimicrobial activity for a prolonged time period.

1.5.3.3 Anti-fouling or Anti-biofilm Modifications

Understanding how biofouling happens is especially important. It is now generally believed that hydration force is the key to surface fouling. In the past, the modeling for interaction of surfaces and foulants only considered electrostatic and electrodynamic (van der Waals) forces. Unfortunately, this model is not applicable for strong interactions within the 3-nm range [127, 128]. Within this range, the hydration force predominates [129] because the interactions between water-soluble moieties are best seen in terms of their interaction with water itself. When largely surrounded by water molecules, they repel each other, and the strength of this force was found to be independent of the charge. Thus, it is essential to create a hydration force barrier at short distances to prevent fouling. Two types of polymeric materials have been developed to address the fouling of biomaterials. The materials exhibit ultra-low fouling properties.

PEG and OEG

The ability of polyethylene glycol (PEG) and oligo ethylene glycol (OEG) to prevent protein absorption has been reported [130]. The hydration force barrier of

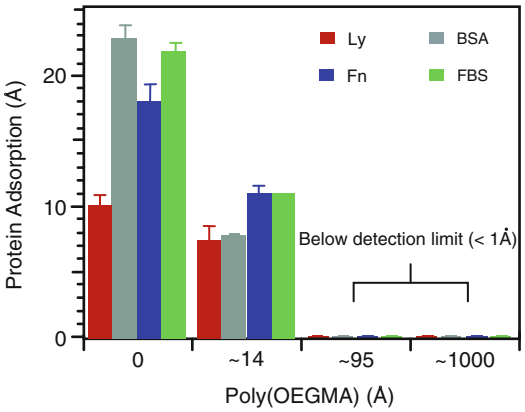

Fig. 1.19 Protein adsorption comparison of the poly(OEGMA) coating on SiO_2 and SiO_2 with only ATRP initiator but no polymer coating (control) measured by ellipsometry. The x-axis is the thickness of poly (OEGMA) coating (14, 95 or 1,000 Å; Control is labeled as a film with 0 Å thickness), and the *y-axis* is the thickness of the adsorbed protein. *Ly* lysozyme, *Fn* fibronectin, *BSA* bovine serum albumin, *FBS* fetal bovine serum (Reprinted from [135]. With permission from American Chemistry Society)

highly water-soluble PEG chains is believed to be responsible for the resistance to protein fouling because a large entropy penalty must be paid to force the PEG chains to collapse for proteins to adhere to the coated surface [131, 132]. Both self-assembly membranes and surface-initiated ATRP coating techniques have been employed to treat glass for anti-fouling applications [133, 134]. Protein absorption is substantially reduced by a PEG coating comparing to untreated glass [135] (Fig. 1.19).

Zwitterionic Polymers

One obvious disadvantage of PEG-type anti-fouling materials is that PEG chains are subject to oxidation by oxygen or transition metal ions, which are commonly present in the physiological environment. This prevents long-term application of PEG-type materials [136–138]. While PEG uses hydration force to avoid protein fouling, zwitterionic polymers such as phosphobetaine, sulfobetaine, and carboxybetaine can interact with water molecules even more strongly through electrostatically induced hydration [139, 140], The first generation of anti-fouling zwitterionic polymers are phosphobetaine-based polymers. They are considered as biomimetic fouling-resistant materials, since they contain phosphorylcholine head-groups, which are abundantly present in the outside layer of cell membranes [141, 142]. However, the complicated synthesis process limits its application. Hence, polymers with similar structures, such as

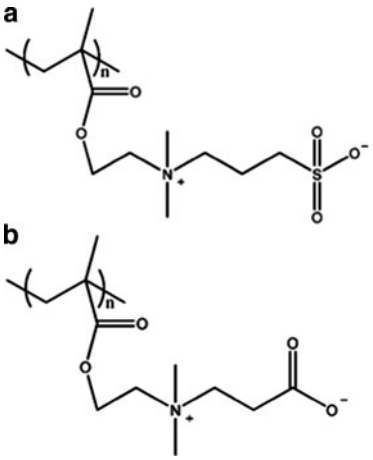

Fig. 1.20 Zwitterionic polymer structure: (**a**) polysulfobetaine, (**b**) polycarboxybetaine

polysulfobetaine (PSB) and polycarboxybetaine (PCB) (Fig. 1.20), are used instead and have been experimentally proved to avoid non-specific protein absorption [143].

Jiang [144] compared the anti-fouling properties of PEG, PSB, and PCB polymers formed by both self-assembly and surface-initiated ATRP techniques to coat Au-coated glass slides. Ultra-low protein absorption on PSB and PCB polymer coatings was observed (Fig. 1.21) by SPR (Surface Plamson Resonance). Jiang [145] also explored the optimum film thickness to achieve almost "zero-adhesion" (Fig. 1.22).

Anti-biofilm Materials

The concepts "anti-fouling" and "anti-biofilm" have different definitions and sometimes cause confusion. The term "anti-fouling" usually means resistance to adhesion of proteins and normal cells. However, "anti-biofilm" means the inhibition of adhesion of bacteria and the formation of biofilms. It is generally assumed that a surface that avoids protein adhesion can prevent biofilm formation. However, this correlation is not always true [137].

The PEG, PSB, and PCB polymers are able to avoid biofilm formation in addition to their ability to prevent protein fouling. Coatings created by both ATRP and SAM of PEG and PSB polymers on Au surface were subject to anti-biofilm tests [142]. Short-term adhesion (3 h) of Gram-positive *Staphylococcus epidermidis* and Gram-negative *Pseudomonas aeruginosa* were reduced compared with untreated glass surfaces. When long-term anti-biofilm experiments (18–24 h) were carried out, SAM coatings were less effective in inhibiting biofilm formation,

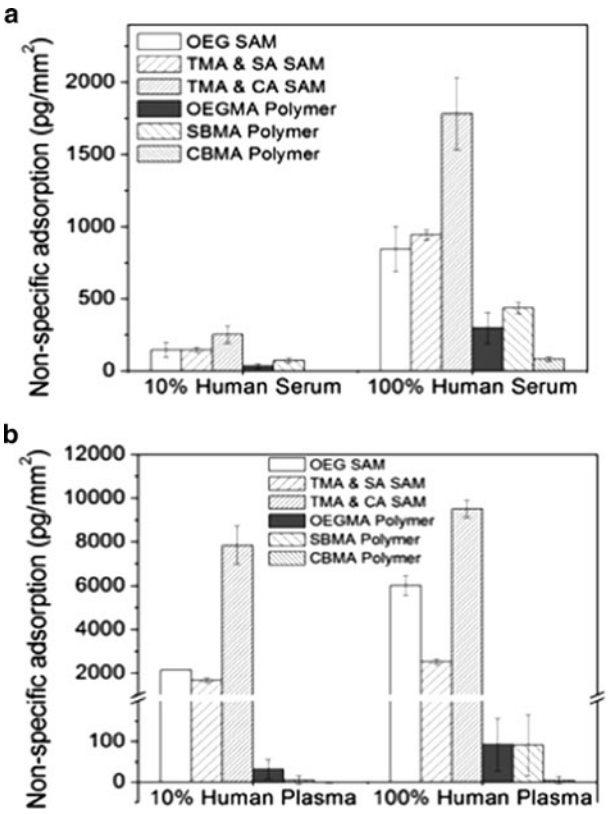

Fig. 1.21 Non-specific adsorption comparison for (**a**) 10% human serum in PBS and 100% human serum and (**b**) 10% human plasma in PBS and 100% human plasma. A full monolayer of protein on the surface is equivalent to 2,700 pg/mm^2 standard error of the mean. Surfaces used in these experiments are self-assembled monolayers of oligo(ethylene glycol) (OEG SAM), mixed trimethylamine and sulfonic acid (TMA&SA SAM), mixed trimethylamine and carboxylic acid (TMA&CA SMA), and ATRP-created poly(OEGMA) (OEGMA polymer), sulfobetaine methacrylate (SBMA polymer), and carboxybetaine methacrylate (CBMA polymer) (Reprinted from [144]. With permission from American Chemistry Society)

probably because of their long-term instability. The anti-biofilm capability of PCB polymer in prolonged period was studied [146], and the biofilm formation by *Pseudomonas aeruginosa* was considerably hindered by a PCB polymer coating with a thickness of 30 nm. At 25°C, an untreated glass surface was completely covered by biofilm within 48 h whereas a PCB surface exhibited less than 5% accumulation in 10 days. At 37°C, the optimum growth temperature of *Pseudomonas aeruginosa*, the glass surface showed 100% biofilm coverage in 15 h. In comparison, less than 7% biofilm buildup was observed for the PCB-treated glass surface (Fig. 1.23).

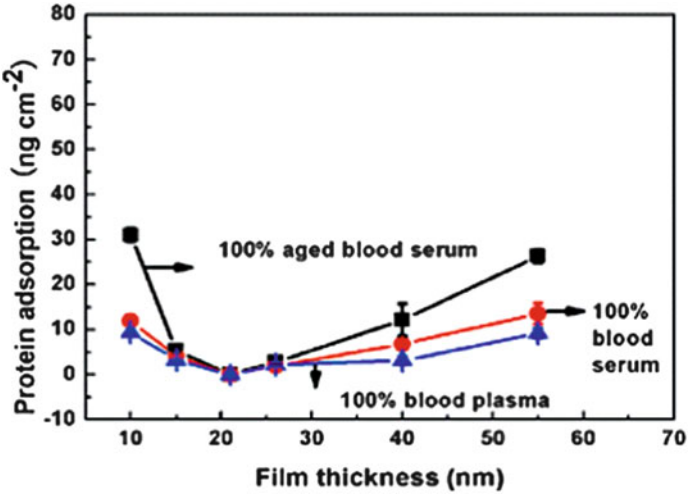

Fig. 1.22 Adsorption of undiluted blood serum, undiluted aging blood serum, and undiluted blood plasma versus film thickness of PCBAA grafted surfaces at 37°C as measured by SPR (Reprinted from [145]. With permission from American Chemistry Society)

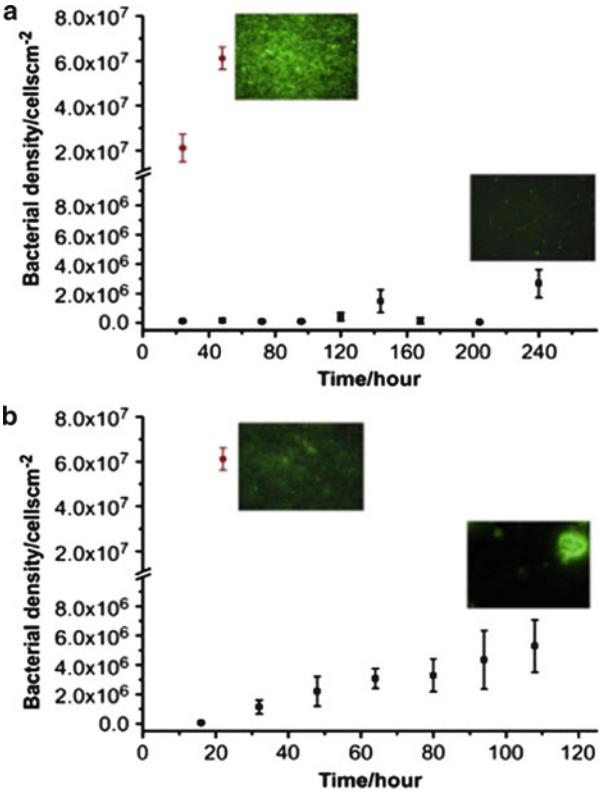

Fig. 1.23 *Pseudomonas aeruginosa* accumulation on PCB surface (■) and glass (●) surfaces as a function of time (**a**) tested with Method I at 25°C, (**b**) tested with Method II at 25°C, (**c**) tested with Method I at 37°C, and (**d**) tested with Method II at 37°C reported as the mean ± SD ($n = 20$) (Reprinted from [146]. With permission from Elsevier)

1.5.4 Perspectives on Silver Antibacterial Materials

Currently, highly effective silver-containing antimicrobial materials are used in various ways, many of which are already commercialized. However, the warfare between humans and pathogenic microorganisms does not end. New threats to our health emerge, such as drug-resistant or silver-resistant pathogens and fouling of biomedical devices. To address these challenges, new approaches and novel materials are needed. The strategies may include (1) the search for novel anti-fouling or anti-biofilm modifications, (2) combining different antimicrobial agents, (3) better recognition and utilization of the antibacterial mechanisms of silver, and (4) the employment of new modification techniques to hitherto non-antibacterial materials. By combining modern material sciences and technologies, silver, this ancient antimicrobial agent, will broaden its current applications.

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