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Covalent immobilization of lysozyme onto stainless steel as an anti-biofilm coating

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Abstract

Covalent immobilization of an enzyme for use as antimicrobial biocompatible surface applications was performed utilizing the sol-gel chemistry, illustrated by chicken egg white lysozyme. The sol-gel network, which contains amine functional groups, provides covalent attachment sites for the enzyme via the carbodiimide reaction. As an alternative method, these free amine groups of the sol-gel layer were utilized for the immobilization of lysozyme from its N-terminus by using poly(acrylic acid) brushes as the linker molecule. The enzymatic activity of covalently immobilized lysozyme with both of the procedures has been investigated.

Keywords: Biofilm, sol-gel chemistry, polyacrylic acid (PAA), lysozyme, *Pseudomonas aeruginosa* PAO1

Özet

Antimikrobiyal ve biyouyumlu yüzey uygulamalarında kullanılmak üzere enzimlerin bir yüzeye kovalent olarak bağlanması yeni geliştirilen bir sol-jel yöntemi kullanılarak, tavuk yumurtası beyazından elde edilen lizozim enzimi çalışılarak gösterilmiştir. Amin fonksiyonel grupları içeren sol-jel ağı, enzimin karbodiimid reaksiyonu aracılığıyla bağlanabilmesi için kovalent bağlanma bölgeleri sağlamaktadır. Alternatif bir metod olarak, sol-jel tabakasındaki bu serbest amin gruplarından lizozim enziminin N-terminus bölgesinden poliakrilik asit katmanına immobilize etmek amacıyla da faydalanılmıştır. Her iki prosedür ile de immobilize edilmiş lizozim enziminin aktivitesi incelenmiştir.

Anahtar Sözcükler: Biyofilm, sol-jel yöntemi, poliakrilik asit, lizozim, *Pseudomonas aeruginosa* PAO1

Introduction

A biofilm is essentially composed of microbial cells attached to a surface and covered completely with an extracellular polymeric matrix produced by biofilm-forming bacteria (Costerton, 1995). When these bacterial cells approach inert surfaces, they first get bound to these surfaces utilizing their external structures such as flagella, fimbriae or capsular components by weak forces (Xavier et al., 2005). As the cells remain attached to the surface for some period, they start to secrete extracellular polymeric substances (EPS) in order to compose a biofilm matrix that embeds many layers of bacterial cells when the biofilm develops (Orgaz et al., 2006). Bacterial EPS are generally composed of a wide variety of materials like polysaccharides, proteins, nucleic acids, uronic acid and humic substances (Xavier et al., 2005; Orgaz et al., 2006). The EPS has several vital functions such as providing an adhesive foundation, structural integrity, bacterial protection and intercellular communication (Zhang et al., 2005; Ploux et al., 2007; Leroy et al., 2008).

Bacteria can communicate and form biofilm nearly on all surfaces including the materials of medical devices and instruments through the

quorum sensing pathway. Even these medical devices and instruments have been sterilized with gaseous agents for many years (Carvalho, 2007; Bukshpan, 2010), the vast majority of the contamination occurs after the adhesion of the microorganisms and they sustain their growth when placed inside the human body (Carvalho, 2007).

Several attempts have been made to protect the surfaces of materials, instruments and equipments by addition of antimicrobial, biocidal and non-adhesive substances for coating, addition of diffusible toxic agents and changing surface roughness. In one of these studies, physical and chemical properties of biomaterial surface are modified by coating with a hydrogel. This method was effective in reducing bacterial adhesion but it was difficult to cover the surface uniformly (Bayston et al., 2005). In another attempt, cuffs on catheters were coated with silver (Bong et al, 2003). The drawback of this approach was degradation of the cuff, which results in diffusion of silver ions and loss of antimicrobial activity (Raad, I., 1998). Antibiotics have also been coated onto surfaces but with the emergence of microbial resistance, this kind of applications had short-lived effect (Raad et al., 1995; Schierholz et al., 1997; Lelievre et al., 1999). Due to their hazardous effects, using of biocides in paints or coatings on metallic surfaces is restricted and hence, new strategies have to be considered to protect metallic surfaces from biofilm growth (Dafforn, 2011).

One of the solutions to overcome the problem with biofilm formation could be the replacement of biocides with non-toxic alternatives, such as enzymes (Kristensen et al., 2008) which have been used in several industries ranging from food industry to large scale biocatalysis. They can also be used for the degradation and the removal of the bacterial biofilms (Orgaz et al., 2006; Kristensen et al., 2008, Leroy et al., 2008). Extracellular Polymeric Substances (EPS) in the biofilm matrix is the essential part of the biofilm development. The complexity and variability of biofilm polymers in the matrix described above could be the utility to use several enzymes like hydrolases and lyases, individually and/or their combinations. This could achieve a sufficient disintegration of the polymeric networks composing the biofilm matrix and detachment of the biofilm from the surface it was attached (Kristensen et al., 2008, Leroy et al., 2008).

Sol-gel technology presents numerous advantages including high biocompatibility, non-toxicity, low-temperature processing and easy application to any kind of substrate including enzymes. Moreover, sol-gel procedure is carried out in low temperature which gives considerable advantage for preparation of materials in industry (Gupta, 2008). The composite materials of silica in the aqueous solutions have drawn attention to the propensity toward entrapment and covalent attachment of enzymes to the solid-liquid interface of materials. Hydrolytic enzymes such as lysozyme and other glycosidases are environmentally friendly and they are likely to appear as alternative additives in antifouling coatings. Therefore, sol-gel chemistry has been widely used for immobilization of enzymes (Pasunooti, 2010; Tomin, 2011).

The main objective of the present article was to perform the covalent immobilization of a hydrolytic enzyme, lysozyme, for obtaining an antimicrobial surface. The strategy for this application was the utilization of sol-gel technology and carbodiimide chemistry. The amine groups of the sol-gel silicate network provides functional sites for covalent bonding of enzyme via the carbodiimide reaction (Yang, 2003). Alternatively, carboxyl functional groups are used as an active site for protein immobilization (Cullen, 2008; Dong, 2007; Kurosawa, 2004; Ying, 2003). It is mentioned that polymer brushes, like polyacrylic acid, are capable of immobilizing 30 times more enzyme compared to self-assembled monolayers (Cullen, 2008) and as much as 80 monolayers of protein in the case of BSA (Dai, 2006). Both coating steps were characterized by means of the activities of the immobilized enzymes compared with each other and their anti-biofilm activities were tested towards *Pseudomonas aeruginosa* PAO1 strain, which is a widely used biofilm-forming bacteria for studying bacterial biofilm formation.

Materials and Methods

Cleaning and conditioning of stainless steel substrates

316L stainless steel (SS) coupons (1cm in diameter, 0.2 cm in thickness), purchased from Gama Metallurgy, were cleaned and pre-conditioned according to literature methods (Minier, 2005; Chovelon, 1995). The coupons were ground with SiC sandpaper and polished using a 6 µm diamond suspension and rinsed with ethanol, then ultrasonically washed 15 minutes in cyclohexane, 10 minutes in water (three times) then 20 minutes in acetone. They were etched by sulfochromic acid (6 gr of potassium bichromate, Merck, in 100 ml of sulfuric acid 95-97%, Merck) at 60°C for 10

minutes to generate a reactive oxide/hydroxide layer (SS-SC). They were extensively washed with water and dried under a flow of nitrogen.

Preparation of stock sol–gel solution and spin coating

A homogeneous stock sol–gel solution was prepared for the application of spin coating. 20 ul of this solution was applied on the coupon, so the stock solution was prepared according to number of coupons to be coated. For this, 30 volumes of methanol (Merck), 1 volume of 10 mM HCl (Merck), 30 volumes of MTMOS (Merck) and 20 volumes of APTMOS (Merck) (Yang, 2003) was mixed in a tube, applied on the middle of the previously polished coupons and spin-coated at 2000 rpm for 30 seconds at room temperature. After coating the coupons, they were dried at 100°C for 1 hour (SS-SC-Sol).

Covalent immobilization of lysozyme on sol-gel thin film

Lysozyme from chicken egg white (specific activity 24700 U/mg) was purchased from Applichem. Covalent immobilized of the enzyme was carried out on the sol–gel coated stainless steel coupons by using the free amine groups exposed on the surface of the sol–gel layer by carbodiimide chemistry. The sol–gel coated coupons were placed in a phosphate buffer solution (0.05 M, pH 6.2) which contains 0.015 M EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Sigma-Aldrich) and 0.03 M NHS (*N*-Hydroxysuccinimide**,** Merck) for 90 minutes, and immediately transferred to an enzyme solution of 10 mg/ml lysozyme prepared in the same buffer solution for another 90 minutes. Following the coating steps, the enzyme-coated coupons (SS-SC-Sol-Lys) were rinsed with phosphate buffer solution, pH 6.2, in order to remove the excess unbound and adsorbed enzyme. All the steps for enzyme immobilization experiments were carried out at room temperature (Yang, 2003).

As an alternative method for the immobilization of lysozyme, PAA brushes were utilized as a linker between the sol-gel network and lysozyme (Figure 1). Sol-gel coated coupons which were prepared and used for the covalent immobilization of lysozyme by utilizing their free amine groups on their surfaces were reacted with 0.1 w/v% aqueous solution of PAA (average Mw ~250,000, 35 wt. % in H_2O, Sigma-Aldrich) at pH 4 in order to obtain a PAA layer on their surface. Immediately after immersing the coupons, 5 mg/ml of EDC was added. The sample was incubated overnight (16 h) at 4°C with gentle shaking. The coupons were then extensively rinsed with water to remove non-covalently adsorbed PAA (SS-SC-Sol-PAA). Remaining carboxyl groups were then utilized to immobilize lysozyme molecules. These stainless steel coupons with carboxyl groups were placed in a phosphate buffer, pH 6.2 and then 0.1 M EDC and 0.1 M NHS were added. The coupons were kept in this buffer for the activation reaction to take place for 30 minutes. (Cullen, 2008). Following the activation, the coupons were rinsed and reacted in a 10 mg/mL lysozyme solution in phosphate buffer, pH 6.2 for 90 minutes at room temperature. Lastly, the coupons were again rinsed with phosphate buffer to remove excess unbound/adsorbed enzyme and dried under a flow of nitrogen (SS-SC-Sol-PAA-Lys).

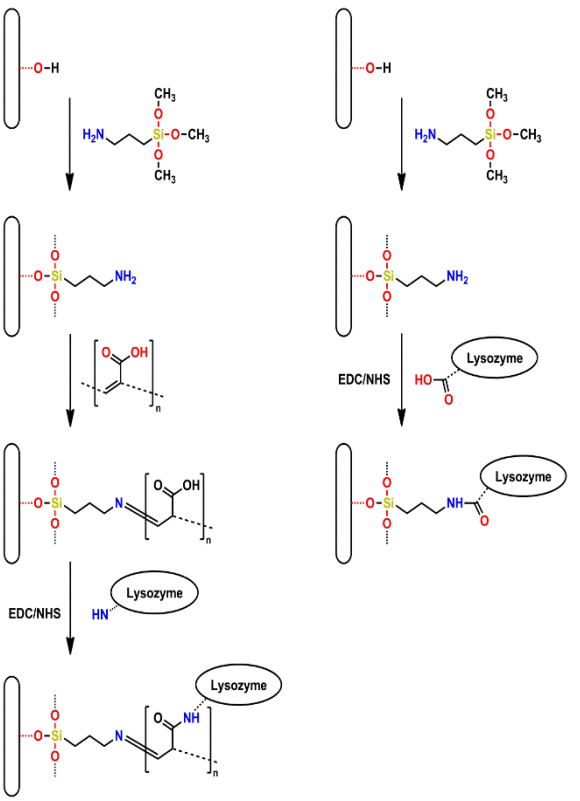

Figure 1. Reaction scheme for the immobilization of lysozyme on steel surface. The amine groups in the sol-gel network are utilized for the addition of carboxyl groups and then the covalent binding of lysozyme from its amine-end (left) or for the direct

immobilization of lysozyme from its carboxyl-end (right)

Enzymatic activity of the lysozyme coated substrates

The enzymatic activity of the stainless steel coupons coated by lysozyme with both of the procedures was measured on *Micrococcus lysodeikticus* (Sigma) cells using a procedure adapted from the classical lysozyme assay (Shugar, 1952). Stainless steel substrates were placed in the wells of a plate and 3 ml of a 0.03 % (w/v) suspension of *M. lysodeikticus* was added. This suspension was prepared by lyophilized *M. lysodeikticus* bacteria in 66 mM phosphate buffer, pH 6.24. The coupons in the wells of the plate were put on a plate shaker. The enzymatic activity of immobilized lysozyme was carried out for 270 minutes. For this, 1 ml of each suspension was aspirated every 15 minutes and the turbidity of these aspirations was measured at 450 nm in a single tube spectrophotometer. These suspensions dispensed back into the wells of the plate after every measurement. Two control experiments were carried out to measure non-enzymatic bacterial lysis (autolysis), where the turbidity of a stirred bacterial suspension alone and in the presence of a sulfochromic acid-treated stainless steel coupon (SS-SC) which was not coated with lysozyme was monitored. The changes in the optical density of different coated coupons versus time were recorded and the graphs were generated (Minier, 2005).

Biofilm studies on coated stainless steel substrates

All the coating steps were also analyzed under Confocal Laser Scanning Microscopy for the detection of their anti-biofilm activity. The coupons were placed in the wells of the flow cell and inoculated with the overnight culture of *Pseudomonas aeruginosa* PAO1 (ATCC 15692) cells. The first inoculation culture was left over the coupons for 24 h for the initial attachment and growth of the cells. After the initial attachment, growth medium was flown over the initially-attached cells. After 24 h incubation with PAO1 cells, confocal microscopy images were obtained.

Results

Enzymatic activity assays

The enzymatic activity of different coated coupons were analyzed spectrophotometrically during 270 minutes by monitoring the turbidity of a suspension of *Micrococcus lysodeikticus* (Figure 2).

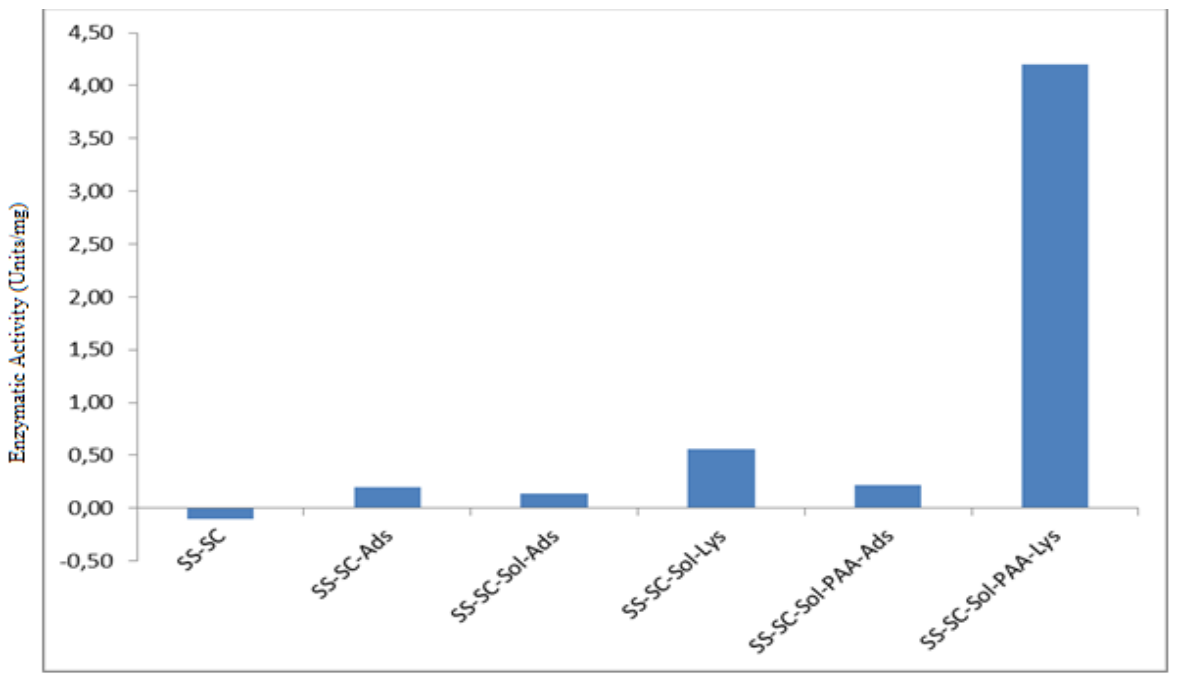

Figure 2. Enzymatic activity of six different stainless steel coupons. (Ads refers to the adsorbed lysozyme on the coupons)

Lysozyme molecules can be physically adsorbed on stainless steel coupons without any covalent attachment. However, these coupons resulted in poor enzymatic activity (SS-SC-Ads: 0.2 ± 0.01 U, SS-SC-Sol-Ads: 0.14 ± 0.02 U and SS-SC-Sol-PAA-Ads: 0.22 ± 0.44 U) whereas the covalent bonding of lysozyme on the coupons yielded a higher activity. When the enzyme is covalently bound on the surface from its amino terminus (SS-SC-Sol-PAA-Lys: 4.2 ± 0.1 U), the activity was 7.5 times higher than that of the enzyme covalently bound on the surface from its carboxyl terminus (SS-SC-Sol-Lys: 0.56 ± 0.37 U).

Biofilm studies on coated stainless steel substrates

Confocal microscopy images of different coated coupons were analyzed for their anti-biofilm efficiencies. There was not an obvious difference between the anti-biofilm activities of enzyme-coated coupons and other coupons (Figure 3), however, the activity of the enzyme on biofilm-forming cells was evident when the results were evaluated together with the other experimental findings.

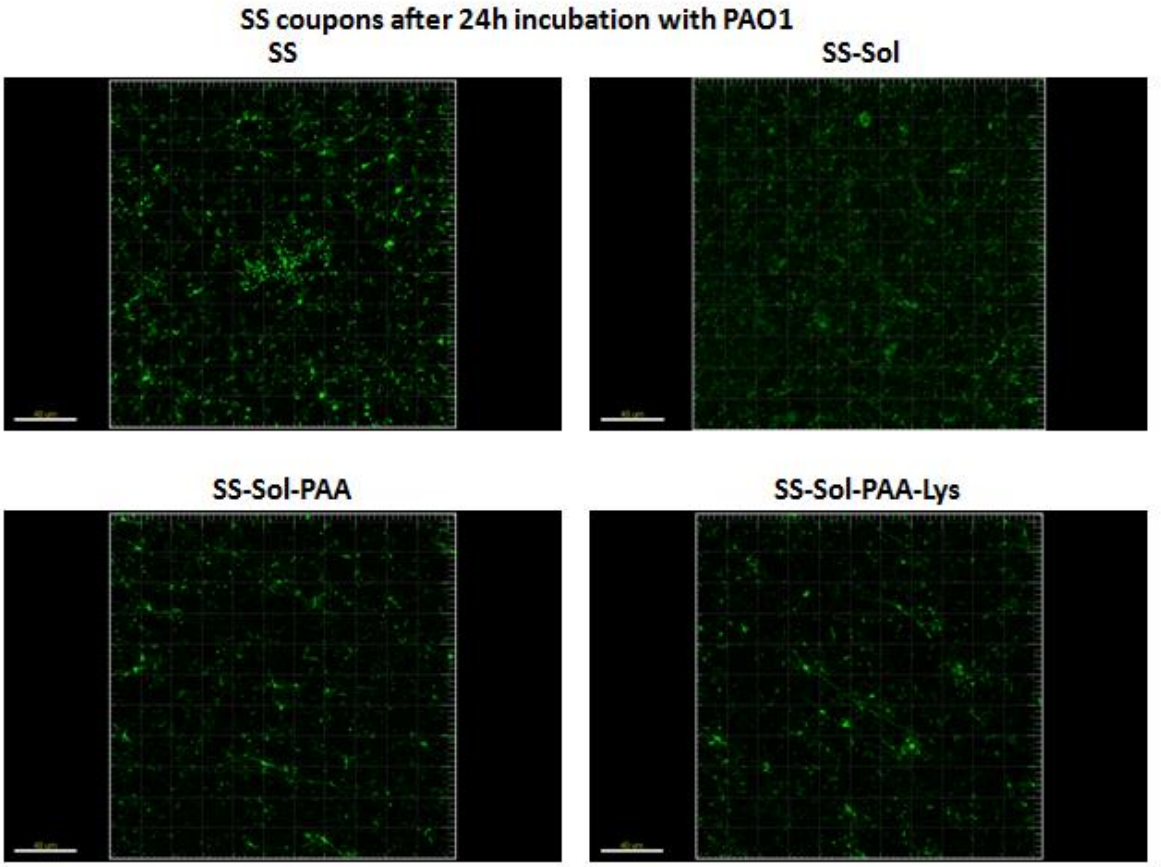

Figure 3. SS coupons after 24h incubation with PAO1

After confocal microscopy imaging of coated surfaces, serial dilutions were done to obtain the colony forming units on these surfaces. Serial dilutions of the incubated cells on several different stainless steel surfaces were prepared and these dilutions were grown on agar plates. Colony forming units were counted on these plates and were compared with the confocal microscopy images.

DISCUSSION

Lysozyme is a hydrolytic enzyme cleaving the polysaccharidic component of the cell wall of most bacteria and so, inducing the lysis of the cells. The enzymatic activity of lysozyme is generally assayed spectrophotometrically by monitoring the decrease of turbidity of a cell suspension of *Micrococcus lysodeikticus.* When the enzyme is covalently

bound on the surface from its amino terminus (SS-SC-Sol-PAA-Lys: 4.2 ± 0.1 U), the activity was 7.5 times higher than that of the enzyme covalently bound on the surface from its carboxyl terminus (SS-SC-Sol-Lys: 0.56 ± 0.37 U). This could be due to the formation of a single layer of amine groups in sol-gel thin film and when the enzyme is covalently bound, it stands very close to the steel surface, where the accessibility of the *M. lysodeikticus* cells to the enzyme becomes limited, causing a hindrance.

On the other hand, when the enzyme is covalently bound to the carboxyl functional groups of PAA, enzyme molecules might become more relaxed with better accessibility to the substrate. Also, the rigidity of the covalent linkage of lysozyme molecules could be another reason. In the case of amine containing surfaces, covalently bound lysozyme molecules are directly linked to the surface with higher rigidity. In the case of covalent binding to the polyacrylic acid layer, the enzyme molecules could be covalently bound along the length of the polymer chain (Cullen, 2008). This layer is generally called as the "polyacrylic acid brush" due to its brush-like structure (Cullen, 2008; Dai, 2006). These brushes are advantageous due to their high binding capacity which originates from their high density of reactive functional groups and their ability to swell in appropriate solvents. They do not also have cross-links between polymer chains and this could increase the accessability of proteins to the functional groups within films (Dai, 2006). So, this brush-like structure could prepare a "multi-layer" of covalently-bound enzyme molecules by supplying more regions for binding the enzyme to the carboxylic acid groups.

These finding were then tested with the biofilm studies. The surfaces obtained by two different methods were used to test their anti-biofilm activity towards *Pseudomonas aeruginosa* PAO1 strain. When consolidating all the data of AFM imaging (data not shown), confocal microscopy imaging and the colony counting of serial dilutions for different coated and uncoated surfaces showed that sol-gel coating and PAA brush coating increased the surface area when compared to the bare stainless steel coupons. This increase in surface area also increases the possibility of bacterial attachment on these surfaces. That could be the reason stainless steel coupons have the least number of attached cells even in confocal images and also the colony forming units. Sol-gel coating is known to be non-toxic and inert, meaning that it could not have an antibacterial effect on PAO1 cells. However, enzyme coated surfaces have a slightly lower number of colonies when compared to PAA-coated surfaces (data not shown) even the confocal microscopy images seem to be the same. The reason of this could be that PAA brush coating increased the surface area when compared to the bare stainless steel coupons. This increase in surface area also increases the possibility of bacterial attachment on these surfaces. Lysozyme coated coupons have an antibacterial activity towards the PAO1 cells when compared to PAA brush coating. This could mean that the active enzyme molecules immobilized on the coupons show activity towards the biofilm forming bacterial cells but even it is effective on bacteria, the number of cells attached on the PAA brush is much more higher than the sol-gel coated coupon so the number of colonies on sol-gel coated coupons are lower. This finding shows that the immobilized enzyme shows activity towards the biofilm-forming bacterial cells.

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