J Mater Sci: Mater Med (2010) 21:2775–2782
DOI 10.1007/s10856-010-4136-z

Surface with antimicrobial activity obtained through silane coating with covalently bound polymyxin B

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Received: 12 January 2010 / Accepted: 17 July 2010 / Published online: 28 July 2010

Abstract Surfaces exhibiting antimicrobial activity were prepared for potential medical application. A polycationic lipopeptide polymyxin B was selected as the bioactive agent for covalent immobilization onto the surface. First, by using sol–gel technology the inert glass substrate was functionalized by a silane coating with epoxide rings to which the peptide was coupled by means of a catalyst. Preparation of the coating and presence of the peptide on the surface were followed by FTIR, XPS and AFM analyses. The obtained material showed antimicrobial effect indicating that in spite of immobilization the peptide has retained its bioactivity. The coated surface was able to reduce bacterial cell counts of the Gram-negative bacterium *Escherichia coli* by more than five orders of magnitude in 24 h of incubation. It can be concluded that bioactive coatings with covalently bound polycationic peptides have potential for application on medical devices where leakage into the surrounding is not allowed in order to prevent bacterial growth and biofilm formation.

1 Introduction

Different materials used in medical applications are exposed to various microorganisms, which tend to adhere to their surface, limit their functionality and serve as infection loci. When microbial cells multiply to a high number they begin to stick together on the material's surface and form a specific sessile community that enables survival in hostile environments [1]. Such microbial biofilms formed on medical devices can lead to serious infections since they are highly resistant to antibiotics [2, 3]. Several approaches have been considered to prevent multiplication of microbes and growth at the surface of different devices. Staphylococcal biofilm formation on prosthetic grafts could be avoided by inhibiting bacterial cell-to-cell communication during the quorum sensing process [4]. Another approach is the design of materials with antimicrobial activity. In such a case, appropriate bioactive agents have to be involved. Several antiseptics and antibiotics [5], metal ions [6] etc. have been used. Most commonly, the active biocidal substance is incorporated into the material [5, 7] or is adsorbed to the surface [8] allowing slow release of the drug into the surrounding medium. The drawback of this approach is loss of the effect with time and potential toxicity of the released biocides to the tissue or development of resistance based on the low concentrations of the released antimicrobials.

Material with permanent antimicrobial activity can be obtained when the active drug is covalently bound onto its surface [9] however immobilized antimicrobial drugs may lose their activity as they may not reach their cellular targets. Many biomaterials such as plastics, metals or ceramics have inert surfaces. This can be functionalized through a silane polymeric layer bearing reactive groups enabling covalent binding of molecules to the surface by methods involving sol–gel technology [10, 11].

The objective of our study was to prepare surface coatings for different materials to supply them with permanent antimicrobial activity. For this purpose polycationic

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amphiphilic peptides seem to be suitable biocidal agents, since they are active against a broad spectrum of microbes and possess antimicrobial and/or endotoxin-neutralizing activity [12, 13]. In addition, they can act through more than one mechanism [14] and the development of bacterial resistance to them is very low [9, 13]. Polycationic peptides can be conjugated to the activated surface through their free amino groups. For our purpose the immobilized peptide should not be released into the environment. Additionally, the immobilization should not affect the peptide's biological activity. The coatings with biocidal activity could be deposited on surfaces of different materials used in medicine or wherever bacterial contamination must be avoided. Such coatings can be beneficial on medical devices such as catheters and implants, protecting them from microbial biofilm formation.

Here we show that a lipopeptide polymyxin B can be covalently bound to the solid surface via a silane coating with epoxy groups and the surface thus obtained exhibits high bactericidal activity towards Gram-negative bacterium *Escherichia coli*, without leaching into the environment.

2 Materials and methods

2.1 Preparation of coating

Square slides (4 cm^2) of indium-tin-oxide (ITO) glass as a substrate were cleaned with isopropanol and dried in N_2 flow. They were coated by a thin film as follows. A 50% solution of 3-glycidyloxypropyl-trimethoxysilane (GPTMS, 95%; purchased from ABCR) in isopropanol was hydrolysed with 0.1 M HCl at a molar ratio GPTMS:H_2O = 1:3 for 15 min. The solution was diluted with isopropanol to 0.3 M GPTMS which was used for deposition of the coatings by applying the dip-coating technique. The deposited coatings were thermally treated at 120°C for 30 min and later used for binding the peptide.

2.2 Antimicrobial peptide binding

Polymyxin B (PMB, Fluka) was used as an antimicrobial polycationic lipopeptide. Silanized glass slides were immersed for 1–4 h in a 1.25–2.5 mg/ml solution of PMB in Milli-Q water. Catalysts for opening the epoxy ring were added to improve the peptide binding, according to Azizi and Saidi [15]. Catalysts WCl_6 (purchased from ABCR) and phosphoric tungsten acid $H_3P(W_3O_{10})_4 \times H_2O$ (PWA; purchased from Aldrich) were used at a concentration of 0.6 mg/ml. After immobilization, the samples were immersed in Milli-Q for 20 h and then ultrasonically cleaned in fresh Milli-Q water for 10 min in order to remove all noncovalently bound PMB. The slides were dried in the N_2 flow.

2.3 Analyses of the surface

2.3.1 Fourier transform infrared (FTIR) spectroscopy

IR transmission and near-grazing incidence angle reflection–absorption (IR RA) spectra were recorded on a Bruker Model IFS 66/S spectrometer (Germany) [16]. IR transmission spectra were obtained from samples deposited on silicon wafers, while the IR RA spectra were recorded with the help of a monolayer grazing angle (80°) specular reflectance accessory (Specac) equipped with a standard polarizer for obtaining P-polarized IR light. A substrate of ITO glass was used to record the background.

2.3.2 Atomic force microscopy (AFM)

Morphology of the surface was observed by the AFM microscope; model Solver Pro, produced by NT-MDT Company (Russia). Analyses of two series of samples were performed. On each sample two sites of 20 × 20 μm^2 surface distant about 2 mm one from another were analysed. Since the topography was similar we assumed that the results were representative. AFM images of the surfaces were recorded over the area of 1 × 1 μm^2 to 20 × 20 μm^2 using a silicon tip. From the AFM images the root mean square (RMS) roughness of the surfaces was calculated after proper background subtraction.

2.3.3 X-ray photoelectron spectroscopy (XPS)

Analyses were performed under an ultra-high vacuum of 10^{-7} Pa in the XPS spectrometer Physical Electronics Inc.; model TFA XPS (USA). Al-monochromatic X-ray source was used for surface excitation. The X-ray beam energy was 1486.6 eV; the energy resolution during the analysis was around 0.6 eV. The analysed surface had a diameter of 0.4 mm, the signal during the XPS analysis came from 1 to 3 nm thick surface layer. Two types of XPS spectra were taken. First, we took an overview spectrum over a wide energy area in which we identified peaks of the elements C 1s, O 1s, Si 2p and N 1s. Using the model of homogenous matrix we calculated elemental concentrations by dividing the measured XPS intensities by their relative sensitivity factors as reported by the XPS spectrometer's producer [17]. We have also taken high-energy resolution XPS spectra of the elements C 1s, O 1s, Si 2p and N 1s over narrow energy regions. The spectra were processed with the software tool Phi Multipak, version 8.1 (Physical Electronics, Inc., USA). Two series of samples obtained in two different experiments of material preparation were analysed.

2.4 Assay of antimicrobial activity

The bacterial strain *Escherichia coli* (NCTC 8007, serotype O111 K58 H2) has been stored at −80°C. For the experiments it was cultivated on agar plates containing Luria–Bertani (LB) medium (Sigma) at 37°C for 16 h. From a single colony an overnight culture of *E. coli* in LB medium (10 ml) at 37°C and 180 rpm was prepared and diluted with fresh, sterile Tryptic Soy Broth (TSB) medium to gain bacterial suspension of $\sim 10^7$ cells/ml. Glass slides with the immobilized PMB, along with controls without the PMB, were sterilized by autoclaving for 20 min at 121°C (after having experimentally confirmed that autoclaving did not affect activity). The slides were placed into the wells of sterile 6-well tissue culture plates. Aliquots (4 ml) of bacterial culture were added into the wells and the plates were incubated at 37°C for 24 h at 60 rpm on reciprocal shaker. Antimicrobial activity of the immobilised PMB was quantified by a spectrophotometric analysis of optical density (OD) at 600 nm and by viable cell enumeration on agar plates. Viable cell counts were estimated by spreading aliquots (100 µl) of serial dilutions of bacterial culture suspension onto the LB agar plates. After overnight incubation at 37°C the number of colony-forming units (CFU) was counted. Two series of experiments were performed. Within each, samples and controls were prepared in duplicates.

2.5 Leaching test

In order to confirm that the PMB was covalently bound to the surface and that it did not leak into the liquid a test with a dialysis tube was performed. It based on the assumption that unlike the free peptide the immobilized PMB could not cross the membrane and therefore its effect could only be detected inside the dialysis tube. The TSB medium, the dialysis tube (3,500 Da cut-off) and the Erlenmeyer flasks (100 ml) with wide neck were autoclaved prior to use. From an overnight culture of *E. coli* a bacterial suspension in TSB medium containing $\sim 10^2$ cells/ml was prepared. The glass slides were placed inside dialysis tube together with 15 ml of bacterial culture. The dialysis tube was immersed in 35 ml of bacterial culture in Erlenmeyer flasks and incubated overnight (18 h) at 37°C and 90 rpm on a rotary shaker. As a negative control, glass squares without immobilized PMB were treated the same way. As a positive control, the dialysis tube with 15 ml of bacterial culture and 0.05 mg/ml free PMB was prepared and incubated as described. Growth of the bacterial culture inside and outside the dialysis tube was quantified by measurements of OD_{600} and by viable cell enumeration of the samples. Experiments were performed in two separate series with two parallels of each sample.

3 Results

3.1 Preparation of coating for peptide immobilisation

Indium-tin-oxide (ITO) glass was selected as a substrate since it has a surface reflective for infra-red (IR) light, enabling us to track immobilisation of the peptide by measuring IR reflection–absorption (IR-RA) spectra. To bind the PMB to the ITO glass slide we deposited the hydrolysed GPTMS on its surface and left to polymerize after evaporation of the solvent. A schematic procedure is shown in Fig. 1. When silane coatings are used for surface modification of ITO glass it is essential to establish the optimal hydrolysis conditions of GPTMS sol. The silanol (SiOH) groups which are formed by hydrolysis are required for the condensation of the hydrolysed GPTMS to form a sol–gel network, and also for the interfacial silane-ITO glass bonding. The hydrolysis reaction of GPTMS was confirmed by IR transmission spectra as shown in Fig. 2a. IR transmission spectra, recorded 5 min after the start of hydrolysis show practically no changes in the bands 2840, 818 and 783 cm^{-1} regarding the initial spectrum (Fig. 2a, inset) except for small but distinct decrease in intensity of the corresponding bands. The IR spectra recorded 10 min after the addition of acidified water showed a loss of these bands confirming that the hydrolysis was completed. Additional evidence for hydrolysis reactions was inferred from the increase of the band at 908 cm^{-1} attributed to the

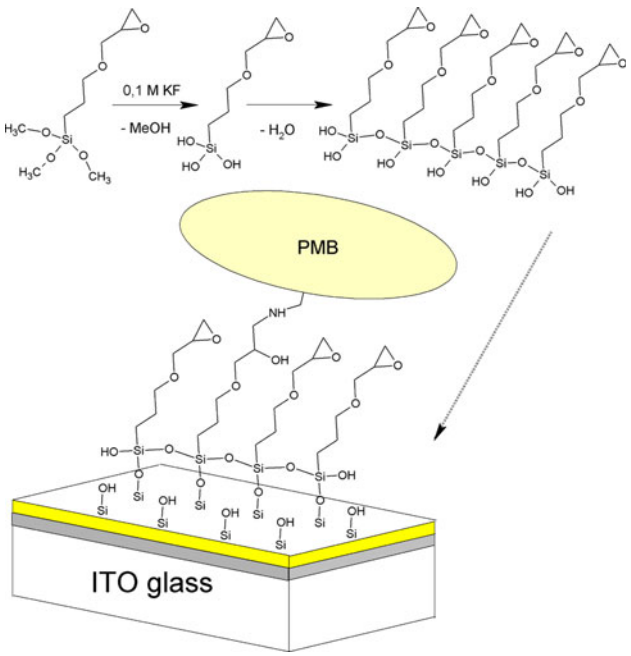

Fig. 1 Schematic presentation of linker preparation and immobilisation of polymyxin B (PMB) on solid support (coated ITO glass slide)

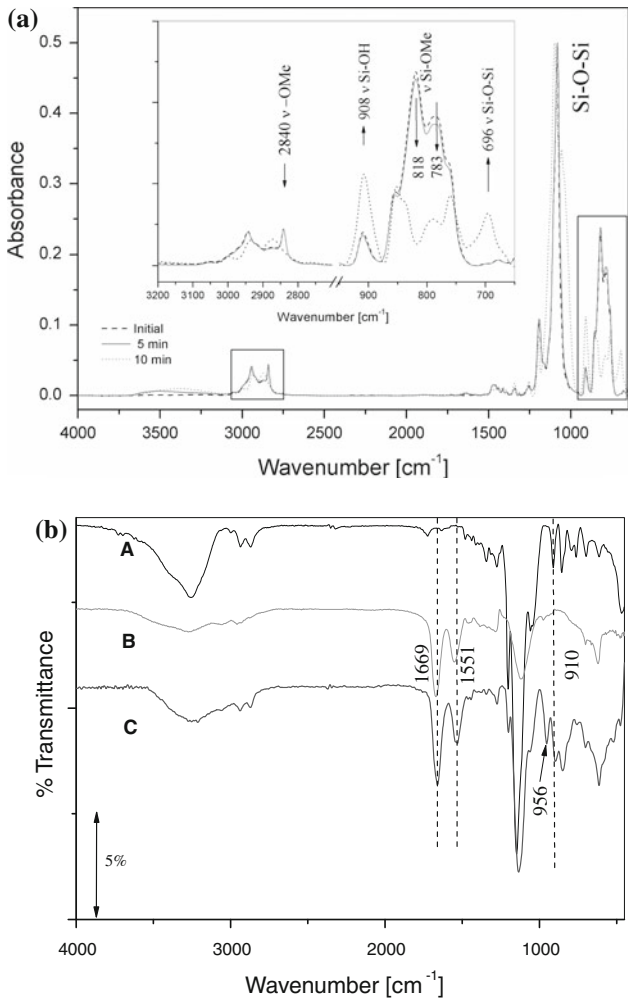

Fig. 2 **a** Time dependent IR transmission spectra of hydrolysis and condensation reactions of GPTMS. The inset shows enlargement of the two segments. **b** IR-reflection absorption spectra of GPTMS on ITO (spectrum A), PMB deposited from aqueous solution (spectrum B) and surface after immobilization of the peptide on the glass surface coated by a thin film of GPTMS (spectrum C). Peaks at 1551 and 1669 are characteristic for the PMB

SiOH groups. This band coincides with the epoxy ring mode and becomes quite intense during the course of drying (10 min) and levels off at longer drying times (2 h, not shown). As expected, drying caused appearance of the Si–O–Si bands in the spectral region of 1100–1000 cm^{-1} which signalled the condensation of silanol groups and establishment of the Si–O–Si sol–gel network. The appearance of two bands in dried films (1099, 1056 cm^{-1}) strongly suggested the presence of short linear and branched oligomers [15] linked with the Si–O–Si bonds. The persistence of the band at 908 cm^{-1} attributed to either Si–OH or epoxy ring mode, does not contradict the condensation of GPTMS because the disappearance of both bands could be expected only after opening of the epoxy ring (see the following section).

3.2 Peptide binding to the surface

PMB binding to the silanized surface was followed by IR-RA spectra. In Fig. 2b, spectra of the silanized surface (spectrum A), free PMB (spectrum B) and of the bound peptide (spectrum C) are shown. The band at 910 cm^{-1} (indicating either Si–OH or epoxy ring) present in the coating without the peptide was not detected anymore in the spectrum of immobilised PMB on the GPTMS layer. Opening of the epoxy rings and the establishment of the covalent bonding with the $-NH_2$ groups of PMB was achieved due to the addition of WCl_6 as a catalyst. In the spectrum B the bands at 1669 and 1551 cm^{-1} are attributed to the amide I and II vibrational modes which are normally found in the region between 1850 and 1350 cm^{-1} [18]. Bonding of the PMB onto the GPTMS layer was substantiated from the IR-RA spectra of the GPTMS/PMB which did not change even after washing in Milli-Q water (20 h) followed by ultrasonic cleaning (10 min) (Fig. 2b, spectrum C).

The reaction of the opened epoxy rings with the amino groups of the PMB was not directly apparent from the GPTMS/PMB spectrum, presumably due to weak R–N–R′ bands. The band at 956 cm^{-1} (Fig. 2b, spectrum C) appeared in the IR-RA spectra of GPTMS/PMB which could not be attributed either to GPTMS or PMB. The origin of the corresponding band is difficult to assign but indicates structural changes expected to appear due to the binding of PMB to the linker surface groups.

3.3 Surface analyses by AFM and XPS

The surfaces with bound peptide, along with controls without the PMB, were analysed by AFM. A topographic image obtained over the surface of 10 μm^2 is presented in Fig. 3.

It was shown that peptide binding to GPTMS layer slightly increased the RMS roughness of the surface from 5 to 6 nm $\pm$ 0.05.

We analysed the surface of the material with covalently bound PMB using the XPS method before and after incubation with the bacterium *E. coli*, compared to the glass slides coated with GPTMS layer without the peptide. Chemical composition and chemical bonding of the elements on the surface were analysed. It was found that the relative amounts of C, O and Si were similar in all three samples (Fig. 4a). Nitrogen, which was not detected in the control sample, was present in the samples with the immobilized PMB. These results confirmed that the peptide was bound to the GPTMS surface. After incubation with the bacterium, an increase in N content from 2.9 to 4.1 atomic % was detected, however, the difference was within the experimental error and could hardly be ascribed to some bound bacterial components.

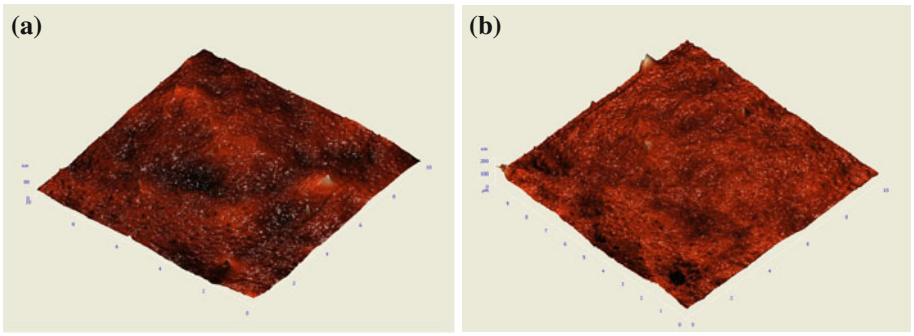

Fig. 3 Atomic force micrographs: 3D topographic scan of the surface before and after peptide binding: surface coated with GPTMS (**a**), and surface coated with GPTMS and PMB (**b**). On each sample two sites of 20 × 20 μm^2 surface distant about 2 mm one from another were scanned. A representative topography is shown. (RMS—root mean square roughness)

Analysis of chemical bonds on the surface of the material was also performed by XPS. As shown in the high-resolution XPS spectrum N 1 s of the sample with the peptide layer two peaks were detected, one at 400.0 eV and the other one at 402.0 eV (Fig. 4b). These peaks can be attributed to bonds in $–NH_2$ groups and the amide bonds, respectively. The peak of the amide bond is attributed to the covalent binding of the peptide molecule to the underlying surface [19, 20].

3.4 Antimicrobial activity of the surface and optimisation of peptide binding

Antimicrobial activity was determined by incubation of the coated glass slides in the medium inoculated with the bacterium *E. coli*. The first results of antimicrobial tests allowed us to retrospectively optimise the immobilization procedure. Varying pH value of the reaction conditions from 8.0 to 9.5 and PMB concentration in the PMB immobilization step gave surfaces with very low antimicrobial activity (data not shown). Therefore, a catalyst was introduced for opening the epoxy rings of the GPTMS layer to enable subsequent peptide binding. Viable bacterial counts after the exposure to the surfaces with the immobilized PMB obtained after using two catalysts, WCl_6 and PWA, are shown in Fig. 5. With WCl_6 we found that 1-h incubation of the slides with the peptide resulted in a

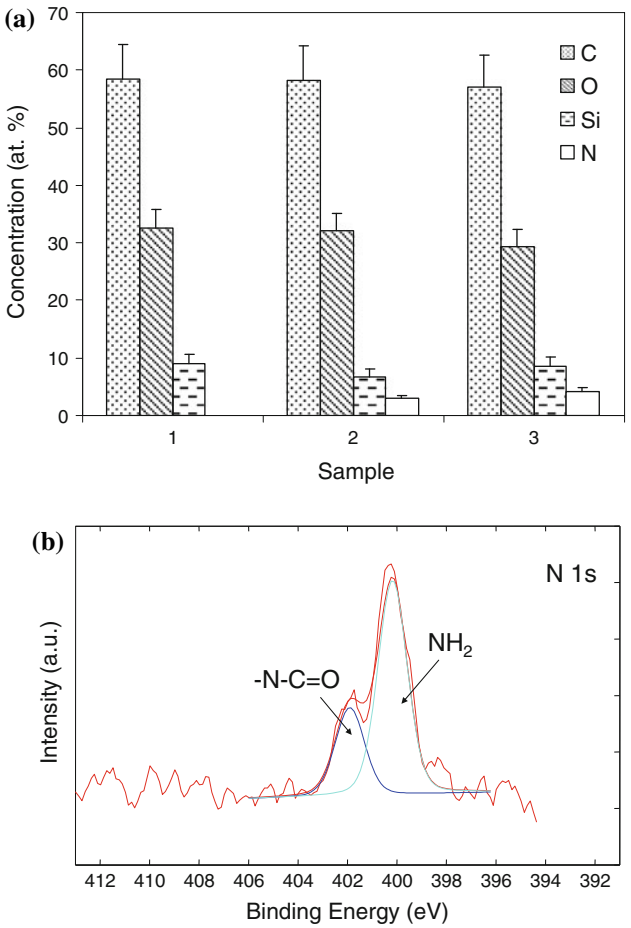

Fig. 4 **a** XPS analyses of the elemental composition of the surfaces of different samples. Sample 1—Glass/ITO/GPTMS, sample 2—Glass/ITO/GPTMS/PMB, sample 3—Glass/ITO/GPTMS/PMB after incubation with *E. coli*. **b** A high-resolution energy spectrum of N 1s obtained on the surface of the sample with the peptide layer

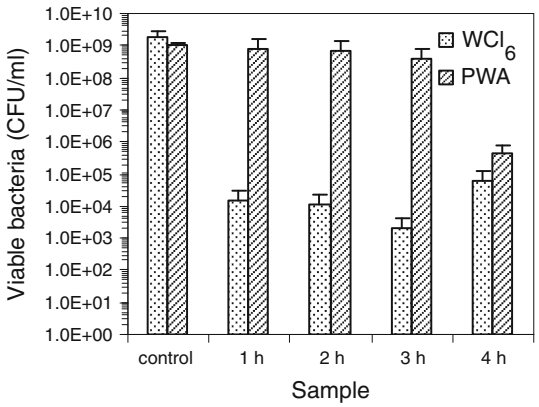

Fig. 5 Viable bacteria after exposure to the surface coated with GPTMS and immobilized PMB using two different catalysts for opening the epoxy ring in order to bind the peptide. WCl_6—tungsten hexachloride (0.6 mg/ml), PWA—phosphoric tungsten acid (0.6 mg/ml). Bacterial reduction represents the difference between the control and the sample growth, determined by counting CFU on agar plates

drastic decrease in number of viable bacteria and prolonged time did not improve much. On the contrary, in the case of PWA longer incubation time was needed for better PMB binding to the silane coating. Based on the results of antimicrobial activity we selected the following conditions for peptide binding: peptide concentration of 2.5 mg/ml, WCl_6 as the catalyst at the concentration of 0.6 mg/ml, the incubation time between 1 and 3 h. Using such conditions for peptide immobilization the surfaces were obtained which were able to reduce the bacterial cell counts for more than five orders of magnitude per ml of bacterial suspension.

3.5 Confirmation that the antimicrobial activity is due to the immobilized peptide

The generally accepted mechanism of action of PMB is that it permeabilizes the inner bacterial membrane, which could be difficult with a rigidly immobilized peptide. Therefore, further experiments were required to confirm that the antimicrobial activity is due to the bound peptide and not due to its release into the solution. The slides with the bound peptide were inserted inside a dialysis tube with the bacterial culture. The sealed tubes were then immersed into the *E. coli* culture. In such a way the immobilized peptide was separated from the bacterial suspension outside the dialysis tube. The antibacterial effect outside the dialysis tube would only be detected in the case of released PMB, which could pass through the dialysis tube. In order to detect even small amounts of the released PMB we used a low concentration of bacterial cells ($\sim 10^2$ CFU/ml). When using larger bacterial concentration the effect of small amounts of the PMB, eventually released into the solution, could not be detected. Slides without the peptide represented negative controls, while positive controls contained free PMB in the dialysis tube (Fig. 6a). After 18 h of incubation, viability of bacteria inside and outside the dialysis tube was determined. Results showed that the free PMB was able to migrate through the dialysis tube into the bulk liquid and exhibited bactericidal activity on both sides of the membrane (Fig. 6b). In contrast, the immobilized peptide showed activity only inside the dialysis tube while no activity was detected outside. These results confirmed that antimicrobial activity was due to the covalently bound PMB and it was not a consequence of peptide leaching into the solution.

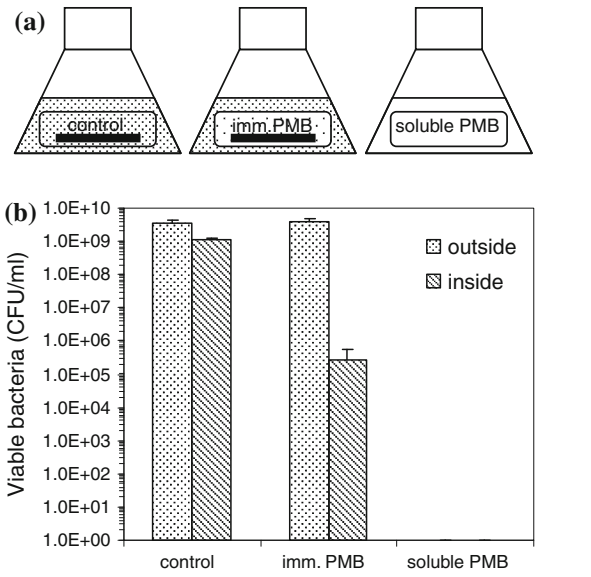

Fig. 6 Leaching test confirmed that bactericidal activity of the surface was due to the immobilized PMB and not to the free PMB, eventually released into the solution. **a** Schematic presentation of the experiment. Slides were inserted into the dialysis tube with a bacterial culture, and the tube was placed into the Erlenmeyer flask with a bacterial culture. From left to right: negative control—slide without PMB; sample—slide with surface immobilized PMB; positive control—free PMB in the dialysis tube. **b** After 18 h of incubation viable bacterial counts inside and outside the dialysis tube were determined

4 Discussion

Recently, materials containing bioactive molecules such as proteins, antibodies, peptides etc. were the subject of many studies. Such materials can improve cell adhesion, regulation of cell growth, biological identification, detection of enzyme activity etc. [21]. There is a large demand for materials that have surfaces with antimicrobial activity, and in most cases they were prepared using biocidal molecules, which were incorporated into the materials in order to be slowly released into the surrounding and to act in a free form [5, 7]. In such a case, the biological activity fades with time. In contrast, our idea was to design surfaces with covalently bound biological agent which would allow permanent activity. For this purpose, peptide antibiotics seemed suitable bactericidal agents [12, 14, 22, 23]. Antimicrobial peptides can be either endogenous in all species, acting as their host defence molecules, or synthetic. It is believed that, due to their membrane-disturbing mode of action, the development of microbial resistance is less possible than against other types of commonly used antibiotics [9].

In order to bind the peptides onto the inert surfaces these can be functionalized through silane coatings prepared by sol–gel technology [24, 25]. Such coatings increase hydrophobicity, which can already reduce adherence of bacteria to the surface. In our study, the model substrate was coated with a silane film bearing epoxy rings as the reactive groups. Epoxy-activated supports have several advantages for covalent protein binding, such as high stability and formation of strong linkages with nucleophilic

residues [26]. The antimicrobial peptide was covalently bound to them via its amino groups. We selected a catalyst to enhance the coupling reaction. The coating thus obtained exhibited biocidal activity, which can not be ascribed to hydrophobicity but can only be due to the antimicrobial activity of the bound PMB. The *E. coli* cell counts were reduced for 5 log units per ml of bacterial suspension and per cm^2 of the surface. Comparison with the results of other studies shows that our surface was more effective than the one reported by Perni and co-workers [27] who obtained up to 3.5 log reduction in the viable *E. coli* cells/ml after exposure to reactive oxygen as a biocidal agent. On the other hand, Harney and co-workers [28] reached up to 7 log units reduction of *E. coli* cells per cm^2 using a coating with amphiphilic quarternary ammonium biocides. These, however, have strong haemolytic activity unlike the PMB, which showed very low haemolysis even at 500 μg/ml concentration [29].

Studies with covalently bound antimicrobial peptides to prevent biofilm formation have also been reported [9, 30–32]. However, direct comparison of the activity is difficult due to different methods used. Willcox and coworkers [32] succeeded in reducing the number of *Pseudomonas aeruginosa* and *Staphylococcus aureus* on contact lenses with bound synthetic peptide by more than 70%. In our experiments the reduction of viable *E. coli* bacteria was even higher (more than 99%).

Some research groups reported that for ensuring the activity of the immobilized peptide it had to be bound to the surface via a long spacer molecule. Gabriel and co-workers [30] conjugating the peptide LL37 to titanium surface found that the activity was present only if the peptide was bound site-specific at the N-terminus via a long spacer to allow lateral mobility. Similarly, Bagheri and co-workers [9] reported that the peptide flexibility and the length of the spacer are crucial. In contrast to the above mentioned findings, in our experiments random coupling of the peptide without the spacer was suitable to confer the surface with bactericidal activity, probably due both, to the selected sol–gel material and the selected peptide.

PMB does not attack a single target within bacteria [14] and therefore the development of resistance by the bacteria is not very common [14, 33]. However, some pathogens possess intrinsic resistance to polymyxins and even acquired resistance has been observed in some bacteria [34]. Polymyxins, as cationic peptides, when free in the solution, increase permeability of the Gram-negative bacterial cells. In the initial step, electrostatic interactions with the anionic lipopolysaccharide molecules in the outer membrane of bacteria lead to the displacement of Ca^{2+} and Mg^{2+} ions from the lipopolysaccharides and thus to the destabilisation of the membrane. Subsequently, the peptides penetrate through the outer membrane to permeabilize the inner membrane and dissipate the membrane potential [22, 35]. In the case of surface bound PMB only electrostatic interactions with the structurally critical divalent cations can be involved. The second phase, penetration through the inner membrane, may not be a prerequisite for antimicrobial activity. In our experiments covalent immobilization of PMB through its amino groups to the glycidoxysilane coating did not disturb its biocidal activity.

5 Conclusion

Surfaces with biocidal activity can be obtained by preparing a glycidoxysilane coating with reactive groups to which antimicrobial peptides can covalently bind. The use of a catalyst, such as WCl_6, improves the coupling reaction between the epoxy rings of the silane layer and the amino groups of the peptide. Presence of the peptide on the surface was confirmed by IR-RA and XPS. The obtained surface exhibits biocidal activity indicating that for the antimicrobial effect the peptide does not need to have a long linker to reach its target. We propose that high density of positive charge created by immobilized PMB at the surface is responsible for the antimicrobial activity. It can be concluded that coatings with covalently bound antimicrobial peptides show potentials for the application in biomaterials for medical devices, such as catheters, implants etc.

Acknowledgment The authors wish to thank Dr Janez Kovač, Jožef Stefan Institute, Ljubljana, Slovenia for AFM and XPS analyses and Prof Ignacio Moriyon, University of Navarra, Pamplona, Spain, for kindly providing the *E. coli* strain O111 K58 H2. Financial support by the Slovenian Research Agency is gratefully acknowledged.

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