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Bacterial-growth inhibiting properties of multilayers formed with modified polyvinylamine

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ABSTRACT

New methods are needed to fight antibiotic-resistant bacteria. One alternative that has been proposed is non-leaching, permanently antibacterial surfaces. In this study, we test multilayers formed with antibacterial cationic polyvinylamine (PVAm) and polyacrylic acid (PAA) in a growth-inhibition assay. Both hydrophobically modified and native PVAm were investigated. Multilayers did reduce the bacterial growth, as compared to single layers. However, the sampling time in the assay was critical, as the treated surface area is a capacity-limiting factor. After 2 h incubation, a maximal growth inhibition of more than 99% was achieved with multilayers. In contrast, after 8 h we observed a maximal growth-inhibition of 40%. At longer incubation times, the surface becomes saturated, which explains the observed time-dependent effectiveness. The polymers giving multilayers with the strongest growth-inhibiting properties were native PVAm and PVAm modified with C_8, which also were the polymers with highest charge density. We therefore conclude that this effect is mainly an electrostatically driven process. Viability staining using a fluorescent stain showed a high viability rate of the adhered bacteria. The multilayers are therefore more bacteriostatic than antibacterial.

1. Introduction

Bacteria are a natural element in our ecosystem. In some sensitive situations and environments, such as hospitals, it can though be desirable to control bacterial growth. This control has traditionally been achieved by direct application of disinfectants and biocides. The approach is not without problems. Given the wrong dosage or wrongly chosen biocide, biocide resistant strains can evolve. In some cases, as with triclosan or silver, antibiotic resistance can be induced [1]. This approach leads to the leaching of toxic compounds into the environment, where they are harmful to both beneficial microorganisms and higher organisms.

An alternative method of bacterial control is non-leaching, permanently antibacterial surfaces [2,3]. By immobilising cationic polymers via covalent modification, it is possible to achieve a high, constant biocide concentration [2]. The use of cationic substrates also offers another advantage, as the positive charges electrostatically attract negatively charged bacteria [4]. Since the cationic polymers are immobilised, the bactericidal effect of these polymers must be based on physical disruption of the bacterial cell envelope [5]. Two major biocidal mechanisms have been proposed for antibacterial surfaces. The first claims that polycations with hydrophobic modifications interact with the outer bacterial membrane of Gram-negative bacteria, thereby causing bacterial lysis [2,6]. To accomplish this, the polymers must have sufficiently long branches to be able to penetrate the bacteria [7] as the bacterial cell envelope is around 50 nm [8]. The second proposed mechanism is based on findings of a charge density threshold for antibacterial surfaces [4,8–10]. According to this theory, the surfaces displace the stabilising counterions of bacteria, mainly divalent Mg^{2+} and Ca^{2+} in an ion-exchange process similar to polymer adsorption [4]. The mechanism is known for antibacterial compounds in solution such as EDTA and polylysine [11]. It is also interesting to note that many naturally occurring surfaces are neutral or bear a negative charge.

A drawback of the contact-active surfaces has so far been the chemistry used for surface preparation. Using covalent attachment, the surface preparation is often elaborate and involves organic solvents [2,4,7,9,10,12]. An appealing alternative would instead be physical adsorption, such as the polyelectrolyte multilayer (PEM) technique. The multilayer technique, based on the stepwise adsorption of oppositely charged polymers, was first introduced by Decher in the 1990s [13]. The simple, yet precise, process can be carried out on any charged surface, regardless of geometry, and can be performed in aqueous solutions. Multilayers have been used to construct anti-adhesive surfaces [14,15] as well as to incorporate antibacterial substances for controlled release applications [16–18]. However, considering the advantages of the technique, surprisingly few articles on contact-active antibacterial PEM systems have been published [19,20,21]. Wong et al. [21] was able to reduce bacteria by using multilayers of polycationic *N*,*N*-dodecyl,methyl-polyethylenimine and polyacrylic acid

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(PAA). However, the multilayers were constructed with organic solvents for the cationic polymer. In the study they found the bactericidal effect to improve by higher number of layers. Similarly, in the work of Westman et al. [20], the antibacterial activity of a hydrophobically modified polyvinylamine (PVAm) and PAA against *Escherichia coli* was found to increase with increasing number of layers. The highly charged weak polyelectrolyte PVAm was used together with PAA to form PEM-covered surfaces on regenerated cellulose in aqueous solutions in room temperature. The PVAm/PAA multilayer system has been studied in detail [22]. The native PVAm and especially the hydrophobically modified PVAms have been shown to have excellent antibacterial properties in solution [23,24]. In the study by Westman et al. [24] the antibacterial properties of PVAm in suspension were increased more than tenfold by hydrophobically modifying the polymer backbone.

In the present contribution, the work by Westman et al. and Illergård et al. is continued by screening immobilized polymers with different modifications and different numbers of layers to develop mechanism of the observed growth-inhibiting effect.

2. Experimental

2.1. Materials

Polyvinylamines with different substituents and degrees of modification (Table 1) were supplied by BASF SE (Ludwigshafen, Germany). The molecular weight was 250 kDa for the unmodified PVAm and 340 kDa for the modified PVAm. The details of the polymer synthesis can be found elsewhere [25]. The polymers were dialysed and freeze-dried prior to use. Polyvinylamines are weak polyectrolytes; *i.e.* their charges depend on pH and charge densities have been previously determined for all polymers used except PVAm-C_4 [24]

Anionic polyacrylic acid (PAA) (Sigma) with a molecular weight of 240 kDa according to the supplier was used without further purification.

MilliQ water (MQ) (Millipore, Solna, Sweden) was used to prepare all polymer solutions.

Commercially available cover slides (VWR, Stockholm, Sweden) were used as substrates for multilayer formation. The slides were made out of pure, white glass and had a diameter of 13 mm. Prior to use, the slides were cleaned by rinsing with a sequence of MQ–EtOH–MQ and hydrolysed in 10% NaOH for 30 s. Finally, the slides were plasma treated at 10 W for 30 s at reduced air pressure. Untreated glass slides were used without additional cleaning as a negative control in the growth experiments.

Two commercial antibacterial fabrics treated with octadecyldimethyl(3-trimethoxysilylpropyl) ammonium chloride (AEGIS Microbe shield, Midland, USA), hereafter designated Fabric1 and Fabric2, were used as positive reference samples. Fabric1 consisted of 100% polyester fabric for clean room use (Precision fabrics, Bamberg, Germany). Fabric2 consisted of cotton terry intended for household usage (LaRedoute, Borås, Sweden). As the terry absorbed water, it was first saturated with 100 mM NaCl before testing (Fig. 1).

Table 1
The different PVAm polymers used in the present study. The polymer properties are according to the supplier's specifications.

Polymer	Substituent	Hydrolysis	Degree of substitution
PVAm	–	100%	–
PVAm-C_4	C_4	92.5%	100%
PVAm-C_6	C_6	90.7%	30%
PVAm-C_8	C_8	90.7%	10%

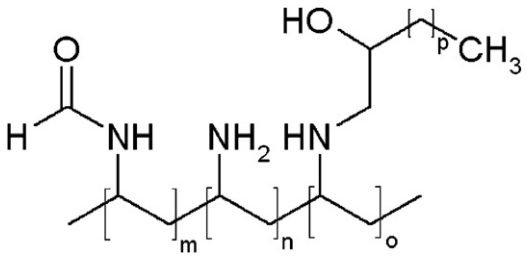

Fig. 1. The principal polyvinylamine polymer structure. In this study, the hydrophobically modified PVAm corresponds to a p of 1, 3, or 5.

2.2. Bacteria

The test organisms for the growth-inhibition assay were Gram-negative *E. coli* ATCC 11775 obtained from SIK (Göteborg, Sweden) and Gram-positive *Bacillus subtilis* (MERCK, Solna, Sweden). The bacteria were grown in tryptone glucose extract broth (TGE) (BD Difco, Stockholm, Sweden) at 37 °C with continuous shaking.

2.3. Methods

2.3.1. Polyelectrolyte multilayers

Polyelectrolyte multilayers were formed on negatively charged cover slips using PVAm as the cationic polymer and PAA as the anionic polymer. The conditions for the multilayer build-up were chosen for maximal adsorption, based on results from a previous study [22]. The polymer concentration was 0.100 g/L, and a salt concentration of 100 mM NaCl was used. The pH was adjusted to 7.5 for PVAm and 3.5 for PAA. Salt solutions with the same salt concentrations and pH as the polymer solutions were used to rinse the surfaces after each polymer adsorption step. The samples were rinsed with salt solution after the final adsorption step and were thereafter air dried. All future references to the multilayer thin films in this report will be denoted as "Polymer name–*x*", where *x* is the number of monolayers. Uneven numbers are capped with PVAm, whereas even implies that PAA is in the outer layer.

2.3.2. Contact angle analysis

The contact angles for water were measured with a CAM 200 (KSV, Helsinki, Finland).

2.3.3. Growth-inhibition experiments

Growth medium consisting of 10% TGE and 90% 100 mM NaCl was inoculated with *E. coli* or *B. subtilis* to a final bacterial concentration of 10^3 colony forming units (CFU)/mL. This growth-medium mixture was used to limit the maximal growth, and to facilitate spectroscopic readings. One milliliter of the suspension was added to each test surface placed in a multiwell plate with a bottom diameter of 15 mm (Nunc, Roskilde, Denmark). Each sample condition was tested by two samples. The plates were incubated at 30 °C with 90 rpm shaking. The growth was analysed by transferring two aliquots of 200 μL from each test sample to a transparent 96-well microplate (Nunc) and reading the optical density (OD) of these aliquots at 540 nm (Labsystems Multiskan MCC/340, Thermo Scientific, Sweden). The values were corrected for background adsorption and were compared to the negative control by calculating the relative growth according to Equation 1

$$\text{Relative growth} = \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{reference}}} \quad (1)$$

where $\text{OD}_{\text{sample}}$ is the OD averaged over the two test samples at 540 nm and $\text{OD}_{\text{reference}}$ is the average OD for the reference samples (untreated glass slides) at the same wavelength.