

## Soft nanotechnology: the potential of polyelectrolyte multilayers against *E. coli* adhesion to surfaces

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Preventing bacterial attachment to surfaces is the most efficient approach to controlling biofilm proliferation. The aim of this study was to compare anti-adhesion potentials of 5 and 50 mmol/L polyelectrolyte multilayers of poly(allylamine hydrochloride)/poly(sodium 4-styrenesulfonate), poly(4-vinyl-*N*-ethylpyridinium bromide)/poly(sodium 4-styrenesulfonate), and poly(4-vinyl-*N*-isobutylpyridinium bromide)/poly(sodium 4-styrenesulfonate) against *Escherichia coli*. Glass surface was covered with five polyelectrolyte layers and exposed to bacterial suspensions. Poly(4-vinyl-*N*-ethylpyridinium bromide)/poly(sodium 4-styrenesulfonate) was the most effective against bacterial adhesion, having reduced it by 60 %, followed by poly(4-vinyl-*N*-isobutylpyridinium bromide)/poly(sodium 4-styrenesulfonate) (47 %), and poly(allylamine hydrochloride)/poly(sodium 4-styrenesulfonate) (38 %). Polyelectrolyte multilayers with quaternary amine groups have a significant anti-adhesion potential and could find their place in coatings for food, pharmaceutical, and medical industry.

**KEY WORDS:** bacterial adhesion; poly(allylamine hydrochloride)/poly(sodium 4-styrenesulfonate); poly(4-vinyl-*N*-ethylpyridinium bromide)/poly(sodium 4-styrenesulfonate); poly(4-vinyl-*N*-isobutylpyridinium bromide)/poly(sodium 4-styrenesulfonate) surface hygiene

Bacterial attachment to surfaces and proliferation under favourable conditions can lead to biofilm growth, lower product quality, a failed industrial process, and eventually adverse health issues (1). This bacterial surface attachment depends on several factors, including bacterial cell properties (surface charge, flagella, and extracellular polymeric substances), surface properties (roughness, surface charge, and chemical composition), and environmental conditions (nutrient, temperature, pH, and the presence of antimicrobial substances) (2). During the initial stage of cell adhesion, bacterial cells interact with substrate surface chemically and physically. Interactions typically contributing to bacterial adhesion are electrostatic, van der Waals, and hydrophobic or hydrophilic (3).

The most effective approach to bacterial management is to prevent cell adhesion rather than to treat it. Some have tried to accomplish this with antimicrobial coating and others by modifying surface properties (4). The anti-adhesive effect can be achieved by inhibiting close contact between the cell and the surface with hydrophilic polymer-based polyelectrolyte multilayers (PEMs) or by stiffening the surface so that the cells cannot attach (5).

The last decade saw a remarkable progress with PEMs (6–9), which have good antibacterial properties but are not toxic to eukaryotes, humans included. Another appealing advantage of PEMs is that their layered structures can be

tuned with nanoscale precision to obtain desired surface properties (9). PEMs owe their antibacterial effects to hydrophobicity and charge interaction, which destabilises and disrupts bacterial cells (10–15).

The aim of this study was to compare the anti-adhesion potential of newly synthesised poly(4-vinyl-*N*-ethylpyridinium bromide) (PVP-ethyl Br) and poly(4-vinyl-*N*-isobutylpyridinium bromide) (PVP-isobutyl Br) with a well-established poly(allylamine hydrochloride) (PAH) of earlier generation to identify the most efficient against bacterial adhesion.

## MATERIALS AND METHODS

### *Bacterial strain*

Standard strains of *Escherichia coli* ATCC 35218 were obtained from the Czech Collection of Microorganisms (Brno, Czech Republic). *E. coli* is often used as a model organism and indicator of faecal contamination and is, therefore, included in hygiene assessment (16).

### *PEM preparation and characterisation*

PAH ( $M_w=15000$  g/mol, Sigma-Aldrich, St. Louis, MO, USA), (PVP-ethyl Br), and (PVP-isobutyl Br) were used as polycations, and poly(sodium 4-styrenesulfonate) (PSS;  $M_w=70000$  g/mol, Sigma-Aldrich, USA) was used as polyanion. PVP-ethyl Br and PVP-isobutyl Br were prepared with the nucleophilic substitution of alkyl bromide

on poly(4-vinylpyridine) ( $M_w \approx 60000$  g/mol, Sigma-Aldrich) as described below.

PVP-ethyl Br was prepared using a modified procedure described by Okubo and Ise (17); 2.08 g of poly(4-vinylpyridine) were dissolved in 20 mL of nitromethane and added 30 mL of ethyl bromide with vigorous stirring. The mixture was heated to 45 °C and stirred overnight. The obtained precipitate was filtered, dried, and ground in a mortar. The obtained powder was solved in the filtrate, and 10 mL of ethyl bromide added. After another 24 h of heating at 45 °C, the volatiles were evaporated. The residue was dissolved in 40 mL of ethanol and added to 250 mL of dioxane. The precipitated product was filtered and dried under vacuum, yielding 3.73 g of polymer.

PVP-isobutyl Br was prepared by dissolving 3.00 g of poly(4-vinylpyridine) in 60 mL of nitromethane, and 40 mL of isobutyl bromide was added with vigorous stirring. The mixture was heated to 60 °C and stirred for seven days. During the first 24 h, the product was separated as oil. Volatiles evaporated after seven days, and the obtained residue was dissolved in 100 mL of ethanol. The precipitation of the product was induced by abrupt addition of ether (300 mL). The product was filtered, washed with ether, and dried under vacuum, yielding 4.30 g.

Polycation monomer functionalisation degrees ( $f$ ) of PVP-ethyl Br and PVP-isobutyl Br were determined with potentiometric titration, either with a standardised solution of sodium hydroxide or with an  $\text{AgNO}_3$  solution of known concentration. The monomer functionalisation degree of PSS was determined spectrophotometrically. The molar absorption coefficient of PSS(aq) in 5 % KCl(aq) by weight

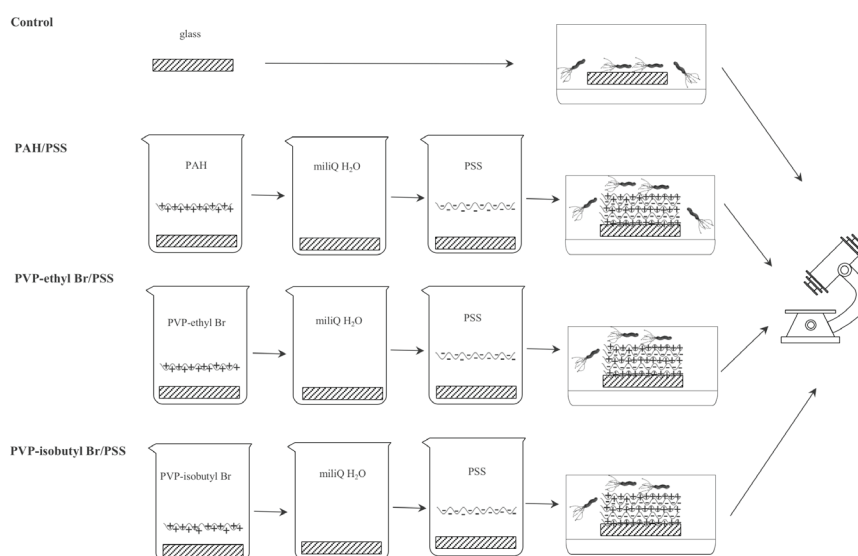
( $\epsilon=420$  L/cm/mol at 261.5 nm) used for this purpose was taken from literature (18). The following values were obtained:  $f(\text{PAH})=0.89$ ,  $f(\text{PVP-ethyl})=0.91$ ,  $f(\text{PVP-isobutyl})=0.57$ , and  $f(\text{PSS})=0.83$ .

#### Multilayer preparation

Stock solutions (100 mmol/L) of polyelectrolytes were prepared by dissolving appropriate amounts of solid polyelectrolyte in miliQ water and then diluting it to 5 mmol/L or 50 mmol/L solutions. Borosilicate glass (1 mm thick, Isolab, Eschau, Germany) was cut in  $1 \times 1$  cm squares, cleaned with ethanol and miliQ water, and then autoclaved before use. The glass was then coated with PEMs using a layer-by-layer method that is highly versatile for surface modification. It involves dip coating to deposit complementary molecules on the surface (14). First, the glass surface was soaked in  $\approx 50$  mL of polycation solution (PAH or PVP-ethyl Br or PVP-isobutyl Br) for five minutes and then rinsed with water. After that, it was soaked in PSS for another five minutes and rinsed with water. These two steps were repeated until five layers were deposited, and the polycation was the top layer (Figure 1).

#### Monitoring bacterial adhesion on PEMs

*E. coli* from the collection were transferred to nutrient agar and incubated at 37 °C for 24 h. After that, a single colony was transferred to nutrient broth (Biolife, Milan, Italy) and incubated in the same conditions. Adhesion was tested with a modified method described by Bohinc et al. (19). Overnight *E. coli* culture was diluted with fresh



**Figure 1** Sample coating and anti-adhesion assessment

nutrient broth in a 1:300 ratio, and the newly inoculated medium poured over the PEM-coated glass samples. Control samples were those not coated with PEMs. The samples were incubated at 37 °C for 1 h to achieve irreversible bacterial adhesion (20). After incubation, the medium was removed. Loose cells were then removed with phosphate-buffered saline (PBS) (0.026 g KH<sub>2</sub>PO<sub>4</sub>, 0.047 g K<sub>2</sub>HPO<sub>4</sub> in 1 L) and the remaining attached bacteria stained with 0.1 % crystal violet (Merck, Darmstadt, Germany) suspension for five minutes. The dye was removed and the cells counted with a Olympus CX40 light microscope (400x magnification) with a CCD CMOS camera (Olympus, Tokyo, Japan). Each counting was done in triplicate and cell counts expressed as log number of cells per square millimetre (Figure 2).

*Statistical analysis*

Average bacterial counts (of 15 samples per PEMs or control) were compared on R software version 3.1.3 (Bell Laboratories, New Jersey, NJ, USA) with the paired Student's *t*-test between control and PEM-coated surfaces. Differences were considered significant at *p*<0.05.

RESULTS AND DISCUSSION

Surfaces coated with PAH/PSS PEMs showed significantly lower bacterial counts than uncoated control glass surfaces (*p*<0.05). Moreover, bacterial counts decreased with higher polyelectrolyte concentrations (50 mmol/L). The best anti-adherent effect was achieved with PVP-ethyl Br/PSS at 50 mmol/L, which reduced bacterial adhesion up to 60 %, followed by PVP-isobutyl Br/PSS (38.4 %), and PAH/PSS (38.1 %) (Table 1).

Light microscopy confirmed significant reduction in *E. coli* adhesion for all PEMs, which again was especially pronounced for PVP-ethyl Br/PSS (Figure 2).

As expected, our findings have confirmed anti-adhesion properties of polyanion top layers in PEMs (22–25). Kovačić et al. (23), in fact, reported that only 20 % of *Pseudomonas aeruginosa* stuck to the surface. Similar 80 % reduction was reported for *E. coli* by Richet et al. (25) for chitosan/hyaluronan PEMs.

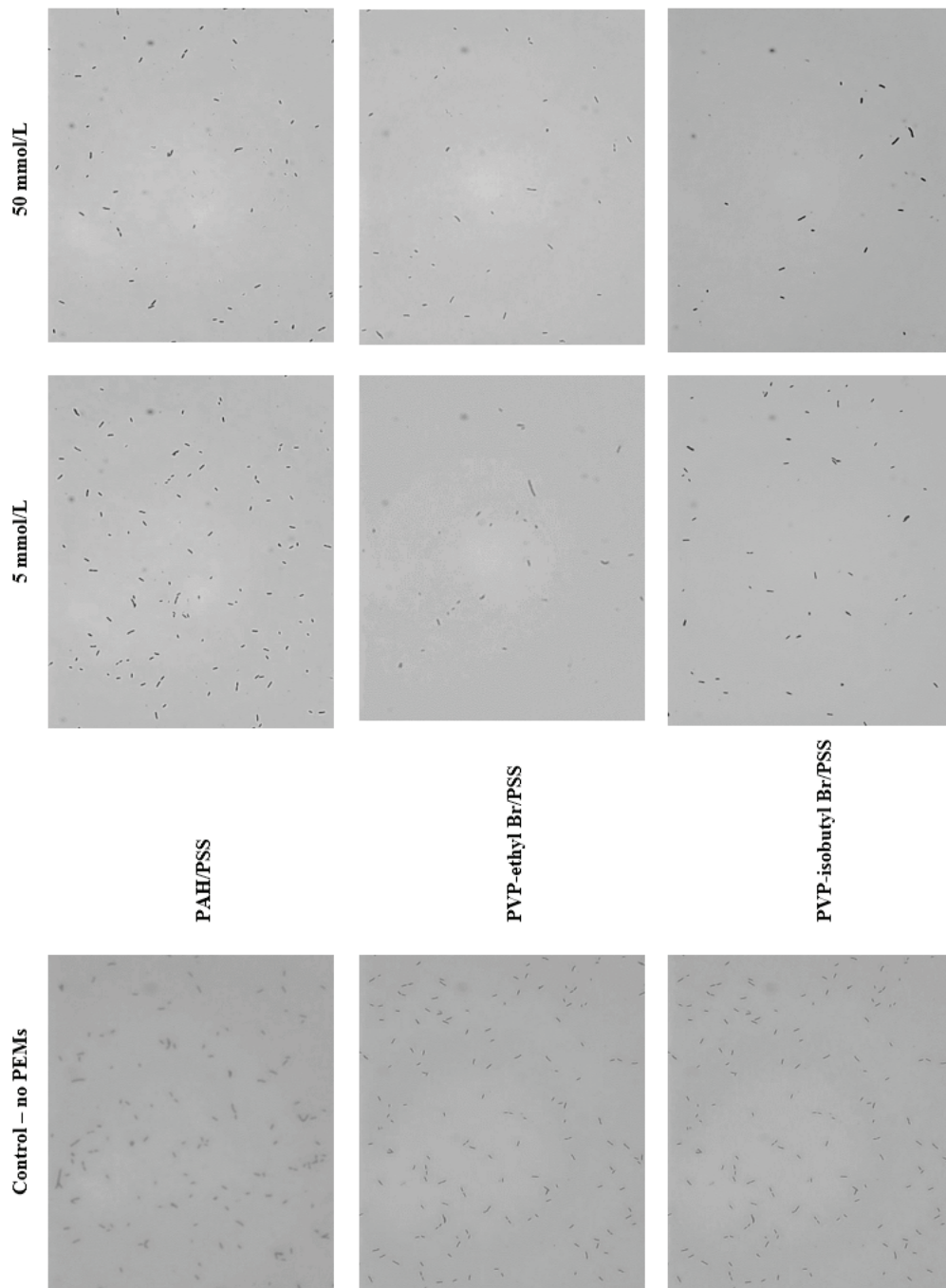
Our study has also demonstrated the efficacy of polycation top layer against bacterial adhesion. Guo et al. (8) reported that polycations with quaternary ammonium groups have sufficient charge density on flexible backbones to prevent adhesion. The interaction between positively charged molecules and negatively charged cell membranes can cause leakage of intracellular constituents and, consequently, cell detachment.

Besides concentration, the structure of polyelectrolytes seems to have a significant role in antibacterial properties of PEMs. We observed that polycations with quaternary amine groups (PVP-ethyl and PVP-isobutyl) had greater anti-adhesive potential than polycations with primary amine

Table 1 Comparison of bacterial cell counts on control surfaces and surfaces coated with PEMs

Polyelectrolyte multilayers	Bacterial count (log/mm <sup>2</sup> )	Difference to control (Δ log no. bact./mm <sup>2</sup> )	Bacterial count (no./mm <sup>2</sup> )	Difference to control (%)	t-value	p-value
PAH/PSS 5 mmol/L	3.784	0.079	6,316	17.3	3.577	0.0010*
PAH/PSS 50 mmol/L	3.651	0.212	4,771	38.1	7.942	0.0000**
PVP-ethyl Br/PSS 5 mmol/L	3.589	0.274	4,058	47.3	11.743	0.0000**
PVP-ethyl Br/PSS 50 mmol/L	3.466	0.397	3,075	60.0	16.637	0.0000**
PVP-isobutyl Br/PSS 5 mmol/L	3.726	0.137	5,504	29.8	6.163	0.0000**
PVP-isobutyl Br/PSS 50 mmol/L	3.641	0.222	4,750	38.4	6.138	0.0000**
<b>Control – no PEMs</b>	<b>3.863</b>	/	<b>7,708</b>	/	/	/

\* *p*<0.05; \*\* *p*<0.001



**Figure 2** Microscopy of *E. coli* cells on control glass and glass coated with PEMs (400x magnification)



groups (PAH). This may be related to different charge density of the polyion, which in PAH depends on pH. Furthermore, some steric factors and hydrophobicity of the monomers could have influenced the observed behaviour of PEMs. It is expected that polyelectrolytes with longer and more hydrophobic side chains would have stronger anti-adhesion properties (13).

However, the development of anti-microbial coatings should rely on the safe-by-design concept. This includes precautionary measures and tools to identify uncertainties and potential risks at the earliest feasible stage of development. Understanding antimicrobial toxicity of applied coatings or their production and surface application and durability is needed to assure safety. For example, the same antimicrobial coating if applied inappropriately may on different surfaces lead to a release of biocides due to incomplete chemical binding (26).

Science is facing new challenges in creating surfaces that would allow systematic management of the attachment of living cells. PEMs provide numerous coating opportunities that can be used to control bacterial attachment. Our study has demonstrated that the selection of proper PEM and proper layering and concentration can optimise anti-adhesive efficiency. In this respect, the best result (60 % reduction) was achieved with top-layer polycations with quaternary amine groups. This may encourage synthesising and evaluating new polyelectrolytes for better anti-adhesion coatings. At the same time, future research should assess potential toxic effects of PEMs.

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### Mehka nanotehnologija: Anti-adhezivni potencial polielektrolitskih premazov proti adheziji *E. coli* na površine

Preprečevanje adhezije bakterij na površine je najbolj učinkovit način obvladovanja rasti biofilmov. Namen te raziskave je bil analizirati anti-adhezivni potencial 5 in 50 mmol/L polielektrolitskih plasti poli(alilamin hidroklorid)/poli(natrijev 4-stirensulfonat), poli(4-vinil-*N*-etilpiridin bromid)/poli(natrijev 4-stirensulfonat) in poli(4-vinil-*N*-izobutilpiridin bromid)/poli(natrijev 4-stirensulfonat) na bakterijo *E. coli*. Pet zaporednih plasti polielektrolitov je bilo sestavljenih na steklenih površinah in izpostavljenih bakterijski suspenziji. Rezultati kažejo, da 50 mmol/L poli(4-vinil-*N*-etilpiridin bromid)/poli(natrijev 4-stirensulfonat) najbolj učinkovito prepreči adhezijo bakterij 0,4 log bakt./mm<sup>2</sup> (60 %), sledi mu poli(4-vinil-*N*-izobutilpiridin bromid)/poli(natrijev 4-stirensulfonat) 0,3 log bakt. mm<sup>-2</sup> (47 %) in poli(alilamin hidroklorid)/poli(natrijev 4-stirensulfonat) 0,2 log bakt. mm<sup>-2</sup> (38 %). Ta raziskava dokazuje, da polielektrolitske plasti z kvartarne amino skupinami igrajo pomembno vlogo pri preprečevanju adhezije bakterij in zato predstavljajo pomembno uporabo v živilski in farmacevtski industriji ter v medicini.

KLJUČNE BESEDE: anti-adhezivni potencial; bakterijska adhezija; *E. coli*; higiena površin; polielektrolitske plasti