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Short Communication

Application of cationic polymer micelles for the dispersal of bacterial biofilms

Contamination of surfaces in hospitals and food industry by bacterial biofilms is a serious health risk. Of concern is their resistance to routine antibacterials and disinfectants. This requires the development of novel approaches to biofilm detachment. The study evaluates the effectiveness of cationic polymer micelles (CPMs) against pre-formed biofilms. CPMs based on different polycations were used. The hydrodynamic radius of the particles ranged from 16 to 360 nm. Biofilms of *Escherichia coli* 420, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* 29213 and *Bacillus subtilis* 168 were cultivated for 24 h then the pre-formed biofilms were treated with the CPMs for 2, 4 or 6 h. Biofilm biomass was evaluated by the crystal violet assay, and live/dead fluorescence test was applied for bacterial viability. The ability of CPMs to interact with pre-formed biofilms of the model strains was evaluated. We observed that the most effective CPMs were those based on poly(2-(dimethylamino)ethyl methacrylate) copolymers which reduced the biofilm biomass three- to four-fold compared with the treatment of the biofilm with water. Significantly reduced vitality of the bacteria in the biofilms was registered by the live/dead stain. The results indicate the applicability of the CPMs for disinfection of biofilm-contaminated surfaces and the treatment of wounds.

Keywords: Bacterial biofilm dispersal / Cationic polymer micelles

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It is presently well recognized that bacteria in natural environments or during infection colonize surfaces and form biofilms - sessile communities of microorganisms embedded in a complex mixture of extracellular polymeric substances (EPS) [1–3]. Biofilms are a serious risk factor for human health. They are significantly more resistant to antibacterial treatments than the microbes living in liquid media [4,5]. The development of biofilms during infection is reported to be between 60 and 80% [6,7]. This, together with the often occurring contamination of surfaces in hospitals and food-processing benches by sessile bacteria [8] puts forward the urgent need for the development of novel anti-biofilm reagents.

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Abbreviations: CPMs, cationic polymer micelles; CV, crystal violet assay; EPS, extracellular polymeric substance(s); PQVP, poly(quaternalized 2-vinylpyridine); PDMAEMA, poly(2-(dimethylamino)ethyl methacrylate); PS-PQVP, polystyrene-b-poly(quaternalized 2-vinylpyridine); PDMAEMA-PCL-PDMAEMA, poly(2-(dimethylamino)ethyl methacrylate)-b-poly(ϵ -caprolactone)-b-poly(2-(dimethylamino)ethyl methacrylate); PGA, poly-N-acetyl glucosamine; PI, propidium iodide

Biofilms are dynamic and develop via initial attachment, growth, and differentiation to a final step of detachment from the surface and dispersal of the bacteria to new niches. Biofilm control can be performed during each one of these steps however the predominant amount of studies in this area have been directed to suppression during the attachment and growth stages. For this purpose, nanoparticles, either alone or loaded with drugs, proved quite successful. Different metal (silver, gold, zinc oxide and copper oxide) or polymeric nanoparticles were effectively applied against biofilm development [4,8–11].

We know much less on how pre-formed biofilms can be removed. The limited diffusion through the EPS and the capacity of the latter to bind antibacterial substances [2,12] create serious obstacles for the effectiveness of antibiotic and sanitizer treatments. A novel direction of study is the potential of nanoparticles to interfere with pre-formed biofilms [13,14]. The success of such a task depends on the capacity of the nanoparticles to penetrate through the EPS, loosen and disrupt it, reach the cells, interfere with their integrity and/or deliver antibacterials to them [3]. Polycations, on the other hand, are well known biocides easily passing through the negatively charged bacterial cell walls [15]. The conjugation of hydrophilic polycations with hydrophobic polymers results in amphiphilic cationic block copolymers. They

are able to self-assemble forming positively charged polymeric micelles.

Here we report on the effectiveness of cationic polymer micelles (CPMs) based on newly synthesized di- and tri-block copolymers to destroy pre-formed biofilms of Gram-negative and Gram-positive bacterial strains. Block copolymers based on quaternized poly(2-vinylpyridine) (PQVP) and poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) cationic moieties were used. The micelles formed thereof have previously been used as gene delivery vector systems manifesting low cytotoxicity to eukaryotic cells and high cells internalization [16, 17]. Therefore, they were considered as promising systems for the dispersal of bacterial biofilms.

For CPMs, all reagents and solvents were purchased from Sigma-Aldrich. The polystyrene-*b*-poly (quaternized 2-vinylpyridine) (PS-PQVP) block copolymers were synthesized by anionic polymerization following the procedure described elsewhere [18]. The poly(2-(dimethylamino)ethyl methacrylate)-*b*-poly(ϵ -caprolactone)-*b*-poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA-PCL-PDMAEMA) triblock copolymers were synthesized by ATRP according to procedures reported previously [17]. The molecular characteristics of the copolymers were as follows: PS-PQVP block copolymers ($M_n = 69000$ g/mol, $\bar{D} = 1.08$, PQVP content 77 wt% and $M_n = 202000$ g/mol, $\bar{D} = 1.02$, PQVP content 75 wt%) denoted as PS-PQVP 35K and PS-PQVP 115K; PDMAEMA-PCL-PDMAEMA triblock copolymers ($M_n = 14300$ g/mol, $\bar{D} = 1.78$, PDMAEMA content of 44 wt% and $M_n = 17100$ g/mol, $\bar{D} = 1.20$, PDMAEMA content of 53 wt%), denoted as MKPa4 and MKPa12, respectively.

Preparation of CPMs: Micellar dispersions were obtained by dissolving the block copolymers in dimethylformamide, followed by addition of appropriate quantity of deionized water, in order to give a final concentration 1 mg/mL. After dialysis against water (SpectraPore7 dialysis membranes, MWCO 8000) for several days, the organic solvent was removed.

The hydrodynamic radius (R_h) and ζ -potential of CPMs were determined at 25°C on a Zetasizer NanoBrook90Plus PALS, equipped with a 35-mW red diode laser ($\lambda = 640$ nm), at a scattering angle of 90°.

Transmission electron microscopy was applied in order to estimate the form and dimensions of the CPMs. For this reason, 5 μ L of the CPMs suspensions were applied onto formvar-coated electron microscopy grids, allowed to dry and counterstained with 1% uranyl acetate in 70% methanol. The observations were made on Opton 10C electron microscope.

For bacterial biofilm experiments, the following strains were used: *Escherichia coli* 420 (NBIMCC), *Pseudomonas aeruginosa* PAO1 [19], *Bacillus subtilis* 168, kindly provided by Dr D. Galabova, Section of Biochemistry, Institute of Microbiology, BAS, Sofia [20–22] and *Staphylococcus aureus* 29213 (ATCC). For comparative biofilm biomass estimation, the crystal violet assay (CV) was applied. Overnight bacterial cultures in TSB (Sigma) were diluted 1:100 in M63 medium (0.02 M KH_2PO_4 , 0.04 M K_2HPO_4 , 0.02 M $(\text{NH}_4)_2\text{SO}_4$, 0.1 mM MgSO_4 and 0.04 M glucose, pH 7.5) and 150 μ L of the suspensions were inserted into the wells of a 96-well polystyrene plate. The biofilms were allowed to develop for 24 h, then the non-adherent bacteria were removed and the wells were washed with PBS. 150 μ L of the

CPMs were applied to each well followed by incubation for 2, 4, or 6 h. After the incubations, the biofilm was stained with CV, solubilized with 70% ethanol and the optical density (OD) was measured on plate reader at 570 nm wavelength. Each variant was performed in 6 replicates. Two controls were applied in the experiments. To estimate the amount of the pre-formed 24-h biofilms, at the beginning of the treatment experiments separate untreated samples were colored by CV (denominated as “hour 0” in the graphs). The controls C(H_2O) represented biofilms that were treated with sterile dH_2O instead of micelles for 2, 4, and 6 h. The data were processed on MS Excel; statistical significance of the differences between test and control groups was calculated by the *t*-test calculator, GraphPad QuickCalcs (<https://www.graphpad.com/quickcalcs/ttest1.cfm>).

The viability of the bacterial cells in the biofilm was estimated by fluorescence microscopy using the Live/Dead BacLight™ bacterial viability kit L13152 (Molecular Probes). This kit is based on two DNA-binding fluorescent probes with different access to the bacterial cells: the green-fluorescent label Syto™9 that penetrates into bacteria with either unaltered or altered cell envelopes, and the red-fluorescent dye propidium iodide (PI) that enters through altered cell envelopes only. Biofilms were cultivated on polymethyl methacrylate-coated glass slides for 24 hours and then treated with CPMs as described above. The viability staining was performed in accordance with the producer's instructions. The observations were performed on Nikon Eclipse TiU fluorescence microscope.

Polymers with quaternary ammonium groups are probably the most explored kind of polymeric biocides [15]. However some polycations having a strong positive charge in water also exhibit antimicrobial properties. The selected copolymers belong to the above mentioned groups as PS-PQVP owns quaternized 2-vinylpyridine moieties, while PDMAEMA blocks of MKPa copolymers have positively charged amino groups in every repeating unit. In aqueous solution, the block copolymers form micelles composed of hydrophobic core formed from PS or PCL and a hydrophilic positively charged corona based on PQVP or PDMAEMA. The hydrodynamic radius, R_h , as well as the surface charge of the resulting CPMs were determined. As evident from Fig. 1A MKPa copolymers form small in size CPMs around 20 nm. The size of the micelles formed from PS-PQVP copolymers was strongly dependent on polymer molar mass. The R_h of micelles based on PS-PQVP 35 K, was 65 nm while PS-PQVP 115 K form rather large particles of mean hydrodynamic radius of 360 nm. Transmission electron microscopy analysis confirmed the formation of nanosized particles of spherical shape (Fig. 1A, inset). The obtained CPMs possess strong positive surface charge as ζ -potential values range from 40 to 60 mV (Fig. 1B).

The results from the CV assay on hour “0” showed that all four tested bacterial strains had formed significant amount of biofilm within the 24-h cultivation in the plates. The treatment with the CPMs results in reduction of the biomass of the pre-formed biofilms. Dependent on the Gram staining pattern, two distinct of outcome could be differentiated (Fig. 2). For the two Gram-negative bacteria, statistically significant reduction in comparison with the controls C(H_2O) of MKPa4, MKPa12 ($p \leq 0.001$) and PS-PQVP 35K ($p \leq 0.05$) was registered after 2 and 4 h of treatment. After 6 h of treatment a trend of biofilm increase in comparison with hour 4 was detected implying some adaptation

Figure 1. Hydrodynamic radius (A) and ζ -potential (B) of the cationic polymer micelles prepared at concentration of 1 mg/mL. Inset, electron micrograph of MKPa4, scale bar = 0.5 μ m.

Figure 2. Effect of the treatment with the cationic polymer micelles suspensions on the pre-formed biofilms of (A) *E. coli* 420, (B) *P. aeruginosa* PAO1, (C) *B. subtilis* 168 and (D) *S. aureus* 29213. Biofilms were developed for 24 h in M63 medium and then treated for 2, 4, or 6 h with the CPMs. Controls: “O h” represents the amount of the pre-formed 24-h biofilm at hour “0” i.e., before the application of the CPMs; C(H₂O) represents parallel samples treated with distilled sterile H₂O. Each bar represents the average of 6 replicates and the SD.

of the survived bacteria to the presence of the micelles. Eventually, substances from the detached biofilm parts could be used by these strains as a source of nutritive substances. The CPMs were much more successful when applied to pre-formed biofilm of the two Gram-positive models. For them, throughout the experiment there was significant ($p \leq 0.001$) reduction of biofilm biomass in comparison with the respective C(H₂O) control values, and this effect increased with treatment duration. These data imply relationship between the response of the biofilms to the CPMs and the structure of the bacterial cell envelopes.

When discussing biofilms, apart from the bacterial cell envelope type, it should be taken into account that the microorganisms contribute to less than 10% of the dry mass [23]. Much larger is the contribution of the EPS matrix. This is structurally

heterogeneous and contains proteins, extracellular DNA, lipids and polysaccharides [12, 24–28]. The structural heterogeneity of the matrix would greatly influence the nanoparticle-biofilm interactions [2]. As a major difficulty in both predicting and explaining the variety of strain-specific responses of biofilms to nanoparticle treatments should be mentioned the lack of knowledge on the exact composition of the EPS.

For the bacterial species included in our experiments there is some clarity only on the matrix polysaccharides. Thus, *E. coli* strains may release different proportions of colanic acid and poly-N-acetyl glucosamine (PGA) [29]. PGA is also the major exopolysaccharide component of *S. aureus* and *B. subtilis* biofilms [1, 30]. The polysaccharide characterizing the biofilms of *P. aeruginosa* is alginate, a linear co-polymer of β -1,4-linked

Figure 3. Fluorescence microscopy estimation of the vitality of bacterial cells within biofilms of *E. coli* 420, Live/Dead staining. Biofilms were cultivated on cover slides for 24 h followed by 4 h incubation in H₂O (A, B) or 1 mg/mL MKPa4 (C, D). A, C - images combining the red and green signal; B, D - the corresponding areas, with the red signal only, indicating only the bacteria with altered cell envelope integrity colored by PI. Scale bar = 30 μ m.

D-mannuronic acid and variable amounts of C-5 epimer of guluronic acid [31]. The polysaccharides characteristics and their strain-specific proportion in the biofilm matrix [29] in combination with the other EPS components like proteins, lipids and DNA would determine strain-specific physicochemical characteristics. EPS composition may also depend on environmental factors like temperature, acidity, aeration, etc. [32]. All these may determine both the diffusion of the nanoparticles and their further interaction with the biofilm.

Another important factor in the interactions are the physicochemical characteristics of the nanoparticles themselves - size, composition, surface, charge, chemical nature, etc. [see recent reviews 2, 13]. Our results indicate influence of R_h , ζ -potential, as well as the nature of the tested CPMs on the biofilm. As mentioned above one obstacle towards particles activity is the passing through the negatively charged bacterial cell walls. Therefore, it seemed logical the micelles of stronger surface charge to pass the easiest. However, the CPMs of highest ζ -potential (PS-PQVP 115K, 60 mV) had the lowest efficiency, which may probably be due to the largest size of these particles. Accordingly, the smaller micelles of PS-PQVP 35 K that was the polymer of similar composition and lower molar mass (65 nm, ζ = 46 mV), had a better effect for the removal of pre-formed biofilms compared to PS-PQVP 115K. The most effective CPMs were those based on MKPa copolymers. In spite of their lower ζ -potential values (around 43 mV) compared to PS-PQVP micelles, their size appeared to be essential (MKPa4, R_h = 21 nm) and probably allowed them to both penetrate through the EPS, and facilitate their interaction with the bacterial cell envelope. It is also worth noting the different nature of the polycationic segments. Obviously, the PDMAEMA moieties have better anti-biofilm effect than the PQVP. Therefore, MKPa-based CPMs may be recommended

for wider-range applications, against both Gram-negative and Gram-positive biofilms. Using the Live/Dead fluorescence staining kit, we further examined the effect of the CPMs on the viability of the bacterial cells within the biofilm. Figure 3 illustrates the results for *E. coli* 420 which are representative for the results obtained with the other model bacteria. In control samples incubated with water alone the cells in the biofilm appeared mostly green, and we found only few PI-labeled bacteria (Fig. 3A, C). In the presence of the CPMs there was a dramatic increase of the red cells (Fig. 3B, D). Studies by other authors have shown that nanoparticles loaded with antibiotics alter the viability of resistant microorganisms within the biofilm [14,33]. The present results with the Live/Dead stain indicate the capacity of the tested CPMs themselves to alter the permeability of the bacterial cells in the absence of antibiotic.

The promising effects of the here tested CPMs have been confirmed on the model of four single-species biofilms, two – of Gram-negative, and two – of Gram-positive strains with good biofilm-forming potential. This implies the applicability of the CPMs to medically important biofilms. It has to be taken into account however that in real environments biofilms are represented by multi-species communities, with variable composition. Such communities are difficult to explore under laboratory conditions, in the first place – due to the lack of well-standardized models of multispecies biofilms composed of bacterial strains attaching to hospital or food-industry surfaces. Within natural biofilm systems, species might exert different interactions, including synergism or antagonism [34] and together with this biofilm characteristics may vary with environmental conditions. The effort in future studies should be directed to the elaboration of appropriate models of multi-species biofilms. As recent advances in this direction, model communities of

multi-species biofilms relevant to dental medicine should be mentioned [35,36]. We expect that further work in this direction will provide novel laboratory models useful for the tests of anti-fouling substances.

In summary, our results show for the first time that the here tested CPMs, previously applied by us as gene delivery vectors with low cytotoxicity [16, 17], not only can be successful for biofilm dispersal and removal, but exert also activity as bacterial cell-envelope permeabilizing agents. For internal medical applications, the low stability of the particles in saline or microbiological media (data not shown) appears an obstacle. Nevertheless, the present results show that they can be successfully applied for the removal of bacterial biofilms from contaminated surfaces in hospitals and in food processing industry, for the decontamination of medical devices, and for the treatment of surface-exposed biofilm-related skin lesions.

Practical application

The results from the study show that polymeric cationic micelles can be applied for the removal of bacterial biofilms from contaminated surfaces in hospitals and in food processing industry, the decontamination of medical devices, and for treatment of surface-exposed biofilm related skin lesions. Out of the tested micelles, MKPa4, MKPa12 are applicable against both Gram-negative and Gram-positive bacteria, while PSPQ2VP 35 and PSPQ2VP 115 are effective against Gram-positives only. The micelles are also expected to have potential for drug delivery within biofilms.

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