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Original Article

Effects of Cationic Polymers on the Viability of Microbial Biofilms

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Abstract

Introduction: The number of published biofilm studies and novel ways for studying them has risen dramatically in recent years, owing to the broad application of biofilms in medicine. Some bacteria develop biofilms that are highly resistant to antimicrobial agents, resulting in persistent infections. This necessitates the development of alternative methods for combating biofilms. In this regard, the application of cationic polymers is a good candidate for realization of this strategy.

Aim: The aim of our study was to investigate the potential of a newly synthesized covalently attached star copolymer of N,N'-dimethylaminoethyl methacrylate and hydroxyl-bearing oligo(ethylene glycol) methacrylate [P(DMAEMA-co-HOEGMA)] to silica surfaces and its quaternized version [P(QDMAEMA-*co*-HOEGMA)] for destruction of biofilms formed by *Bacillus subtilis* or *Pseudomonas aeruginosa*.

Materials and methods: Model strains representing different genera and taxonomic groups were selected for the study. The anti-biofilm activities of two different newly synthesized cationic polymers were investigated by observation (live/dead staining) of the viability of bacterial cells within the biofilm.

Results: The results obtained by the live/dead labeling of bacterial biofilms show a substantial decrease in the viability of population in the presence of cationic polymers, better expressed at *B. subtilis*.

Conclusions: The studied two immobilized on silica wafers newly synthesized star copolymers exhibited potential for anti-biofilm effects. The results demonstrated combined potential for reducing the viability of bacterial cells within the biofilms and probably for loosening the biofilm matrix. The effect was better expressed in *B. subtilis*.

Keywords

biofilm destruction, *B. subtilis*, *P. aeruginosa*, star cationic polymers

INTRODUCTION

Pathogenic microorganisms resistant to antibiotics and antimicrobial drug therapy are an increasingly serious public problem. Furthermore, the increase in health problems is caused not only by the antibiotic resistance of free-living bacterial cells but by their ability to form structured communities (biofilms) as well. Biofilms are surface-attached communities of bacteria embedded in an extracellular matrix.[1] Biofilms formed by large part of bacterial strains are highly resistant to the action of antimicrobial agents, which results in persistent infections and failure in their

treatment. The bacteria in the biofilm are often more resistant to antibiotics than the same bacteria in plankton form.[2,3] The drug tolerance of biofilms is often due to the presence of tolerant and persistent cells in their community.[4] These cells can survive during the antibiotic treatment and recover when antibiotic treatment is discontinued.[5] The biofilm formations are a key factor for survival of bacteria in diverse environments. However, often biofilms underlie major problems in medicine, in industry, and agriculture. Hence, finding alternative ways for either prevention or eradication of biofilms as well as searching for new materials to overcome the biofilm resistance with both industrial and biomedical applications is necessary. In recent years, interest in surfaces with antibacterial properties has significantly increased.

Studies on the potential of novel high-performance polymeric materials as antimicrobial drug carriers[6] or as a bactericidal polymer on its own[7,8] already include investigations on their possible application in novel anti-biofilm strategies.[7,9] Cationic polymers capable of interacting with the biofilm's extracellular matrix are of special interest. Series cationic polymers show sustainable antibacterial activity[10,11] and to enhance their bactericidal activity functional amino groups of these polymers are often quaternized.[12,13]

AIM

The aim of the present study was to evaluate the capacity of the newly synthesized covalently attached star polymers of N,N'-dimethylaminoethyl methacrylate (PDMAEMA) and its quaternized version to destroy biofilms of *P. aeruginosa* and *B. subtilis* and to kill the bacteria embedded in the biofilm.

Reference strains of *Pseudomonas aeruginosa* (important opportunistic pathogen, highly resistant to large number antimicrobials) and *Bacillus subtilis* (adopted as a model organism for pathogenic microbes such as Staphylococcus aureus) were used for the biofilm experiments.

MATERIALS AND METHODS

Microorganisms

The strains *Bacillus subtilis* 168 (Culture Collection, Institute of Microbiology, Bulgarian Academy of Sciences), *Pseudomonas aeruginosa* PAO1 - obtained from the International Reference Panel[14] were used throughout this study. The cultures were maintained at 4°C on Bacto agar (Difco) and Tryptic Soy Agar (TSA, Sigma) slants.

Culture medium and growth condition

Inoculum was prepared by transferring the cells from agar slants in Mueller Hinton Broth (MHB) (HiMedia) and grown overnight at 37°C with agitation at 200 rpm.

Bioflim cultivation

Bacterial cells were diluted in MHB to 1.5–3×10^8 colonies forming units (CFU/ml). Appropriate dilutions were made to obtain working solution of approximately 1.5–3×10^5 CFU/ml. In one test, under sterile conditions and by duplicate, the samples and the control were placed in Multiwell Plate, 6 well containing 5 mL of the working solution. The samples were incubated in temperature-controlled incubator at 37°C for 24 hours. After 24-hour incubation interval, the thin films were removed from the specimens with cotton swab and the microbial cells embedded in the biofilm were suspended in TBS buffer.

Star copolymer nanolayers

Star copolymers of N,N'-dimethylaminoethyl methacrylate (DMAEMA) and hydroxyl-bearing oligo(ethylene glycol) methacrylate (HOEGMA, Mn=360 g/mol) [P(DMAEMA-*co*-HOEGMA)] and its quaternized version P(QDMAEMA-*co*-HOEGMA) (quaternized using octyl bromide) covalently attached to silicon wafers were synthesized in the Centre of Polymer and Carbon Materials, Polish Academy of Sciences, Zabrze, Poland and were provided by Dr B. Mendrek.

Fluorescence microscopy

For fluorescence microscopy, a fluorescent dye combination "live/dead" BacLight Bacterial Viability Kits (Molecular Probes) was applied according to the manufacturer's instructions. The Viability kit is composed of green-fluorescent nucleic acid stain (Syto 9) and the red-fluorescent nucleic acid stain (propidium iodide). Finally, the samples were dropped on glasses into mounting medium, Fluoromount (Sigma).

Observations were made by means of confocal laser scanning microscope (CLSM) Nikon Eclipse TiU, at excitation wavelengths of 488 nm and 543 nm by epifluorescence mode with 60X oil PlanApo objective. Images were acquired with a CCD Camera Nikon DS-Fil and twenty-five of them were randomly taken from each sample. The images were generated and further processed by NIS-ELEMENTS software and Image J program for image analysis.

Viability evaluation by plate count

To evaluate the viability of bacterial cells within the biofilm, we used the drop-plate technique on agar. After 24-hour cultivation at 37°C the biofilm was collected with sterile swabs and after dilutions in phosphate-buffered saline (PBS). 100 µl of treated bacterial suspension was transferred to Mueller Hinton Agar (MHA) plates in triplicates. The inoculated plates were incubated at 37°C for 24 hours and surviving bacterial cells were counted.

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Statistical analysis

The experiments were performed in triplicate and the average values were calculated. Mean values are given with standard deviations of ≤10%. The OriginPro 6.1 was used for statistical analysis.

RESULTS

Observation of the viability of bacterial cells within the biofilm

Evaluation of biofilm formation and estimation of the vitality of bacterial cells within biofilms by fluorescence microscopy were performed. The observed biofilm in *B. subtilis* on P(DMAEMA-*co*-HOEGMA) star nanolayers was made up of predominantly dead bacterial cells **(Fig. 1A)**. There were also single surviving living ones. Dense biofilm monolayer of dead cells, without the presence of separate island formations, was observed in the presence of P(QDMAEMA-*co*-HOEGMA) star nanolayers **(Fig. 1B)**. Relatively small numbers of single surviving cells were registered. In contrast to the control (silicon wafer) developed biofilm, with local accumulations of bacterial cells in intact form were observed **(Fig. 1C)**. Lack of changes in sporulation processes and presence of viable spores were also observed **(Fig. 1D)**.

Data show that in the presence of P(DMAEMA-*co*-HOEGMA) and P(QDMAEMA-*co*-HOEGMA) star nanolayers, the cells in *B. subtilis* biofilm formations are significantly interfered. Most of the bacterial cells in the control biofilm were green, indicating that it consisted mainly of viable bacteria. The presence of P(DMAEMA-*co*-HOEGMA) nanolayers and especially of P(QDMAEMA-*co*-HOEGMA) nanolayers in the growth media caused predominantly red coloring of the cells, indicating the death of the predominant part of the bacterial population.

As also shown by the live/dead staining data, a substantial decrease in viability was registered for the *P. aeruginosa* biofilm cells in presence of P(DMAEMA-*co*-HOEGMA) nanolayers **(Fig. 2A)**. The biofilm developed in the presence of P(QDMAEMA-*co*-HOEGMA) nanolayers also contains a significantly reduced proportion of viable bacteria, but considerably less than that formed in the presence of P(DMAEMA-*co*-HOEGMA) nanolayer **(Fig. 2B)**.

In both treated samples, the presence of dead, islet cell accumulations was reported. In contrast, in the control sample, we report both clusters and single viable cells **(Fig. 2C)**.

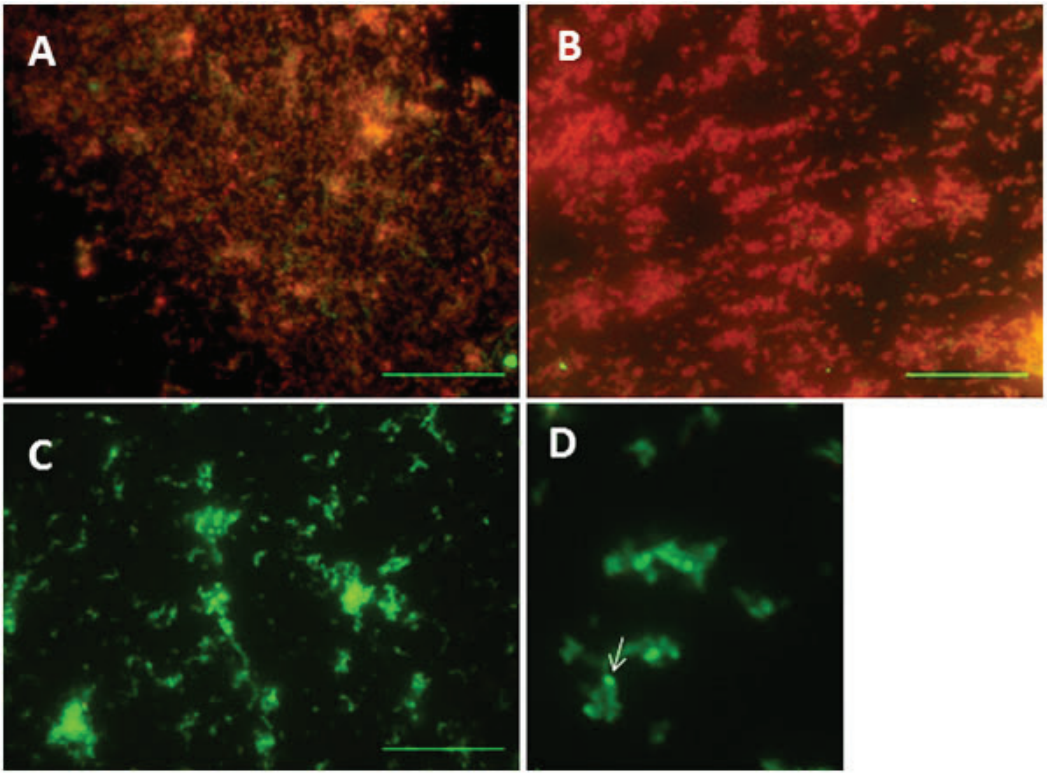

Figure 1. Estimation of the viability of *B. subtilis* bacterial cells within the biofilms. Representative fluorescence microscopy images of *B. subtilis* biofilms (A to C). **(A)** P(DMAEMA-*co*-HOEGMA) nanolayers - predominantly dead, bacterial cells. There are also single, surviving living ones (green color); **(B)** P(QDMAEMA-*co*-HOEGMA) nanolayers - dense biofilm monolayer of dead cells, without the presence of separate island formations; **(C)** silicon wafer (control) - enveloped biofilm, with local accumulations of bacterial cells in intact form; **(D)** the presence of viable spores colored green (white arrow). Scale bar = 30 μm.

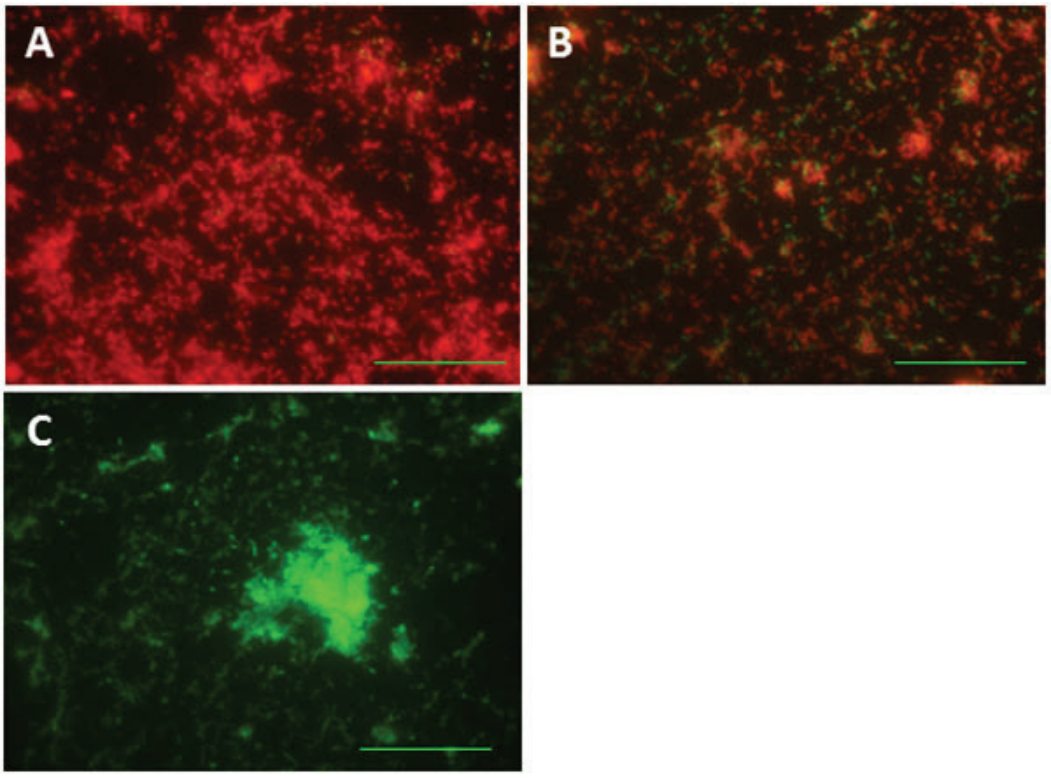

Figure 2. Estimation of the viability of *P. aeruginosa* bacterial cells within the biofilms. Representative fluorescence microscopy images of *P. aeruginosa* biofilms (A to C). **(A)** P(DMAEMA-*co*-HOEGMA) nanolayers - presence of dead, islet cell accumulations and surviving cells; **(B)** P(QDMAEMA-*co*-HOEGMA) nanolayers - dead, islet cell accumulations and higher percentage surviving cells; **(C)** silicon wafer (control) - clusters and single viable cells typical for biofilm cells communities. Scale bar = 30 μm.

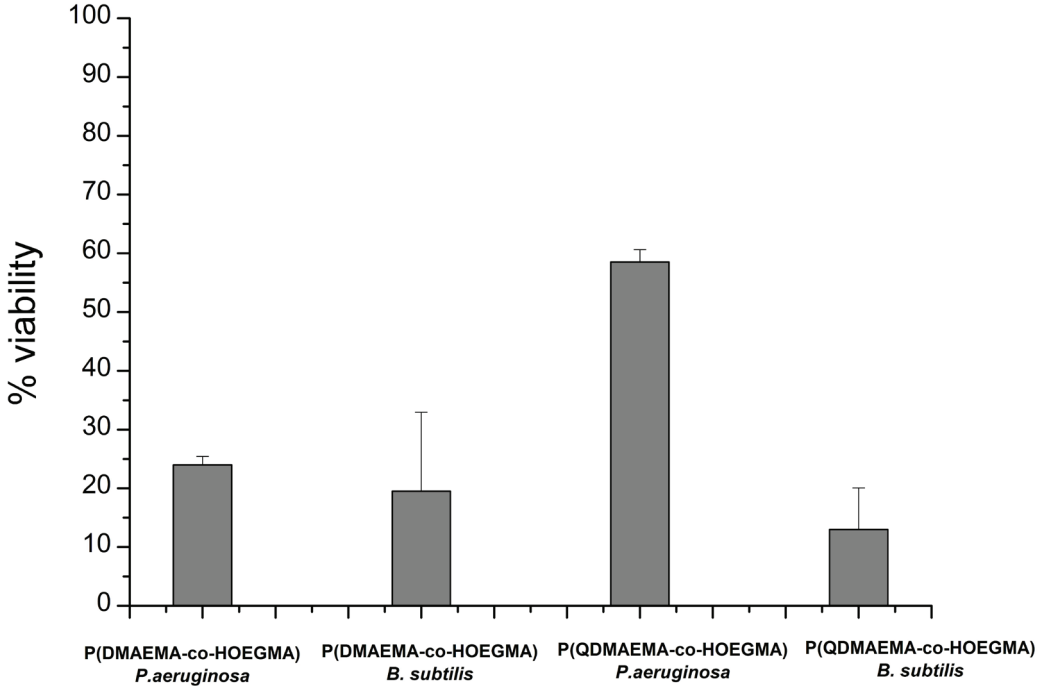

Figure 3. Effects of P(DMAEMA-*co*-HOEGMA) and P(QDMAEMA-*co*-HOEGMA) on the viability of bacterial cells within the biofilms of *P. aeruginosa* and *B. subtilis*. The cells viability was determined as described in the Materials and methods section. Control samples for each strain were accepted as 100 % and the other values were normalized to controls.

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To evaluate the viability of bacterial cells treated with nanolayers we used the classical agar plating method. As also shown by the live/dead labeling, the plate count method verifies a decrease in the viability for the two treated with star nanolayers *B. subtilis* biofilms **(Fig. 3)**. The range of viability was between 15% and 20% of the control values. Regardless of the number of surviving bacterial cells reported in *P. aeruginosa* treated biofilms, the percentage of those in sample P(QDMAEMA-*co*-HOEGMA) was higher within the range of 34% compared to (P(DMAEMA-*co*-HOEGMA) **(Fig. 3)**.

DISCUSSION

Biofilms are microbial communities embedded in an extracellular matrix.[4] The matrix can be composed of exopolysaccharides, extracellular DNA (eDNA), RNA proteins, ions (bound or free), and water.[15] *P. aeruginosa* has the capacity to synthesize three exopolysaccharides implicated in biofilm formation: alginate, Psl, and Pel.[8,9] Pel is a positively-charged chain of amino sugars, and is thought to bind ionically with extracellular DNA (eDNA) in the biofilm matrix.[15]

In *P. aeruginosa* (PAO1), Psl is localized to the periphery of the microcolonies.[16] Peripherally localized exopolysaccharide may be an important general feature of biofilm microcolonies. Psl is a neutral, branched polymer, and increasing Psl production increases the elastic modulus of the biofilm.[17]

Psl and Pel probably cause electrostatic repulsion of the cationic P(DMAEMA-*co*-HOEGMA) and P(QDMAEMA-*co*-HOEGMA) stars and thus do not allow serious damage to the matrix and reach the bacteria embedded in the biofilm.

The weaker effect of P(QDMAEMA-*co*-HOEGMA) star nanolayers is probably due to the increase of positive charge (quaternary ammonium groups) that does not allow interaction with the biofilm extracellular matrix, disintegration of biofilm, and killing the bacteria within biofilm.

In *B. subtilis*, the matrix consists primarily of an exopolysaccharide (EPS), protein fibers (TasA and TapA), and a hydrophobin coat (BslA). These components function synergistically to allow biofilm formation.[18]

Another component that plays an important role in biofilm microstructure is the anionic polymer γ-polyglutamate (γ-PGA).[19] P(DMAEMA-*co*-HOEGMA) and P(QDMAEMA-*co*-HOEGMA) stars are cationic polymers and their efficiency may be due to the presence of positive charges, which allows them contact through electrostatic interaction not only with the negatively charging components of biofilm matrix but with bacterial cell wall too. Probably, the positively charged P(DMAEMA-*co*-HOEGMA) and P(QDMAEMA-*co*-HOEGMA) stars bind to the negatively charged γ-PGA and thus allow interaction with the biofilm extracellular matrix and subsequently exerting antibacterial action by interaction with the bacterial cell wall and killing the embedded bacteria.

CONCLUSIONS

The obtained data from our investigation for *B. subtilis* demonstrate the ability of new star copolymer nanolayers (P(DMAEMA-*co*-HOEGMA) and P(QDMAEMA-*co*-HOEGMA) to reduce the biofilm bacteria viability probably by destroying the extracellular matrix. The positively charged star polymer layers interact with the acidic moieties of the biofilm matrix thus partially solubilizing its structure and killing the embedded bacteria. Therefore, the polymers possess biofilm-eradicating properties which are no less important than preventing biofilm formation. Finally, the results suggested for their possible practical use for successfully reduction in the viability of the biofilm cells formed by Gram (+) bacteria.

In the case of *P. aeruginosa*, there was insufficient viability reduction in the biofilm cells. Regardless the presence of dead islet cell accumulations, the high percentage of surviving bacterial cells was reported especially for P(QDMAEMA-*co*-HOEGMA) star nanolayers. Moreover, the surviving cells could restore the development of the biofilm in the absence of the cationic polymer layers.

However, we do not exclude their possible practical application in the destruction of formed biofilms by other Gram (-) strains.

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Влияние катионных полимеров на жизнеспособность микробных биоплёнок

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Резюме

Введение: Количество опубликованных исследований биоплёнок и новых способов их изучения резко возросло в последние годы благодаря широкому применению биоплёнок в медицине. Некоторые бактерии образуют биоплёнки, обладающие высокой устойчивостью к противомикробным препаратам, что приводит к персистирующим инфекциям. Это вызывает необходимость разработки альтернативных методов борьбы с биоплёнками. В связи с этим применение катионных полимеров является удачным предложением для реализации этой стратегии.

Цель: Целью нашего исследования было изучение потенциала недавно синтезированного ковалентно связанного звездообразного сополимера N,N‘-диметиламиноэтилметакрилата и гидроксилсодержащего олиго(этиленгликоль)метакрилата [P(DMAEMA-co-HOEGMA)] с диоксидом кремния поверхностей и его кватернизованный вариант [P(QDMAEMA-co-HOEGMA)] для разрушения биоплёнок, образованных *Bacillus subtilis* или *Pseudomonas aeruginosa***.**

Материалы и методы: Для исследования были отобраны модельные штаммы, представляющие разные роды и таксономические группы. Антибиоплёночную активность двух различных недавно синтезированных катионных полимеров исследовали путём наблюдения (живое/мёртвое окрашивание) за жизнеспособностью бактериальных клеток внутри биоплёнки.

Результаты: Результаты, полученные методом окрашивания живых/ мёртвых бактериальных биоплёнок, показывают существенное снижение жизнеспособности популяции в присутствии катионных полимеров, лучше выраженное у *B. subtilis*.

Заключение: Два изученных недавно синтезированных звездчатых сополимера, иммобилизованных на кремнеземных пластинах, продемонстрировали потенциал для антибиоплёночного воздействия. Результаты продемонстрировали комбинированный потенциал для снижения жизнеспособности бактериальных клеток в биоплёнках и, вероятно, для ослабления матрикса биоплёнки. Эффект был лучше выражен у *B. subtilis*.

Ключевые слова

деструкция биоплёнки, *B. subtilis*, *P. aeruginosa*, звездчатые катионные полимеры