Available online at www.sciencedirect.com

ScienceDirect

Mendeleev Commun., 2024, **34**, 185–187

Mendeleev Communications

On the quantification of biocidal activity of protective coatings

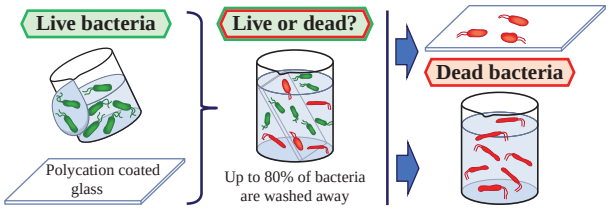
Irina G. Panova,*[a] Anastasiya Yu. Lokova,[a] Timofei A. Pankratov,[b] Yuri A. Nikolaev[b] and Alexander A. Yaroslavov[a]

[a] *Department of Chemistry, M. V. Lomonosov Moscow State University, 119991 Moscow, Russian Federation. Fax: +7 495 939 0174; e-mail: igpan@mail.ru*

[b] *Federal Research Centre 'Fundamentals of Biotechnology' of the Russian Academy of Sciences, 119071 Moscow, Russian Federation*

DOI: 10.1016/j.mencom.2024.02.008

The conventional deposition/drying procedure results in the formation of cationic polymer coatings on glass slides that are capable of binding bacterial and yeast cells. A three-minute water treatment removes up to 80% of cells from the surface, and both the removed and remaining cells are dead. The results obtained clarify the mechanism of the biocidal action of adsorbed layers of poly(diallyldimethylammonium chloride) and the protocol for quantifying their biocidal activity.



Keywords: cationic polymer coating, removal with water, biocidal activity, biocidal surface, poly(diallyldimethylammonium chloride), Live/Dead Cell Viability Assays.

Coatings with cationic groups on the outer surface exhibit high antimicrobial activity.[1,2] Such groups can be chemically attached to the surface[3–5] or immobilized through nonspecific, noncovalent interactions.[3,4] In the latter case, polymers with cationic groups are often used,[5,6] capable of adsorbing on various surfaces due to electrostatic, coordination and hydrophobic interactions, thus forming protective antimicrobial layers.[7] The key step in the conventional procedure for quantifying the biocidal activity of a cationic protective coating is the removal of pre-deposited cells from the surface with water, followed by testing their growth on a solid culture medium and calculating the percentage of surviving cells.[8–11] According to this scheme, the antimicrobial activity of the coating is determined by the viability of cells washed off the surface. This result extends to the entire population of cells initially deposited on the surface.[11,12] A 100% survival rate of washed-away cells means complete survival of all cells initially adsorbed on the test surface; conversely, death of 100% of washed-away cells reflects the complete death of all adsorbed cells. However, quantitative removal of cells from the surface with water is not obvious, especially considering the high negative charge of the microbial cell surface and the high positive charge of the cationic polymer.[13] The extreme affinity of both components to each other was shown in a model study where the cationic polymer was adsorbed on the surface of multiply charged anionic polymer microspheres: the cationic homopolymer was not removed from the surface of the microspheres even in 2 M NaCl solution.[14,15] If so, a question arises: how accurate is the aforementioned protocol in measuring the biocidal efficacy of protective coatings? For a separate but important case: can the 100% survival of washed-away cells be extended to the survival of the entire cellular population, *i.e.*, washed-away cells and cells retained on protective coatings?

In this communication, we describe the behavior of cells deposited on a coating formed by cationic poly(diallyldimethylammonium chloride) (PDADMAC). This polycation exhibits good antibacterial properties both in solution and in solid coating.[16,17] Additionally, PDADMAC is an inexpensive, biocompatible polymer approved by the Food and Drug Administration for use in the food industry.[17] Cells of three types were applied to the PDADMAC coating. The cell-covered coatings were washed with water and the cell desorption efficiency and the viability of the removed and remaining cells were examined. The results make it possible to evaluate the contribution of cells of all types to the overall bactericidal activity of polymer coatings.

To prepare the coating, a solution of PDADMAC (M_w = 470 kDa)[18] was deposited onto glass slides to cover the entire surface and dried at room temperature to constant weight (for details, see Online Supplementary Materials). The coatings were then loaded with three types of microorganisms: the gram-positive bacterium *Staphylococcus aureus* strain 209, the gram-negative bacterium *Pseudomonas aeruginosa* strain 481 and the yeast strain *Yarrowia lipolytica* Y-3322. The slides were then immersed in stirred sterile distilled water for 0.5, 1 or 3 min and dried at room temperature. Cells on the surface were counted using an optical microscope in 40 fields of view and converted to the average number of cells per field. In control experiments, polymer coatings were loaded with cells but not washed with water. The result of the first experiment N_{exp} was compared with the result of the second experiment $N_{control}$, thus obtaining the content of adherent cells $Q = (N_{exp}/N_{control}) \times 100\%$; the Q value in control experiments was taken as 100%. Protocols for working with bacterial and yeast cultures are given in Online Supplementary Materials.

The dependence of the Q value on the duration of treatment of the slide with water, *i.e.*, the dynamics of cell desorption, is presented in Figure 1 for three types of microorganisms. In all cases, increasing the treatment time resulted in a progressive decrease in the Q down to 20–30% for bacteria and 60% for yeast by the end of the

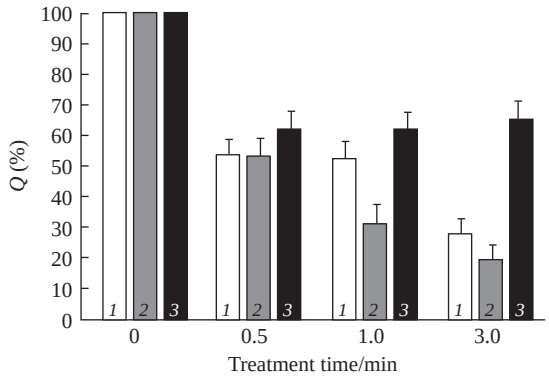


Figure 1 Diagrams of the content of adherent cells (Q) depending on the treatment time for (*1*) *S. aureus*, (*2*) *P. aeruginosa* and (*3*) *Y. lipolytica* cells. Experiments without water treatment served as controls.

experiment. In other words, a significant portion of the microorganisms was removed from the cationic coating during the three-minute water treatment. This is surprising given the high affinity of negatively charged cells for the positively charged coating. It has previously been shown that anionic colloids have extremely high affinity for cationic polymers.[19] The resulting polymer–colloid complexes retain their integrity (do not dissociate to the initial components) even in concentrated aqueous salt solutions.[15] This occurs due to multiple electrostatic interactions between the oppositely charged units of both components.[20] Equally high stability in water could be expected from complexes between cells and the cationic PDADMAC coating; however, the experiments showed a different result.

The reason for this may be as follows. Coatings prepared by deposition/drying of aqueous solutions of cationic polymers are almost quantitatively removed from the surface in several two-minute cycles of water treatment.[18] Obviously, after cells are adsorbed on the coating, part of the cationic polymer should also dissolve in water and leave the surface. Removal of the polymer will be accompanied by desorption of cells, which are likely to leave the surface previously covered with the cationic polymer. This pattern explains the rapid and efficient desorption of all cell types from the PDADMAC coating (see Figure 1).

Thus, the adsorbed cells are divided into two groups: one that can be washed out of the coating with water, and the other that remains on the surface after the washing procedure. The viability of the washed and remaining cells is questionable in this case. Although there are several methods for assessing cell viability,[21–23] we started with Live/Dead Cell Viability Assays.[21,24] The protocol we used typically distinguishes populations of living and dead cells by two colors: live cells are green and dead cells are red.[24] Staining cell suspensions with Live/Dead dye gave bacterial and yeast cells a predominantly green color under a fluorescence microscope, as shown in Figure 2(*a*), indicating that most of the cells remained alive and able to divide.

The situation changed drastically when the Live/Dead protocol was applied to the same cells, but deposited on the polymer coatings and dried. The fluorescence of the adherent cells gradually changed from green to red during the first 15 min, and after 1 h (the standard incubation period in our studies), all cells turned red [Figure 2(*b*)], indicating their death. These data correlate with earlier results[11,18] describing cell viability on surfaces formed by adsorbed cationic polymers. Cell deposition on cationic surfaces resulted in a progressive decrease in cell viability. Thus, according to the Live/Dead protocol, all cells settling on the cationic surface died within the first hour.

Another approach was to use a microcolonial growth method to monitor the condition of cells remained on the PDADMAC-coated surface after washing. Cells were applied to the surface and after 1 h transferred to LB agar nutrient medium and incubated at 30 °C for 24 h. Cell survival was assessed by changes in their number in clusters (microcolonies). As an example, Figure 3(*a*),(*b*) shows two photographs taken using an optical microscope of gram-negative *P. aeruginosa* cells on glass without a polymer coating (control) [Figure 3(*a*)] and glass coated with PDADMAC [Figure 3(*b*)]. The first photograph shows that the cells formed an uneven coating of clusters, while the second photograph shows that they evenly covered the surface.

To assess cell viability, coatings with deposited cells were incubated for 1 h as described above; the samples were then covered with LB agar medium and further incubated for 24 h at 30 °C. The number of cells on the surfaces was counted before and after the 24-h incubation, N_{1day} and N_0, respectively. The same experiment with cells deposited on uncoated glass slides served as a control. The results of experiments on glass and polymer coating are shown in Figure 4 in terms of $Q_{1day} = (N_{1day}/N_0) \times 100\%$. The incubation increased the Q_{1day} value (or number of cells) in the control experiment where cells were deposited on unmodified glass slides, but had little effect on the Q_{1day} value for cells on the polymer coating.

Figure 3(*c*) shows a photograph of *S. aureus* on the surface of a glass slide after one-day incubation. The large clusters in the photo reflect the growth of *S. aureus* cells, which is manifested in their

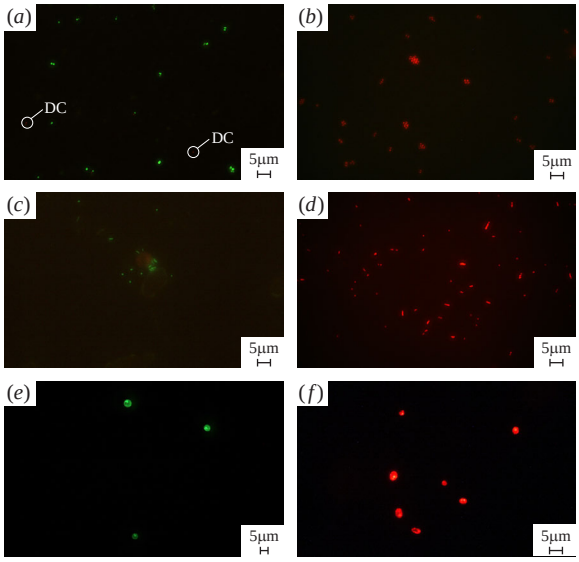


Figure 2 Fluorescence microscopy images of Live/Dead dye staining of (*a*),(*c*),(*e*) initial cell suspensions and (*b*),(*d*),(*f*) cells deposited on the coating for (*a*),(*b*) *S. aureus*, (*c*),(*d*) *P. aeruginosa* and (*e*),(*f*) *Y. lipolytica* cells. Live cells are colored green, dead cells are colored red.

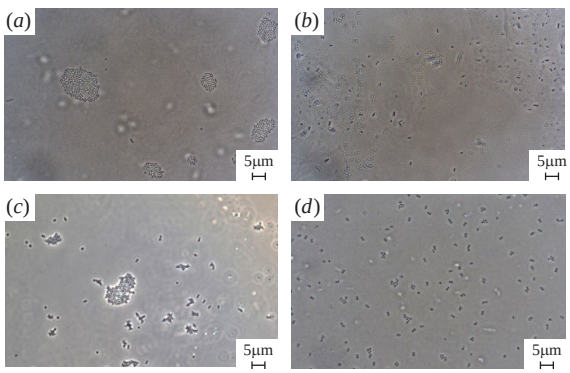


Figure 3 Optical phase contrast microscopy images of (*a*),(*b*) *P. aeruginosa* and (*c*),(*d*) *S. aureus* cells on (*a*),(*c*) uncoated and (*b*),(*d*) polymer-coated glass surfaces. Photographs were taken (*a*),(*b*) 1 h after cell application and (*c*),(*d*) after one-day incubation in LB agar medium. The clusters shown in (*d*) represent their initial size before incubation.

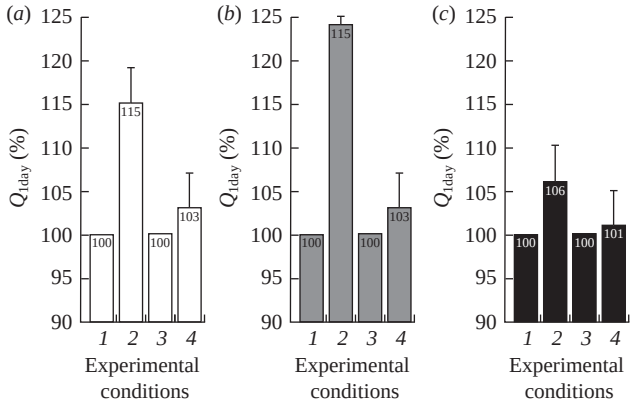


Figure 4 Q_{1day} values for (*a*) *S. aureus*, (*b*) *P. aeruginosa* and (*c*) *Y. lipolytica* cells pre-deposited on (*1*),(*2*) uncoated and (*3*),(*4*) polymer-coated glass slides (*1*),(*3*) before and (*2*),(*4*) after incubation in LB agar medium for 24 h.

ability to divide and form microcolonies. In contrast to this, in Figure 3(*d*) there are no large clusters, but only small groups consisting of 5–10 cells are present, which indicates the inhibitory effect of the coating on the deposited cells. A similar situation was observed in the case of *P. aeruginosa* and *Y. lipolytica* (data will be published elsewhere).

In summary, deposition/drying of an aqueous solution of cationic PDADMAC allows the formation of PDADMAC coatings on glass slides capable of binding bacterial and yeast cells. Following standard antimicrobial testing procedure, 1 h later the cell-covered coatings were washed with water, resulting in the removal of up to 80% of the cells from the coatings formed by PDADMAC macromolecules. According to the data obtained by combining the Live/Dead protocol and the microcolonial growth method, both the removed and the remaining cells were dead. Earlier we have shown the gradual dissolution of PDADMAC coatings in water,[18] which opens the possibility for the polycation to bind to the entire surface of the deposited cells, thereby enhancing the antimicrobial effect. The results obtained allow us to look deeper into the mechanism of the biocidal action of PDADMAC films and refine the protocol for quantifying their biocidal activity.

The work was supported by the Russian Science Foundation (project no. 22-13-00124).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2024.02.008.

References

1 R. Kaur and S. Liu, *Prog. Surf. Sci.*, 2016, **91**, 136.
2 P. Erkoc and F. Ulucan-Karnak, *Prosthesis*, 2021, **3**, 25.
3 C. Adlhart, J. Verran, N. F. Azevedo, H. Olmez, M. M. Keinänen-Toivola, I. Gouveia, L. F. Melo and F. Crijns, *J. Hosp. Infect.*, 2018, **99**, 239.
4 M. Lam, V. Migonney and C. Falentin-Daudre, *Acta Biomater.*, 2021, **121**, 68.
5 L. Timofeeva and N. Kleshcheva, *Appl. Microbiol. Biotechnol.*, 2011, **89**, 475.
6 D. Mitra, E.-T. Kang and K. G. Neoh, *ACS Appl. Polym. Mater.*, 2021, **3**, 2233.
7 Y. Lei, J. Xu, M. Pan, Y. Chen, X. Li, W. Zhu, C. Shu, T. Fang, H. Liao, Q. Luo and X. Li, *J. Mater. Chem. B*, 2023, **11**, 335.
8 J. C. Tiller, in *Developments in Surface Contamination and Cleaning: Fundamentals and Applied Aspects*, eds. R. Kohli and K. L. Mittal, William Andrew Publishing, Norwich, NY, 2008, pp. 1013–1065.
9 Q. Wang, L.Wang, L. Gao, L.Yu, W. Feng, N. Liu, M. Xu, X. Li, P. Li and W. Huang, *J. Mater. Chem. B*, 2019, **7**, 3865.
10 Y. K. Yushina, A. V. Sybachin, O. A. Kuznecova, A. A. Semenova, E. R. Tolordava, V. A. Pigareva, A. V. Bolshakova, V. M. Misin, A. A. Zezin, A. A. Yaroslavov, D. S. Bataeva, E. A. Kotenkova, E. V. Demkina and M. D. Reshchikov, *Coatings*, 2023, **13**, 1389.
11 I. G. Panova, E. A. Shevaleva, I. A. Gritskova, N. G. Loiko, Y. A. Nikolaev, O. A. Novoskoltseva and A. A. Yaroslavov, *Polymers*, 2022, **14**, 4598.
12 M. van de Lagemaat, A. Grotenhuis, B. van de Belt-Gritter, S. Roest, T. J. A. Loontjens, H. J. Busscher, H. C. van der Mei and Y. Ren, *Acta Biomater.*, 2017, **59**, 139.
13 O. V. Morozova, O. A. Levchenko, Z. A. Cherpakova, V. V. Prokhorov, N. A. Barinov, E. A. Obraztsova, A. M. Belova, K. A. Prusakov, K. G. Aldarov, D. V. Basmanov, V. N. Lavrenova, E. R. Pavlova, D. V. Bagrov, V. N. Lazarev and D. V. Klinov, *Int. J. Adhes. Adhes.*, 2019, **92**, 125.
14 A. A. Rakhnyanskaya, I. D. Pebalk, V. N. Orlov, I. A. Gritskova, N. I. Prokopov and A. A. Yaroslavov, *Polym. Sci., Ser. A*, 2010, **52**, 483 (*Vysokomol. Soedin., Ser. A*, 2010, **52**, 761).
15 A. S. Malinin, I. V. Kalashnikova, A. A. Rakhnyanskaya and A. A. Yaroslavov, *Polym. Sci., Ser. A*, 2012, **54**, 81 (*Vysokomol. Soedin., Ser. A*, 2012, **54**, 208).
16 C. Nascimento Galvão, L. Missfeldt Sanches, B. Ideriha Mathiazzi, R. T. Ribeiro, D. Freitas Siqueira Petri and A. M. Carmona-Ribeiro, *Int. J. Mol. Sci.*, 2018, **19**, 2965.
17 G. Ribeiro de Carvalho, A. M. Kudaka, R. A. Netto, C. Delarmelina, M. C. Teixeira Duarte and L. M. Ferrareso Lona, *Int. J. Biol. Macromol.*, 2023, **244**, 125388.
18 I. G. Panova, A. Yu. Lokova, D. V. Bagrov, N. G. Loiko, Y. A. Nikolaev and A. A. Yaroslavov, *Mendeleev Commun.*, 2023, **33**, 562.
19 J. Hierrezuelo, A. Sadeghpour, I. Szilagyi, A. Vaccaro and M. Borkovec, *Langmuir*, 2010, **26**, 15109.
20 J. Forsman, *Langmuir*, 2012, **28**, 5138.
21 D. Olmos and J. González-Benito, *Polymers*, 2021, **13**, 613.
22 K. A. Lusta and B. A. Fikhte, *Metody opredeleniya zhiznesposobnosti mikroorganizmov (Methods for Determining the Viability of Microorganisms)*, ed. V. K. Eroshin, ONTI NTsBI AN SSSR, Pushchino, 1990 (in Russian).
23 O. Braissant, M. Astasov-Frauenhoffer, T. Waltimo and G. Bonkat, *Frontiers in Microbiology*, 2020, **11**, 547458.
24 *LIVE/DEAD Fixable Dead Cell Stains Protocol*, ThermoFisher Scientific, https://www.thermofisher.com/nl/en/home/references/protocols/cell-and-tissue-analysis/protocols/live-dead-fixable-dead-cell-stains.html.

Received: 13th October 2023; Com. 23/7271