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1 **Chemical biofilm dislodgement with chelating and reducing agents in comparison to sonication:**
2 **implications for the diagnosis of implant associated infection**

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10 **Running title:** Chemical biofilm dislodgement for diagnosis of implant infection

11 **Key words**: biofilm, chemical dislodgement, sonication, prosthesis

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20 **Abstract**

21 Sonication of removed devices improved the microbiological diagnosis of infection. Recently, chemical
22 agents have been investigated for dislodgement of biofilms, including the chelating agent
23 ethylenediaminetetraacetic acid (EDTA) and the reducing agent dithiothreitol (DTT). We compared the
24 efficacy of chemical methods (EDTA and DTT) to sonication for biofilm dislodgement. *Staphylococcus*
25 *epidermidis* (ATCC 35984) and *Pseudomonas aeruginosa* (ATCC 53278) biofilms were grown on porous
26 glass beads for 3 days. After biofilm formation, beads were exposed to 0.9% saline, sonication and/or
27 chemical agents. Quantitative and qualitative biofilm analyses were performed by colony counting
28 (CFU/ml), isothermal microcalorimetry and scanning electron microscopy. The colony counts after
29 treatment with EDTA and DTT were similar to those after exposure to 0.9% saline (6.3, 6.1 and 6.0 log
30 CFU/ml, respectively) for *S. epidermidis* biofilms, and (5.1, 5.2 and 5.0 log CFU/ml, respectively) for *P.*
31 *aeruginosa* biofilm. Sonication detected higher CFU counts (7.5 log CFU/ml) for *S. epidermidis;* ($p<0.05$)
32 and 6.5 log for *P. aeruginosa* biofilm ($p <0.05$). Concordant results were detected with isothermal
33 microcalorimetry and scanning electron microscopy. In conclusion, the CFU count after treatment of *S.*
34 *epidermidis* or *P. aeruginosa* biofilms with EDTA and DTT was similar to those observed after 0.9% saline
35 used as control. In contrast, sonication was superior to chemical methods for biofilm dislodgment and
36 detection of microorganisms in sonication fluid. In conclusion, our study showed that sonication is
37 superior to chemical method to dislodge bacterial biofilm from artificial surface and should be considered
38 as standard diagnostic method for biofilm detection in implant-associated infections.

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39 **INTRODUCTION**

40 Orthopedic devices are increasingly used for treatment of degenerative joint disease and for fixation of
41 bone fractures. Infections represent a significant complication of implant surgery, resulting in major
42 challenges in diagnosis and treatment. The crucial step in the management of orthopedic implant-
43 associated infections is the accurate and timely diagnosis (1). However, this represents a considerable
44 challenge, as these infections are caused by microorganisms embedded in a polymeric matrix attached to
45 the device surface. In order to isolate and identify the causing microorganism, the dislodgment and
46 dispersion of the sessile community represent the first step before plating the specimen on solid media
47 (2). To improve biofilm removal from implant surface, different approaches had been described. Among
48 others, sonication is based on a mechanical dislodgement (3), while treatments with the metal-chelating
49 agent ethylenediaminetetraacetic acid (EDTA) (4) and the strong reducing agent, dithiothreitol (DTT) (5),
50 might disgregate biofilm by chemical interactions.

51 Sonication of explanted components as an add-on procedure to routinely conducted microbiological
52 analysis has shown to improve the pathogen detection (3), (6). Sonication reportedly yields rates of
53 bacterial recovery from 70% to 100% compared to 10% to 100% when scraping the prosthetic surface
54 and sensitivity of approximately 65% to 80% depending on prior antibiotic therapy (7), (8).

55 Recent reports considered the treatment of explanted prostheses with a solution containing DTT a
56 potential alternative to sonication to dislodge biofilm-embedded bacteria and allow for subsequent
57 isolation and identification of the microorganisms by conventional laboratory techniques (5). The ability of
58 EDTA to chelate and potentiate the cell walls of bacteria and destabilize biofilms by sequestering calcium,
59 magnesium, zinc, and iron suggests its use suitable for the biofilm detachment (4).

60 The aim of the study was to compare the ability of chemical (EDTA and DTT) and mechanical (sonication)
61 methods alone or in combination to detach biofilm-embedded bacteria.

62 **MATERIALS AND METHODS**

63 **Bacterial strains and biofilm growth conditions.** Biofilms of *Staphylococcus epidermidis* (ATCC
64 35984) and *Pseudomonas aeruginosa* (ATCC 53278) were formed on porous glass beads (4 mm
65 diameter, 60 µm pore sizes, ROBU®, Hattert, Germany). To form biofilms, beads were placed in 2 ml of
66 brain heart infusion broth (BHIb, Sigma-Aldrich, St. Louis, MO, USA) containing $1x10^8$ CFU/ml bacterial
67 inoculum and incubated at 37°C. After 24 h, beads were re-incubated in fresh BHIb and biofilms were let

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68 statically to grow for further 72 h at 37°C. After biofilm formation, beads were washed six times with 2 ml
69 0.9% saline to remove planktonic bacteria.
70 **Biofilm dislodgment by chemical methods (EDTA or DTT) or sonication.** To define the minimal
71 concentration and treatment duration for biofilm dislodging, washed beads were placed in 1 ml of EDTA
72 at concentrations 12, 25 and 50 mM or DTT at concentrations 0.5, 1 and 5 g/l and exposed for 5, 15 and
73 30 min. Untreated beads incubated with 0.9% saline were used as negative control.
74 To evaluate the sonication effect, biofilms were sonicated as described previously (3). Briefly, each bead
75 was inoculated in 1 ml 0.9% saline and sonicated at 40 kHz at intensity 0.1 Watt/cm^2 (BactoSonic,
76 BANDELIN electronic, Berlin, Germany) for 1 minute. One-hundred microliter of serial dilutions of the
77 resulting sonication fluid or the solution obtained after chemical treatment with DTT or EDTA were plated
78 onto Tryptic Soy Agar (TSA) (Sigma-Aldrich, St. Louis, MO, USA). After 24 h of incubation at 37°C, the
79 CFU/ml number was counted.
80 Additionally, the viability of planktonic bacteria in presence of chemical agents was evaluated. Planktonic
81 cells of *P. aeruginosa* and *S. epidermidis* at final concentration of $\approx 10^5$ CFU/ml were exposed to EDTA
82 (25 mM) and DTT (1 g/l) for different time periods (5, 15 and 30 min). All experiments were performed in
83 triplicates.
84 **Isothermal microcalorimetry analysis.** To prove the dislodgment effect of previously described
85 methods and reveal the presence of bacterial cells remained attached on the bead surface, treated beads
86 were washed six times in 2 ml 0.9% saline to remove the dislodged biofilm and placed in 4 ml-glass
87 ampules containing 3 ml of BHIb. The ampoules were air-tightly sealed and introduced into the
88 microcalorimeter (TAM III, TA Instruments, Newcastle, DE, USA), first in the equilibration position for 15
89 min to reach 37°C and avoid heat disturbance in the measuring position. Heat flow (µW) was recorded up
90 to 20 h. The calorimetric time to detection (TTD) was defined as the time from insertion of the ampoule
91 into the calorimeter until the exponentially rising heat flow signal exceeded 100 µW to distinguish
92 microbial heat production from the thermal background (9). Growth media without molds served as
93 negative control.
94 **Scanning electron microscopy.** Beads with biofilm were fixed with 2.5% (v/v) glutaraldehyde in
95 natrium cacodylat buffer and the samples were dehydrated with increasing concentrations of ethanol
96 for 2 min each. The samples were stored in vacuum until use. Prior to analysis by Field emission
97 scanning electron microscope (DSM 982 GEMINI, Zeiss Oberkochen, Germany), the samples were
98 subjected to gold sputtering (MED 020, BAL-TEC). All experiments were performed in triplicate.

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99 **Statistic methods.** Statistical analyses were performed using SigmaPlot (version 13.0; Systat Software,
100 Chicago, IL, USA) and graphics using Prism (version 7.03; GraphPad, La Jolla, CA, USA). Quantitative
101 data were presented as mean ± standard deviation (SD) or median and range, as appropriate. To
102 compare different groups, nonparametric Kruskal-Wallis test and Wilcoxon signed-rank test for
103 independent samples were used. The significance level in hypothesis testing was predetermined at p
104 <0.05.

105 **RESULTS**

106 **CFU counting method**. The dislodged CFU counts after treatment of beads with *S. epidermidis* and *P.*
107 *aeruginosa* biofilms at different concentrations and time points are shown in Figure 1 and 2. Evaluating
108 dislodgement effect of chemical methods, mean colony count obtained after treatment of *S. epidermidis*
109 biofilms with EDTA (25 mM, 15 min) and DTT (1 g/L, 15 min) was similar to those observed after 0.9%
110 NaCl used as control (6.3, 6.1 and 6.0 log CFU/mL, respectively). By contrast, sonication detected
111 significantly higher CFU counts with 7.5 log ($p <0.05$) (Figure 3 A). Similar results were observed when *P.*
112 *aeruginosa* biofilms were treated with chemicals (EDTA and DTT) or saline (5.1, 5.2 and 5.0 log CFU/ml,
113 respectively). By using sonication, CFU count of 6.5 log ($p <0.05$) was observed. (Figure 3 B).
114 **Isothermal microcalorimetry.** The heat produced by samples containing sonicated beads of *S.*
115 *epidermidis* was detected after 11 h from monitoring start (100 µW was set as cut-off value). In contrast,
116 heat production exceeding the threshold of 100 µW was observed earlier (after 6.3 and 6.5 h) for the
117 samples that were previously treated with EDTA and DTT, confirming the presence of a higher number of
118 residual bacteria on beads treated with chemical methods, in comparison to those after sonication. This
119 difference was statistically significant ($p <0.001$). No difference in heat production was observed after
120 treatment with 0.9% saline (control) and EDTA or DTT (6.3 vs 6.5 and 6.4 h, respectively) ($p=0.3$). Similar
121 results were observed with the analysis of *P. aeruginosa* biofilm beads, although the time of heat
122 detection after sonication of beads was significantly higher (11 h) in comparison to EDTA and DTT (6.5
123 and 6.5 h, respectively) ($p <0.001$); no difference between both chemical methods and the control (6.2 h)
124 was observed ($p=0.3$) (Figure 4 A and B).
125 **Scanning electron microscopy.** The use of scanning electron microscopy (SEM) allowed to visualize
126 the biofilms of *S. epidermidis* and *P. aeruginosa* before and after treatments with either chemicals or
127 sonication. For both microorganisms the scanning electron microscope images showed substantial less
128 biofilm biomass remaining on the beads when sonication was applied compared to control as well as both
129 chemical methods (Figure 5 and 6).

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130 **DISCUSSION**

131 Implant-associated infections due to biofilm formation represent a major challenge for the microbiological
132 diagnosis (10), (11). The presence of bacteria aggregated in a biofilm makes the detection of
133 microorganisms challenging when the sample is seeded on standard medium, without any previous
134 dislodgment and dispersion of the sessile community (2). We investigated the ability of different methods
135 to dislodge *S. epidermidis* and *P. aeruginosa* biofilms from an abiotic surface *in vitro*, including sonication
136 and chemical treatment with EDTA or DTT. To compare dislodgement effect of chemical methods, the
137 concentrations of 25 mM EDTA and 1 g/l DTT were chosen as they showed significant increase in CFU
138 count compared to other concentrations at the time point of 15 min. Concentration of 1 g/l DTT was also
139 proposed by Drago et al. (5) The time of 15 min was chosen as a most appropriate time for the routine
140 microbiological examination. (Fig. 1, Fig. 2).

141 Our results showed significantly higher dislodged CFU/ml for *S. epidermidis P. aeruginosa* biofilm when
142 sonication method was applied in comparison to chemical agent DTT. No significant difference in the
143 CFU number was observed after 1 mg/l DTT treatment, as compared to the control. Our results contradict
144 those showed by other authors (5). In this study polyethylene and titanium discs covered with *P.*
145 *aeruginosa*, *S. aureus*, *S. epidermidis* and *E. coli* biofilms were treated with DTT solutions at different
146 concentrations and for different time points. The authors found that a solution of 1g/l DTT applied for 15
147 min was able to dislodge *P. aeruginosa* biofilm with similar yield as obtained by sonication, but the
148 number of *S. aureus* biofilm cells removed by DTT were higher than that dislodged after sonication from
149 the same materials. Similarly, colony numbers for *S epidermidis* were higher after DTT treatment than
150 after sonication, whereas the number of *E. coli* colonies obtained after sonication and DTT were similar.
151 The discordance in the study results might be explained by the influence of biomaterial type on the biofilm
152 formation. In a recent study (12), the influence of biomaterials of retrieved hip and knee prosthesis on
153 microbial detection by sonication was onstrateddem.

154 In our study EDTA was not able to efficiently dislodge bacterial biofilm from artificial surface. Cell colony
155 count was similar to those obtained after treatment with 0.9% saline and significantly lower than those
156 observed when sonication was applied. Previous reports demonstrated that EDTA affects *P. aeruginosa*
157 biofilms (4), (13). Banin et al. suggested that exposure of *P. aeruginosa* biofilms to EDTA triggered
158 detachment of cells from biofilms. They showed the dispersal of cells from biofilms caused by EDTA in a
159 flow cell system. After addition of 50 mM EDTA to the medium reservoir, in 50 min they determined
160 increase two log more in the number of cells in the effluent compared to untreated flow cell which showed

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161 a constant level of viable, dispersed cells in the effluent. They note that activity of EDTA in detachment of
162 cells from the biofilm is mediated by chelation of several divalent cations such as magnesium, calcium,
163 and iron that are required to stabilize the biofilm matrix.

164 Our results from counting the dislodged bacterial counts were confirmed by two additional independent
165 techniques, namely isothermal microcalorimetry and SEM imaging. Isothermal microcalorimetry is a
166 highly sensitive method for bacterial replication due to their metabolic heat production (14), (9). It has
167 been widely employed in different studies to test the viability of either planktonic or biofilm bacteria after
168 antibiotic treatment (15), (16). Here it was used to evaluate bacteria remaining on the glass beads after
169 dislodging treatments. Isothermal microcalorimetry showed a significant delay in the detection of bacterial
170 metabolism-related heat production from the beads with *S. epidermidis* when sonication was applied, as
171 compared to chemical treatments - EDTA and DTT, suggesting that significantly less bacteria remained
172 attached to the beads after sonication. Similar results were observed by the analysis of *P. aeruginosa*
173 biofilm beads. The use of SEM allowed for visualization the biofilms of *S. epidermidis* and *P. aeruginosa*
174 before and after treatments with either chemicals or sonication. In both types of bacterial biofilm SEM
175 images showed less biofilm remaining on the beads when sonication was applied compared to the
176 untreated control as well as both chemical methods.

177 In conclusion, our study showed that sonication is superior to chemical method for dislodgement of
178 bacterial biofilm from surface and should be considered as the standard diagnostic method for biofilm
179 detection in the diagnosis of implant-associated infection. Future studies may investigate a potential
180 synergistic effect of sonication with chemical or mechanical dislodgement techniques, which do not affect
181 the viability of microorganisms.

182 **ACKNOWLEDGMENTS**

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Fig. 1. *S. epidermidis* biofilm after treatment with different concentrations of either EDTA (A) or DTT (B) at different time points. Mean values are shown, error bars represent standard deviation.

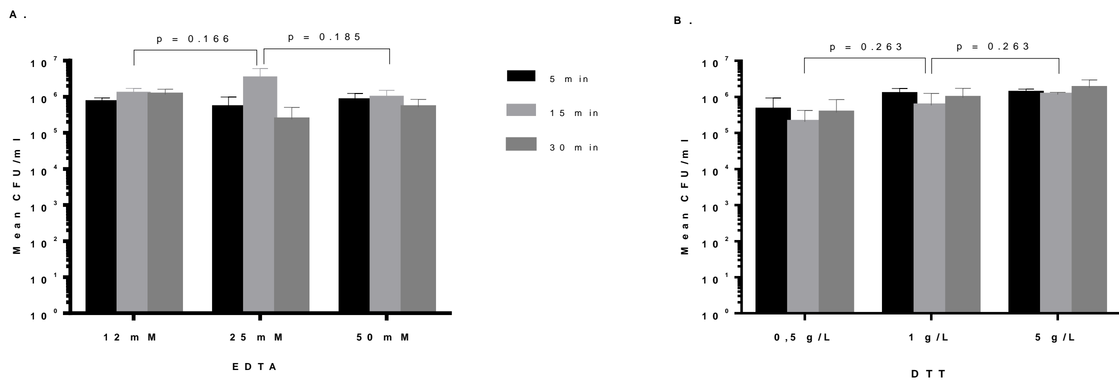

Fig. 2. *P. aeruginosa* biofilm after treatment with different concentrations of either EDTA (A) or DTT (B) at different time points. Mean values are shown, error bars represent standard deviation.

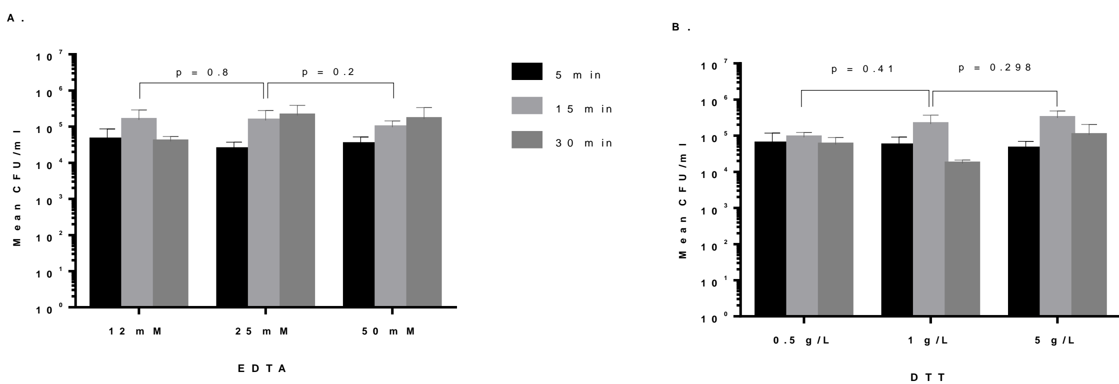

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Fig. 3. Quantitative analysis of biofilm dislodging methods. (A) *S. epidermidis* biofilm. (B) *P. aeruginosa* biofilm.

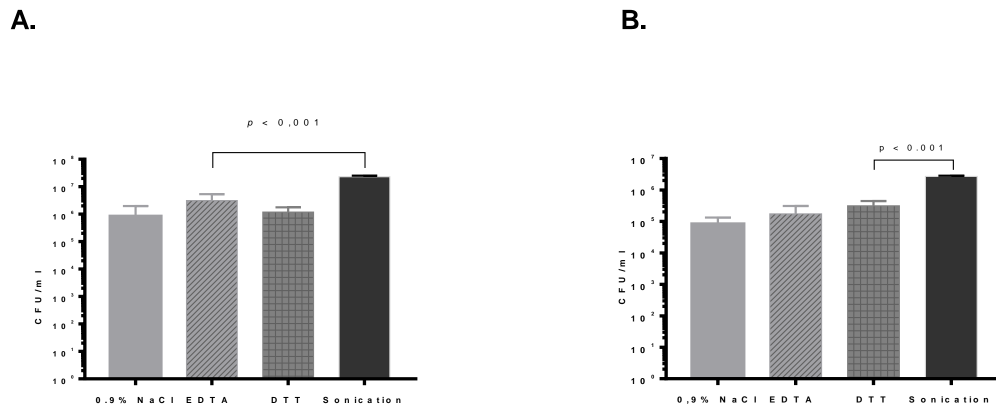

Fig. 4. The microcalorimetric time to detection (TTD) a bacterial growth. A. *S. epidermidis* biofilm. B. *P. aeruginosa* biofilm.

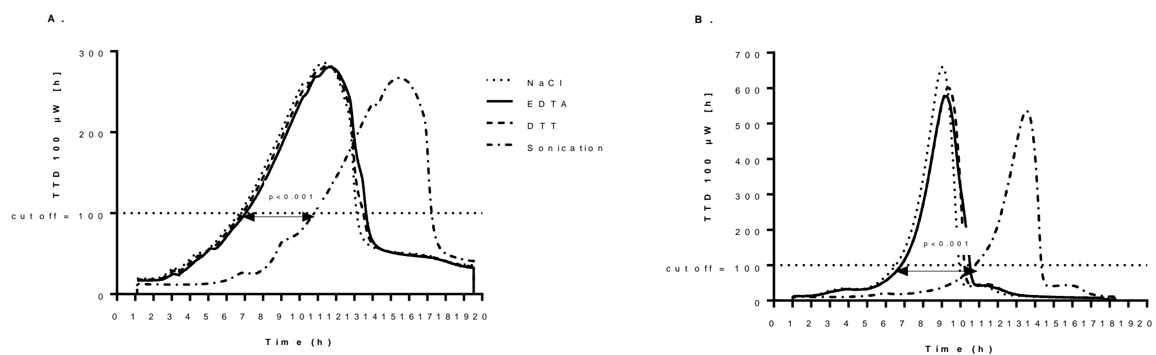

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Fig. 5. Scanning electron microscopy (SEM) of *S. epidermidis* biofilm: (A) beads after 0.9% saline treatment (control); (B) beads after EDTA treatment; (C) beads after DTT treatment; (D) beads after sonication treatment.

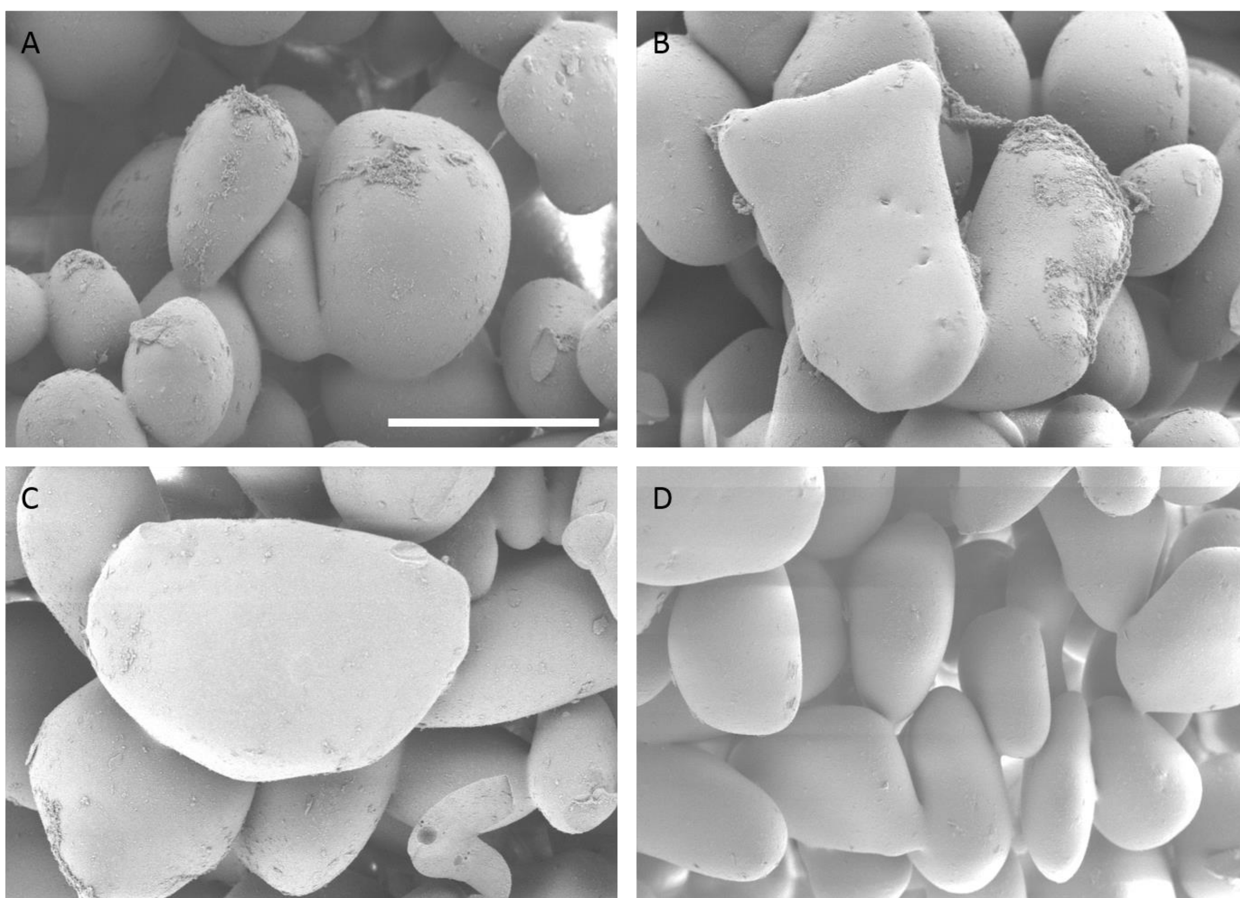

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Fig. 6. Scanning electron microscopy (SEM) of *P. aeruginosa* biofilm: (A) beads after 0.9% saline treatment (control); (B) beads after EDTA treatment; (C) beads after DTT treatment; (D) beads after sonication treatment.