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A Graftable Quaternary Ammonium Biocidal Polymer Reduces Biofilm Formation and Ensures Biocompatibility of Medical Devices

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1 INTRODUCTION:

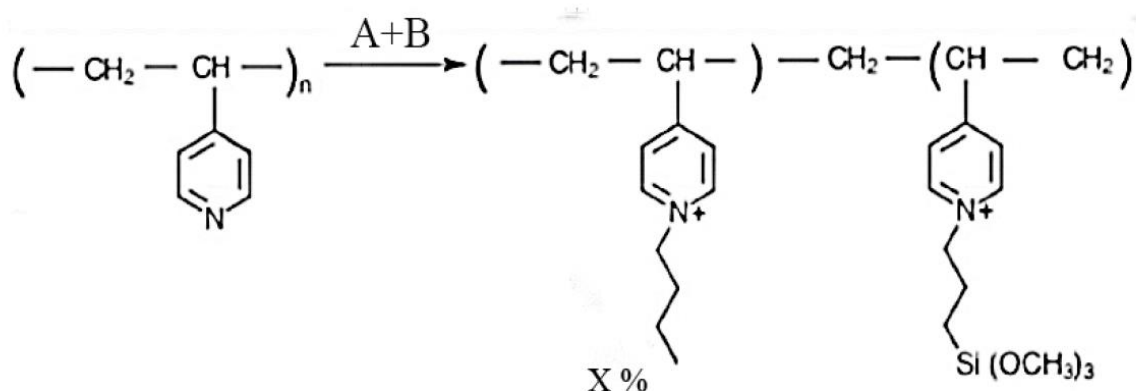
2
3 The use of large biomedical implants has demonstrated a decisive turn over the last 30
4 years with the popularization of hip and knee joint replacement (1). Fast aging in industrial
5 societies, increased life expectancy and quality along with functional demands from the elder
6 population account for the rise of both invasive and non-invasive procedures comprising
7 biomaterial implantation (2). Substantial progress was made in terms of mechanical properties,
8 biocompatibility, and biointegration resulting in excellent functional outcomes in multiple
9 surgical specialties. On the other hand, there is to this date no consensus for an anti-infectious
10 strategy in order to prevent biofilm formation on implanted surfaces. The consequences of
11 nosocomial infections arising from surgical implants still represent a heavy burden on both
12 patients and healthcare systems worldwide (3). In spite of the advancements of antisepsis
13 procedures, a significant and fixed proportion of nosocomial infections are still reported (from
14 1 to 2% respectively for hip and knee joint replacement). Biofilm is an exopolysaccharidic
15 matrix comprising bacteria with reduced antibiotic sensitivity and poor mechanical accessibility
16 (4)(5)(6). Biofilm formation plays a major role in the failure of conservative treatments
17 (antibiotic use, wound debridement and lavage) for implant-related infections (7). A common
18 rule is to perform implant removal to treat biofilm-related infections if a proper conservative
19 treatment has previously failed, which explains the high morbidity caused by the management
20 of infections on implanted devices (8). This represents the rationale behind the development of
21 coating strategies for the prevention of implant-related infections (9). Indeed, since the first
22 stage of biofilm formation is believed to be bacterial adherence, there has been a tremendous
23 enthusiasm about the development of new coatings and surface modifications aimed at repelling
24 bacteria or killing bacteria on contact. Numerous *in vitro* studies showed the interest of biocidal
25 polymers grafted on plastics or metals in the medical field (10). They target bacterial
26 colonization, bacterial encapsulation or both. Polymer-based coatings may either reduce
27 bacterial adherence or directly kill bacteria without altering the mechanical and thermic
28 properties of implanted devices. The agents preventing bacterial colonization are antiadhesive
29 polymers inhibiting bacterial adhesion (polyvinyl sodium sulfonate, polyethylene glycol, PEG)
30 and biocidal substances such as antibiotic coatings and silver-impregnated surfaces. Currently,
31 the most concerning drawbacks of antibacterial polymers which relies on antiadhesive effects
32 are their transitory local efficacy, potential toxicity through progressive leaching and the low
33 or absent bacteriostatic or bactericidal effect. Therefore, bactericidal polymeric agents have
34 been introduced. The most used are high-density quaternary ammonium salts (chitosans (11)),

35 phosphonium salts (12), sulfonium salts (13), pyridinium salts (14) and mono or biguanidium
 36 salts (15). These high-density cationic polymers feature a non-selective bacteria contact-killing
 37 effect (16). While they are extensively described in the literature, the biocidal mechanisms
 38 remain poorly detailed. In fact, most studies focused on killing mechanisms occurring in
 39 solutions of varying densities (18)(19) and not on grafted high-density QAP.

40 A novel ready-to-use and scalable QAP, Q-PVP (quaternized polyvinylpyridine) was
 41 synthesized in a methanolic solvent in a single step. The objective of the study was to
 42 characterize grafted titanium surfaces, measure their *in vitro* antibacterial activity quantitatively
 43 and qualitatively, assess biofilm inhibition and *in vitro* biocompatibility.

44 MATERIAL AND METHODS:

45 1) Polymer synthesis



49
50 **Figure 1.** Quaternized polyvinylpyridine polymer synthesis

51 **n:** 1Eq

52 **A:** iodopropyltrimethoxysilane, 20 to 50mEq

53 **B:** 1-bromobutane, 1Eq

54 **x** depends on reaction time

55
56 A quaternized polyvinylpyridine (Q-PVP) statistical polymer was synthesized following the
 57 introduction of specific chains on the polymeric chain in a single-step reaction in boiling
 58 methanol. Five specific changes were carried out in the synthesis process. Polymers synthesized
 59 with no bromobutane chain were labelled Q-PVP-iodo. Reaction time also varied in order to
 60 modulate the ammonium quaternization rate. Polymers synthesized in 36h, 2 days and 4 days
 61 were respectively labelled Q-PVP-36h, Q-PVP-2days, Q-PVP-4days. When polyethylene

glycol (PEG) replaced 1-bromobutane in the synthesis (24h), the product was labelled Q-PVP-PEG. The iodopropyltrimethoxysilane chain was used as a “hook” in order to immobilize the polymeric chain on the activated surface through a covalent linkage between the surface and the polymeric moiety. The butyl chain was used to quaternize the rest of the Q-PVP chain. A fixed 5% proportion of iodopropyltrimethoxysilane was critical to reach storability of the polymer since higher proportions cause reticulation in volume and therefore prevent long-term storage.

2) Polymer grafting

1cm² titanium plates (99,6% purity, Goodfellow, Cambridge Ltd., Huntington, United Kingdom) were successively polished on 3 grains (P800, P2000 and P4000 grit paper using a Saphir 320, ATA GmbH, Mammelzen, Germany) for respectively 1 minute, 2 minutes and 3 minutes. Following abundant lavage and a 5-minute sonication process with distilled water in order to remove residual particles, the plates were placed in a piranha solution (96% sulfuric acid/30% hydrogen peroxide (2/1, v/v) for two hours so as to obtain hydroxylation and surface chemical abrasion (hence forming activated surfaces). All plates were subsequently rinsed, sonicated in distilled water and dried under nitrogen flux. Polymer grafting was achieved either by one or six spin-coating rounds of a diluted 125μL polymer drop (100μL polymer, 25μL distilled water) on activated titanium plates (40 seconds, 3000 rpm, Laurell WS 650 Spin Coater, Laurell Technologies Corp., North Wales, PA, USA) or by dip-coating (diluted polymer solution with 40% distilled water). Plates were immediately transferred to a pre-heated stove at 110°C (Memmert, model 30, Memmert GmgH, Schwabach, Germany) for 30 minutes for reticulation. The plates were then sonicated in pure ethanol for five minutes for adequate removal of any remaining physisorbed particles. Control plates underwent the same protocol with distilled water instead (spin-coating or dip-coating, heating, sonication).

3) Surface characterization

X-ray Photoelectron Spectroscopy (XPS):

97 XPS analyses were performed using an Omicron Argus X-ray photoelectron spectrometer. The
1 98 monochromated AlK_α radiation source ($h\nu = 1486.6$ eV) had a 300 W electron beam power.
2
3 99 The emission of photoelectrons from the sample was analyzed at a takeoff angle of 90° under
4
5 100 ultra-high vacuum conditions ($\leq 10^{-10}$ Torr). Spectra were carried out with a 100eV pass energy
6
7 101 for the survey scan and 20 eV pass energy for the C1s, O1s, N1s, S2p regions. Binding energies
8
9 102 were calibrated against the aliphatic C1s contribution binding energy at 284.8 eV and element
10
11 103 peak intensities were corrected by Scofield factors. The peak areas were determined after
12
13 104 subtraction of a linear background. The spectra were fitted using Casa XPS v.2.3.15 software
14
15 105 (Casa Software Ltd., U.K.) and applying a Gaussian/Lorentzian ratio G/L equal to 70/30.

16 106 17 18 107 *Polarization Modulation Reflection Absorption Infra-Red Spectroscopy (PM-RAIRS):*

19
20 108
21
22 109 PM-RAIRS analyses were performed in air with the crystal placed in the external beam
23
24 110 of a Fourier transform infrared Nicolet 5700 spectrometer (Nicolet Nexus 5700, Thermo
25
26 111 Electron Scientific Instruments Corporation, Madison ®, WI, USA).; the experimental setup
27
28 112 was described elsewhere (20). All reported spectra were recorded at 8 cm⁻¹ resolution by co-
29
30 113 addition of 128 scans; using a modulation of polarization enabled us to perform rapid analyses
31
32 114 of the samples after immersion without purging the atmosphere or requiring a reference
33
34 115 spectrum.

34 116 35 36 117 *Surface charge determination by fluorescein test:*

37
38 118
39
40 119 Surface cationic density (NH₄/cm²) was calculated by a fluorescein test. It is estimated
41
42 120 that each fluorescein molecule (negatively charged) strongly binds quaternary ammonium
43
44 121 molecules (positively charged) belonging to the polymeric chain. Treated plates were placed in
45
46 122 a 2% fluorescein aqueous solution for five minutes. They were then rinsed and sonicated for
47
48 123 five minutes in order to remove physisorbed fluorescein. Plates were rinsed in distilled water a
49
50 124 second time, immersed in a test tube containing CTAB 0,1% (aqueous solution of cetyl
51
52 125 trimethylammonium bromide) diluted in PBS (Phosphate-Buffered Saline) (90% CTAB/10%
53
54 126 PBS) and vortexed for 10 seconds. Fluorescein was then dissolved, allowing an optical density
55
56 127 measurement using a spectrophotometer at 501 nm wavelength (CamSpec M550, Spectronic
57
58 128 Camspec Ltd, Leeds, UK). Measurements were repeated three times.

58 129
59
60 130 Surface cationic density was estimated according to the following formula:

131

$$\frac{A \times V \times NA}{\epsilon \times S}$$

132

133 A: fluorescein optical density at 501 nm

134 V: volume

135 NA: Avogadro number ($6.022140857 \times 10^{23} \text{ mol}^{-1}$)

136 ϵ : fluorescein molar absorptivity or molar extinction coefficient ($\text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$).

137

138 The impact of coating thickness on fluorescein tests was also assessed. Spin-coating was either
139 performed in a single round (125 μL in 40 seconds) or six rounds (six 125 μL drops, 40 seconds
140 each). Plates were either labelled SLSC (single-layer spin-coating) or MLSC (multi-layer spin-
141 coating).

142

143 *Polymer leaching:*

144 Eight plates spin-coated with Q-PVP-4 days, either SLSC or MLSC, underwent a 1-week
145 leaching test at 37°C and were immersed in two different mediums: SLSC plates in distilled
146 water (n=2), SLSC in rabbit serum (Sigma-Aldrich, Saint-Louis, MO, USA) (n=2), MLSC
147 plates in distilled water (n=2), MLSC plates in rabbit serum (n=2). A fluorescein test was
148 performed to determine the remaining surface charge density percentage (RSCD) after one
149 week. Measurements were repeated three times.

150

151 *Atomic force microscopy (AFM) surface analysis:*

152 Grafted and control titanium plate imaging was performed using a commercial atomic
153 force microscope (Bruker Nano Inc.-Nano Surfaces Division, Santa Barbara, CA, USA)
154 equipped with a J scanner (150 x 150 x 5 μm). Images were analyzed in “QNM ®” mode
155 (Quantitative Nanomechanical Property Mapping) in air or “intermittent-contact” mode in a
156 quartz measure cell. Sharps were made of silicon nitride (Si_3N_4) and had a theoretical stiffness
157 constant of 0.05 N.m^{-1} and a curve ray of 20 nm. Observations were done at a constant speed
158 of 1 Hz, 512 lines of 512 pixels each, being recorded for each image. Data were analyzed using
159 Nanoscope Analysis software (Bruker, Santa Barbara, CA, USA). The displayed images are 3D
160 reconstructions of height and DMT modulus images.

161

162 *Scanning Electron Microscopy with Field Emission Gun (SEM-FEG) surface analysis:*

163

164

165

163

1 SEM images were recorded with a Hitachi SU-70 field emission gun scanning electron
2 microscope. The samples were fixed on an alumina SEM support with a carbon adhesive tape
3 and were observed without metallization. An in-lens secondary electron detector (SE_{Upper}) was
4 used to characterize our samples. The accelerating voltage was 1 kV, and the working distance
5 was around 5 mm. At least five different locations were analysed on each surface, arising to the
6 observation of a minimum of 100 single cells.
7

170

14 *Irradiation test:*
15

172

18 Dip-coated titanium plates were sterilized by gamma irradiation from a ⁶⁰Co source
19 with a total delivered dose of 27kGy (minimum threshold 25kGy, ISO 11137-2: 2013) (21).
20 The irradiation was performed at 0°C for 4 hours (BBF Sterilisationservice GmbH, Kernen,
21 Germany) to prevent denaturation of the polymer during the sterilization process. Infra-red
22 spectra were compared before and after irradiation to verify the integrity of the polymer at the
23 surface.
24

179

31 **4) Biocompatibility Assessment:**

181

34 *MTT Viability Test:*
35

183

38 A murine osteoblast precursor cell line (MC3T3-E1) was cultured in MEM- α (Gibco
39 Invitrogen, France) enriched with 10% FBS (fetal bovine serum), 2mM glutamine, 100 U/mL
40 penicillin and 100 μ g/mL streptomycin. Murine fibroblasts (L929) were also cultured
41 separately in DMEM (Dulbecco's Mod Eagle Medium) supplemented with 10% FBS, 2mM
42 glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin.
43

189

49 5×10^5 cells of each cell line were seeded on each titanium plate in their respective medium
50 (1mL) in 24-well plates. Five triplicate conditions were tested (control, Q-PVP-iodo, Q-PVP-
51 36h, Q-PVP-2days, Q-PVP-4days, Q-PVP-PEG) in order to further elucidate the consequences
52 of the introduction of specific chains in the polymer on cell viability. All tested surfaces were
53 spin-coated. In both cases, following incubation at 37°C in humid atmosphere and 5% CO₂, a
54 trypsin EDTA treatment was applied in order to retrieve adherent cells. The protocol was
55 repeated three times for each cell line. Finally, an MTT assay comparing optical densities at
56

196

6

65

197 550nm was then performed in order to measure cell viability at 72h (Wallac 1420 Victor2™
198 microplate reader, Perkin Elmer, Waltham, MA, USA). 70% cell viability was considered to be
199 the critical threshold. Measurements were averaged on each triplicate and presented with mean
200 ± SEM (standard error of the mean).

202 *SEM-FEG cell adhesion and morphological analysis:*

203
204 L929 and MC3T3 cells were cultured on titanium plates spin-coated and grafted with Q-PVP-
205 iodo, Q-PVP-36h, Q-PVP-2days, Q-PVP-4days until 72h under the conditions described
206 previously. All titanium plates underwent the following fixation protocol: plates were rinsed
207 three times with PBS 5% and fixed in 2,5% glutaraldehyde (2 hours). The fixative was then
208 drained off and plates were immersed in ethanol at room temperature as follows: 25% EtOH (5
209 minutes), 50% EtOH (5 minutes), 75% EtOH (5 minutes), 100% EtOH (5 min) and finally dried
210 under a fume hood. Cell adhesion behavior was assessed by SEM-FEG.

212 **5) Antibacterial activity:**

213
214 An ST 2012-238 MRSA strain (Lyon, France) isolated from a patient with a PJI
215 (prosthetic joint infection) was cultured in Brain Heart Infusion (BHI) at 37°C overnight.
216 According to a modification of the 22196:2011 ISO norm, a 10⁷CFU/mL bacterial suspension
217 of 20 µL in rich medium (BHI) was simultaneously deposited and applied with cover slips on
218 titanium plates (control versus grafted with Q-PVP-iodo, Q-PVP-36h, Q-PVP-2 days, Q-PVP-
219 4 days, Q-PVP-PEG using spin coating or dip coating) inside 24-well culture plates for
220 triplicate. Culture plates were then transferred to a stove at 37°C. Cultures were sequentially
221 stopped after 1h (bacterial “killing” at 37°C) and 24h (growth inhibition at 37°C), diluted in
222 0.9% saline and vortexed for detachment of live bacteria and bacterial counting.

224 **6) Antibiofilm activity:**

225
226 Q-PVP-4days dip-coated titanium plates were placed into 24-well culture plates. A 10⁶
227 CFU/mL bacterial suspension in 1mL of BHI (prepared as described above) was used to fill
228 wells containing titanium plates. Culture plates were then transferred to a stove for culture at
229 37°C. Cultures were then stopped sequentially each time for two control and two grafted
230 titanium plates after respectively 6h, 12h, 24h, 72h, and 7 days (medium was removed and

231 replaced with sterile BHI every 24h). An *in vitro* biofilm of gradual maturity was therefore
232 created on dip-coated titanium plates and controls. In order to determine the influence of BHI
233 on plate surface morphology, two other control titanium plates were prepared: one was
234 immersed in sterile BHI throughout the experiment and underwent fixation while the other was
235 activated by piranha treatment and underwent fixation afterwards. All titanium plates
236 underwent the fixation protocol described above. SEM-FEG imaging on treated and control
237 titanium plates covered with 6 h to 7 day biofilm were obtained.

7) Killing Activity

241 A killing test was performed on Q-PVP-4days plates (controls versus treated) at 20°C following
242 a three-hour contact with a 10⁷CFU/mL MRSA inoculum. For the latter, AFM imaging was
243 performed.

8) Statistical analysis

247 For comparisons of two means, a Student t-test was used. All statistical analyses were
248 performed with Prism 5 (Graphpad software). P-values below 0.05 were considered significant
249 and p-values were represented as follows: *=P<0.05, **=P<0.01.

RESULTS:

1) Surface characterization

X-Ray Photoemission Spectroscopy (XPS)

257 XPS experiments were performed on both dip and spin-coated quaternized surfaces (Q-PVP-
258 4days) compared to bare surfaces before any functionalization. First, one can notice on the
259 control surface that most of layer of the sample is composed of TiO₂ oxidized titanium, with
260 the main contribution of the Ti2p_{3/2} signal being centered at 459.0 eV and only a very small
261 contribution (less than 3 %) of metallic titanium visible at 454.1 eV.

262 The second conclusion that can be drawn from these data is the equivalent thickness of the
263 functionalized surface. In fact, one can estimate that thickness by looking at the decrease of the
264 Ti signal after functionalization, taking into account that the mean free path (λ) of TiO₂

265 electrons onto organic films is around $\lambda=2.85$ nm. In the case of the dip coated method, the
 266 XPS Ti2p signal is completely extinguished suggesting an equivalent thickness of the Q-PVP-
 267 4 days film superior to 3λ , i.e. superior to 8.5 nm, while for the spin coating method, the TiO₂
 268 signal is still faintly visible, suggesting an equivalent thickness of 8 nm for the Q-PVP-4 days
 269 films.

270 Finally, chemical composition details can be obtained when looking at the different atomic
 271 percentages of the different films as compared to non-functionalized control, Table 1, showing
 272 that similar amount of Q-PVP-4days is grafted on the surface, regardless of the deposition
 273 method.

Table 1: Atomic percentage obtained from XPS data for spin and dip coated films as compared
 to non functionalized surface.

| Atomic % | Ti2p | O1s | C1s | N1s |
|----------------------|------|------|------|-----|
| TiO2 control surface | 17.5 | 41.4 | 40.2 | 0.9 |
| Spin-coated surface | 0.3 | 6.5 | 87.6 | 5.6 |
| Dip-coated surface | ND | 4.3 | 89.8 | 5.9 |

277 ND=non detectable; % are obtained as mean of two measurements, the standard deviation of
 278 the measure is around 0.2 %.

Polarization Modulation Infra-Red Reflection Absorption Spectroscopy (PM-IRRAS):

282 Polyvinylpyridine quaternization was modulated depending on reaction time (Q-PVP-
 283 iodo in 12 hours, Q-PVP-36h, Q-PVP-2days, Q-PVP-4days) leading to grafted polymers with
 284 increasing $\frac{N^+}{N}$ ratios along with increased reaction time. The $\frac{N^+}{N}$ ratio was determined through
 285 PM-IRRAS spectra using the area ratio between the stretching mode of the C-N⁺ band (1640
 286 cm⁻¹) and C-N band (1600 cm⁻¹) by looking at the very specific IR marker present at 1640 cm⁻¹
 287 assigned to the protonated amino groups. One can notice that this contribution is increasing
 288 as a function of reaction time (Figure 2).

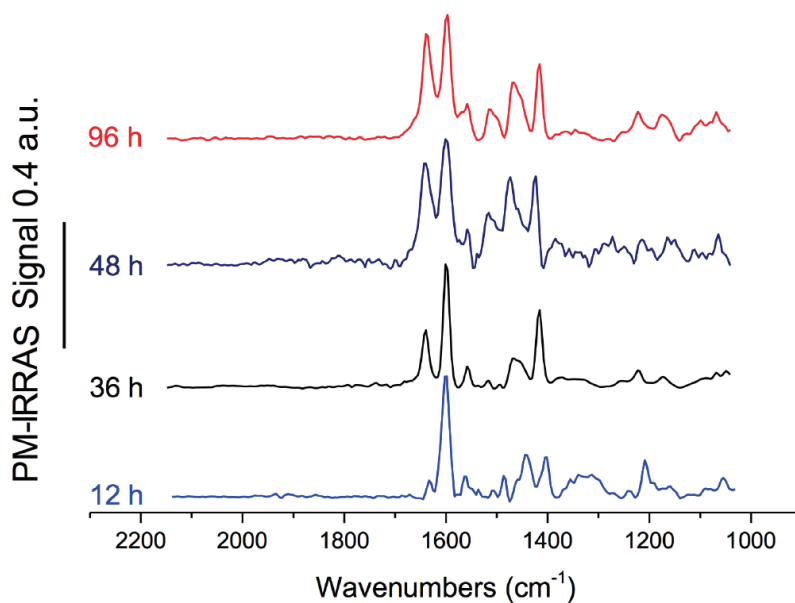


Figure 2. PM-IRRAS spectra following deposition and copolymer grafting on titanium: quaternization increased with reaction time (12 h to 96 h).

The $\frac{N^+}{N}$ ratio are presented on Table 2 for each reaction, and one can see that a stable regime is achieved after 48 hours of reaction time, with no more possible quaternization even with twice longer reaction time, eg. 96 hours.

Table 2: % of quaternization of the copolymer as a function of reaction time.

| Reaction time (hours) | 12 | 36 | 48 | 96 |
|-----------------------------|----------------|----------------|----------------|--------------|
| Ratio : $\frac{N^+}{N}$ (%) | 12.1 ± 0.5 | 31.3 ± 0.7 | 44.6 ± 1.2 | 45.9 ± 2 |

Scanning Electron Microscopy with Field Emission Gun (SEM-FEG):

Grafted surfaces appeared smoother than control plates despite identical polishing. Imperfections in polishing were more easily visible on control plates and grafted plates after BHI sedimentation and fixation. Despite this, all surfaces were relatively homogeneous. Neither the BHI sedimentation nor the fixation protocol seemed to cause further chemical abrasion of titanium surfaces (Figure 3).

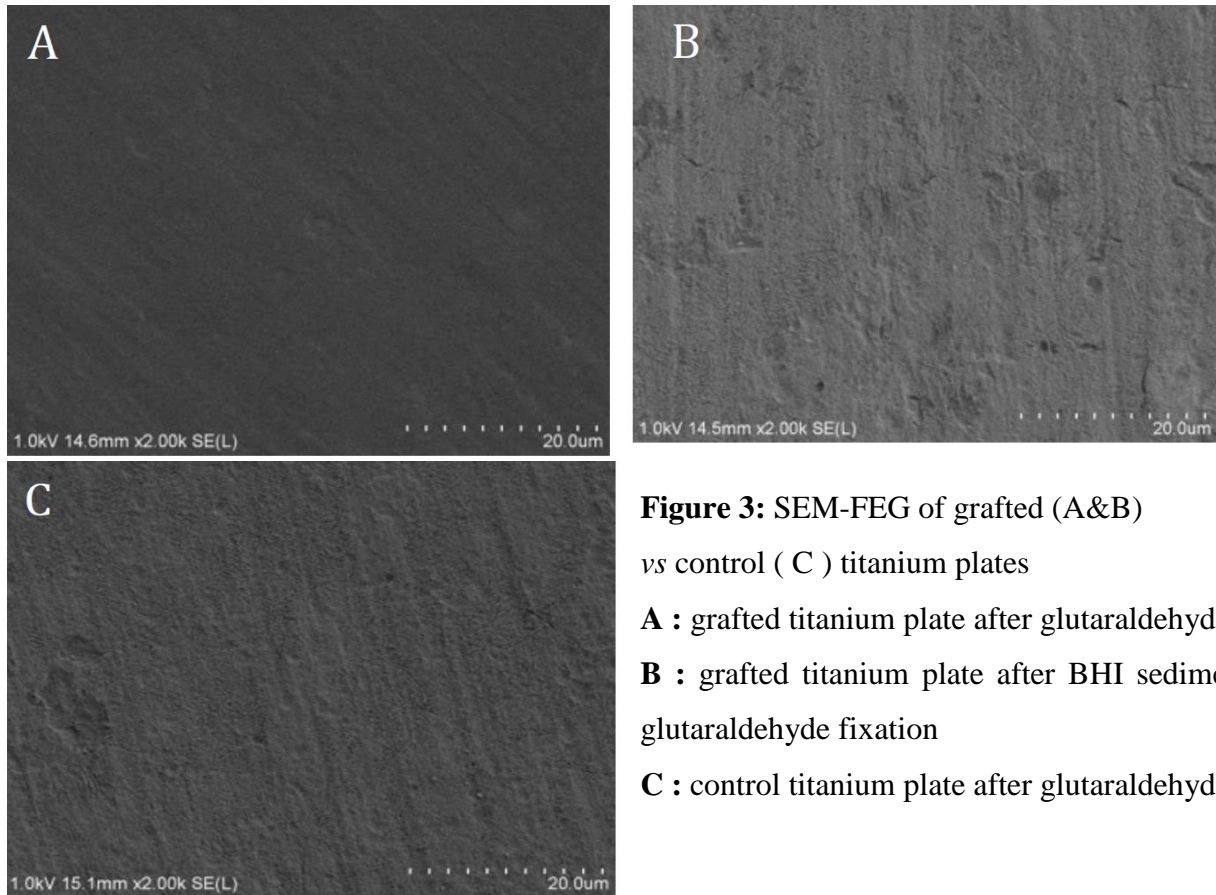


Figure 3: SEM-FEG of grafted (A&B) vs control (C) titanium plates
A : grafted titanium plate after glutaraldehyde fixation
B : grafted titanium plate after BHI sedimentation and glutaraldehyde fixation
C : control titanium plate after glutaraldehyde fixation

Atomic Force Microscopy (AFM):

Grafted and control surfaces presented a near identical roughness (controls: 23.0 nm vs grafted: 22.6 nm). Grafted surfaces were chemically homogeneous.

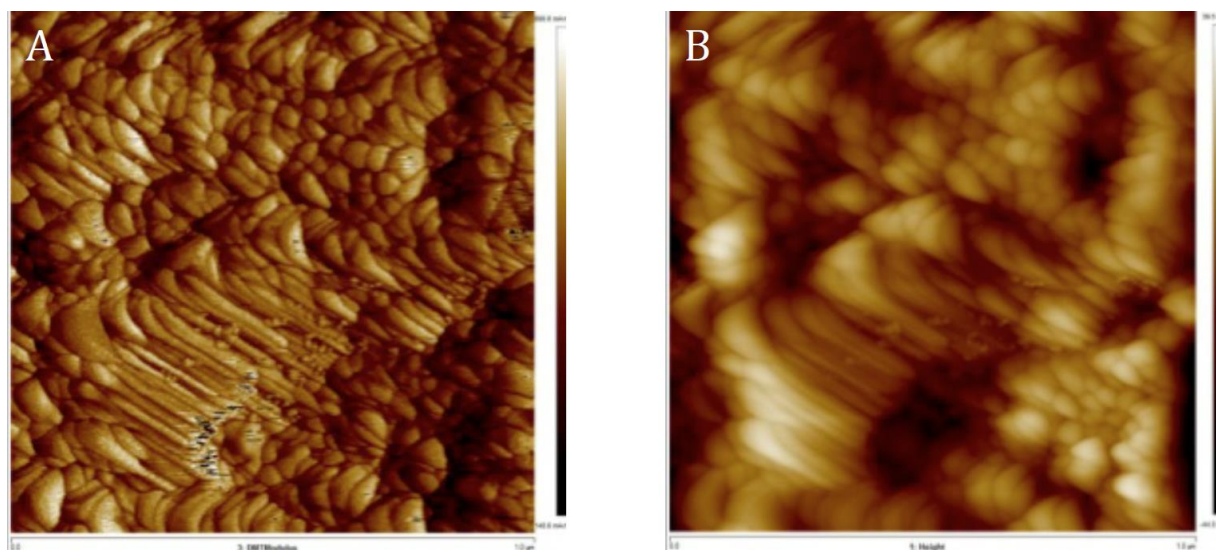
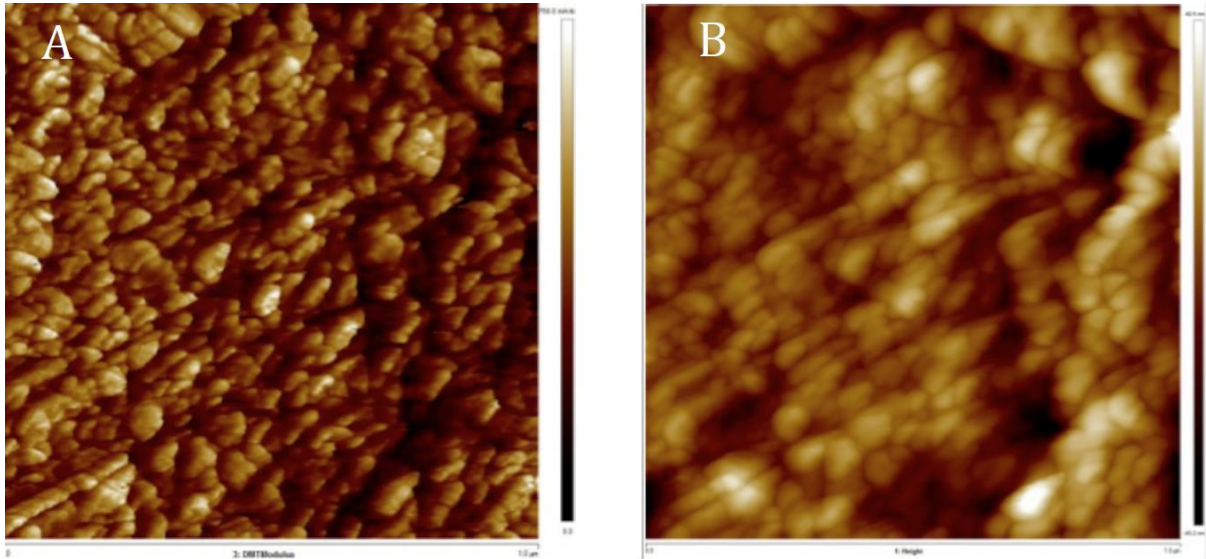


Figure 4: AFM imaging of a control titanium plate (1µm scale)

335 **A:** DMT Modulus: chemically homogeneous sample (pure titanium).

336 **B:** Height mode : sample roughness after polishing: 23nm.



339 **Figure 5:** AFM imaging of grated plates spin-coated with Q-PVP-4days (1μm scale).

340 **A:** DMT (Derjaguin-Muller-Toporov) Modulus: grafted polymer appears chemically
341 homogeneous on the titanium surface

342 **B:** Height mode: grafted polymer layer, roughness : 22,6nm

343 2) Surface charge determination by fluorescein test:

344 SLSC Q-PVP-36h showed systematically higher absorbance than plates that were spin-coated
345 in a single round with Q-PVP-4days (0.296 ± 0.034 or 2.31×10^{17} charges/cm², n=6 vs $0.133 \pm$
346 0.026 or 1.04×10^{16} charges/cm², n=6). SLSC Q-PVP-2days plates averaged 0.130 ± 0.040
347 (n=3) with an estimated surface charge density of 1.02×10^{17} charges/cm².

348 3) Polymer Leaching

349 MLSC plates showed a tendency towards lower leaching than single SLSC plates in rabbit
350 serum though it was more significant in distilled water (respectively $47.55 \pm 22.13\%$ vs 68.95
351 $\pm 13.36\%$ and $72.30 \pm 1.13\%$ vs $88.65 \pm 3.61\%$). Furthermore, leaching in rabbit serum was
352 systematically lower than in distilled water while assessing similar coating thicknesses.

353 Fluorescein test measurements with leaching and RSCD calculations are reported in Table 3.

361

362 **Table 3.** Surface charge determinations on titanium plates grafted with Q-PVP-4 days before
 363 and after a 7-day immersion in either rabbit serum or distilled water.

| | RABBIT SERUM | | | | DISTILLED WATER | | | |
|--------|--------------|-------------|-----------|----------|-----------------|-------------|-----------|-----------|
| | t=0 | t=7 days | Leaching% | RSCD | t=0 | t=7 days | Leaching% | RSCD |
| SLSC1 | 0.125±0.004 | 0.027±0.005 | 78,40 | 2,11E+15 | | | | |
| SLSC2 | 0.101±0.013 | 0.041±0.008 | 59,50 | 3,21E+15 | | | | |
| SLSC3 | | | | | 0.137±0.014 | 0.012±0.005 | 91,2 | 9,382E+14 |
| SLSC4 | | | | | 0.108±0.07 | 0.015±0.003 | 86,1 | 1,173E+15 |
| MLSC 1 | 0.494±0.023 | 0.182±0.006 | 63,20 | 1,42E+16 | | | | |
| MLSC 2 | 0.457±0.009 | 0.311±0.005 | 31,90 | 2,43E+16 | | | | |
| MLSC 3 | | | | | 0.295± 0.008 | 0.084±0.011 | 71,5 | 6,567E+15 |
| MLSC 4 | | | | | 0.360±0.029 | 0.097±0.018 | 73,1 | 7,584E+15 |

4) Biocompatibility assessment (MTT-assay)

Mean MC3T3 absorbance on control titanium plates was 0.42 ± 0.02 , arbitrarily considered 100% viability. MC3T3 cell viability decreased on plates displaying higher surface cationic densities compared with controls (from Q-PVP-iodo ($118\% \pm 5.4\%$ survival) to Q-PVP-4 days ($73\% \pm 5.1\%$). If the initial reaction did not include a bromobutane chain and lasted only 12 hours (Q-PVP-iodo), cells deposited on grafted titanium surfaces persistently showed higher cell survival at 72h than cells on control titanium plates ($118\% \pm 5.4\%$). Of all tested samples, Q-PVP-PEG surfaces displayed the lowest viability rate ($60.1\% \pm 2.3$).

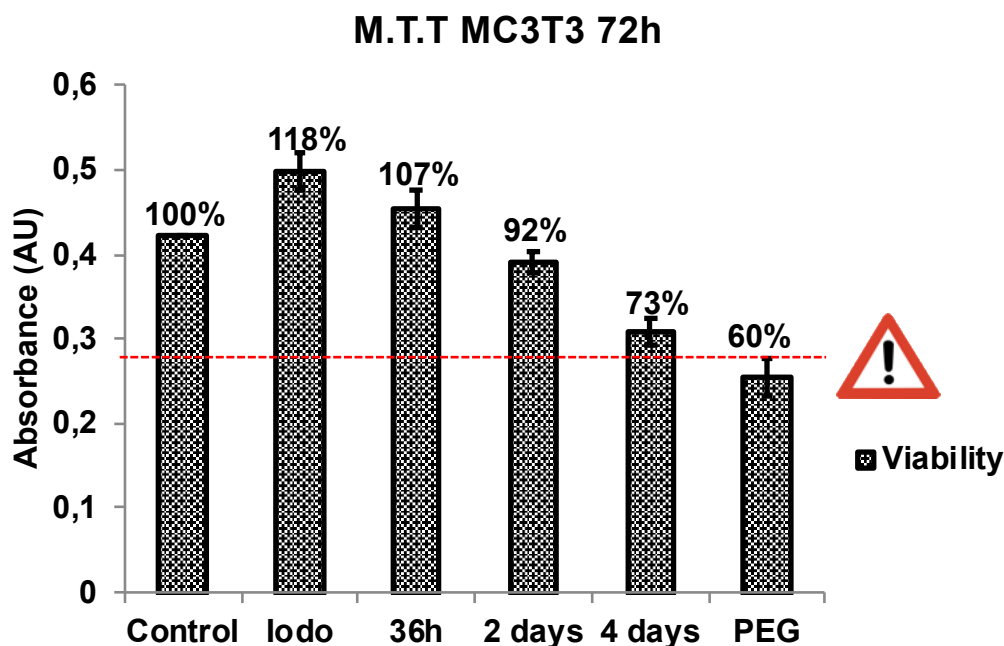


Figure 6. MTT-assay: M3T3 cell survival at 72h on surfaces of various cationic densities

Controls: n=9 ; Q-PVP-iodo: n=9 ; Q-PVP-36h: n=9 ; Q-PVP-2 days: n=8 ; Q-PVP-4 days: 7 ; Q-PVP-PEG: n=3. All data are represented as mean \pm standard error. All treated plates showed statistically significant differences in absorbance values compared with control ($P < 0.05$) except for Q-PVP-36h. The red-dotted line represents the minimum acceptable viability (70%).

Mean L929 absorbance on control titanium plates was 0.27 ± 0.002 , arbitrarily considered 100% viability. L929 cell viability gradually decreased on plates displaying higher cationic surface densities, from Q-PVP-iodo (96.3 ± 2.2 %) survival to Q-PVP-4 days ($87\% \pm 1.6$ %). Q-PVP-iodo did not increase L929 survival at 72h.

For each given surface cationic density, L929 cells demonstrated better survival compared with MC3T3 cells at 72h

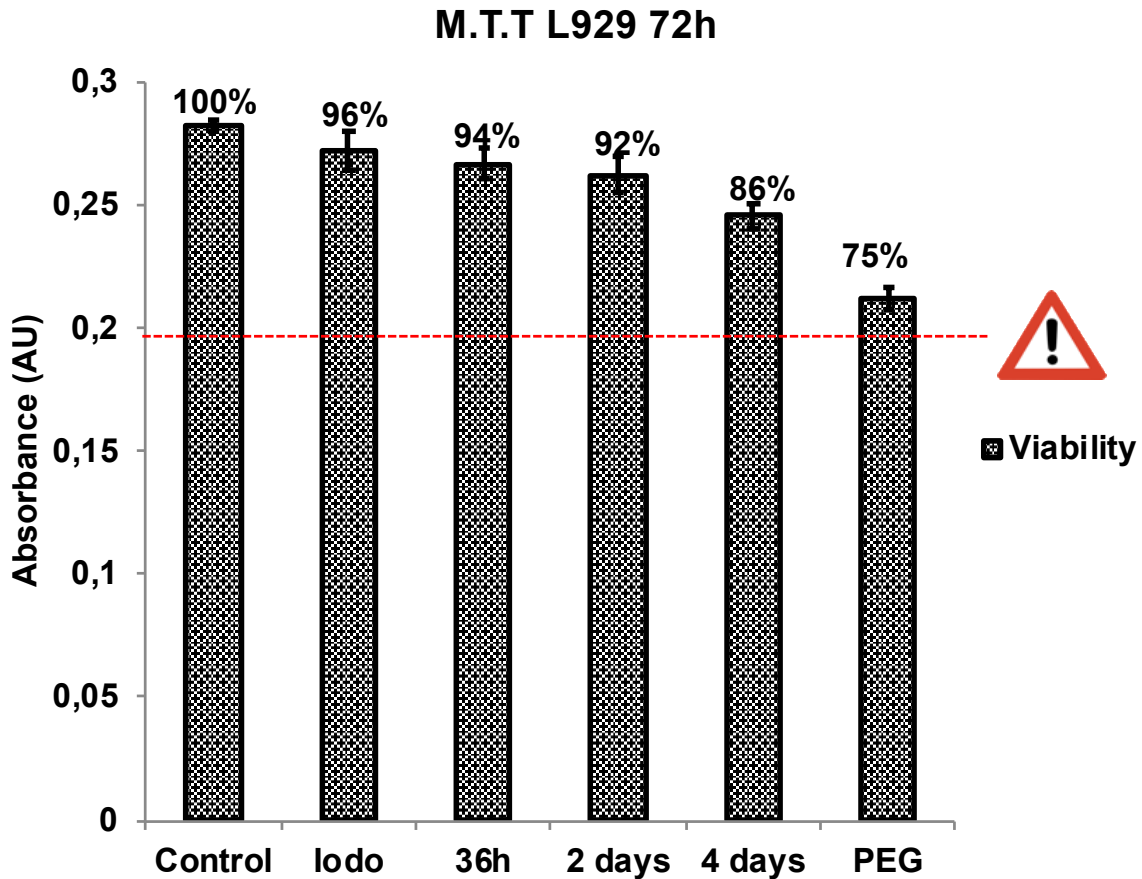


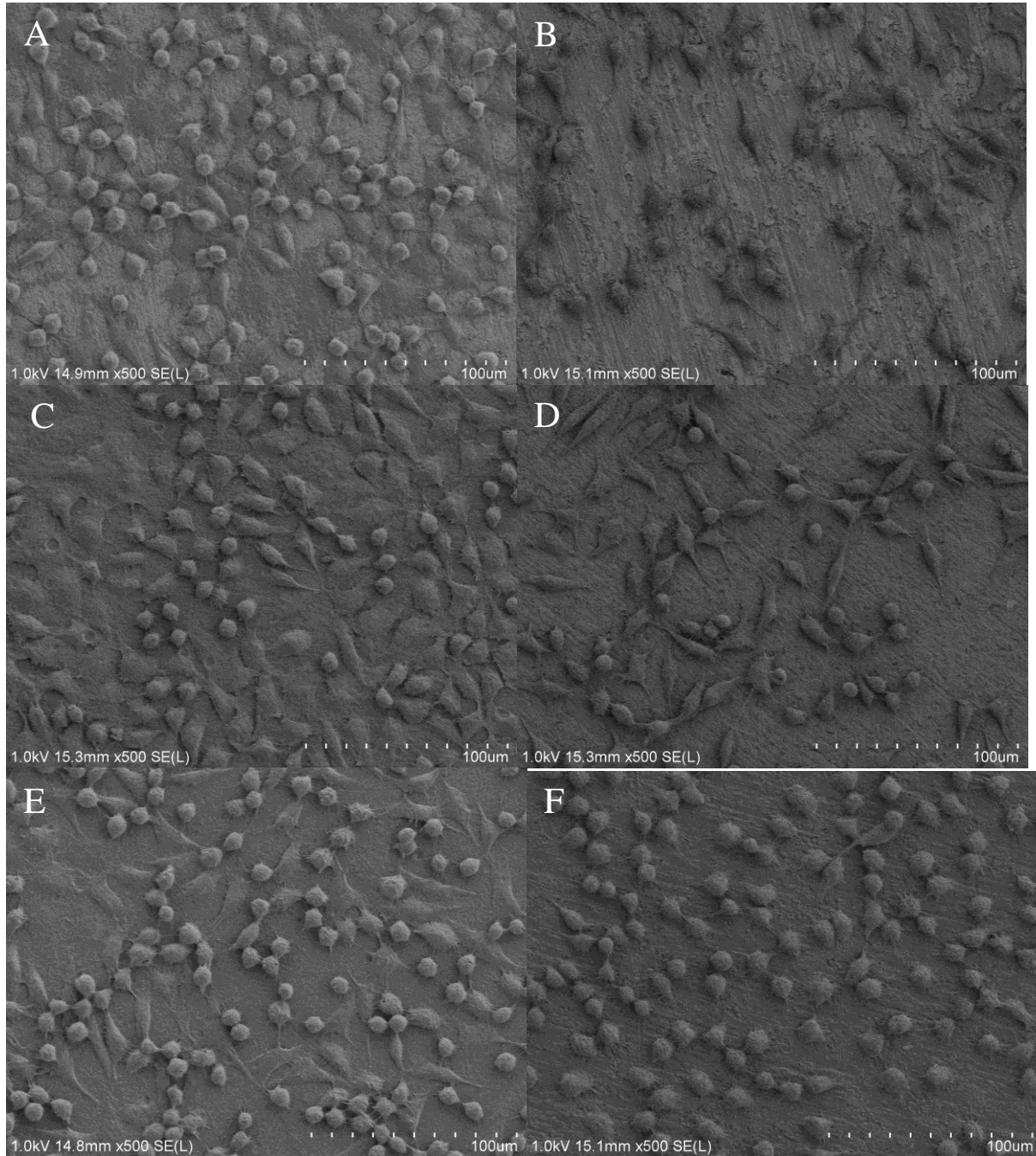
Figure 7. MTT assay: L929 cell survival after 72h on surfaces of various surface charge densities. Controls: n=6 ; Q-PVP-iodo: n=6 ; Q-PVP-36h: n=6 ; Q-PVP-2 days: n=6 ; Q-PVP-4 days: 3 ; Q-PVP-PEG: n=3. All data are represented as mean \pm standard error. All treated plates showed statistically significant differences in absorbance values compared with control ($P < 0.05$) except for Q-PVP-iodo. The red-dotted line represents the minimum acceptable viability (70%).

5) Biocompatibility assessment (SEM-FEG):

Qualitative morphological assessment showed that L929 cell behavior differed in terms of adhesion, morphology and shape on depending on surface charge density. Surfaces of increased charge density decreased cell adhesion and shape. Indeed, a gradual transition in the shape of cells was observed between Q-PVP-4 days, the highest density polymer (cells mostly round, few spindle cells, few pseudopods) and Q-PVP-iodo, the lowest density polymer (mostly spindle cells, numerous pseudopods) (Figure 8). Surfaces were ranked in terms of cell-adhesion promotion: Q-PVP-iodo > Control > Q-PVP-36h > Q-PVP-2 days > Q-PVP-4 days. Plates

408 grafted with Q-PVP-PEG markedly reduced cell adhesion and spreading as most cells were
409 round with few pseudopods. Cell populations on these plates stood out compared with all other
410 polymers as the least viable.

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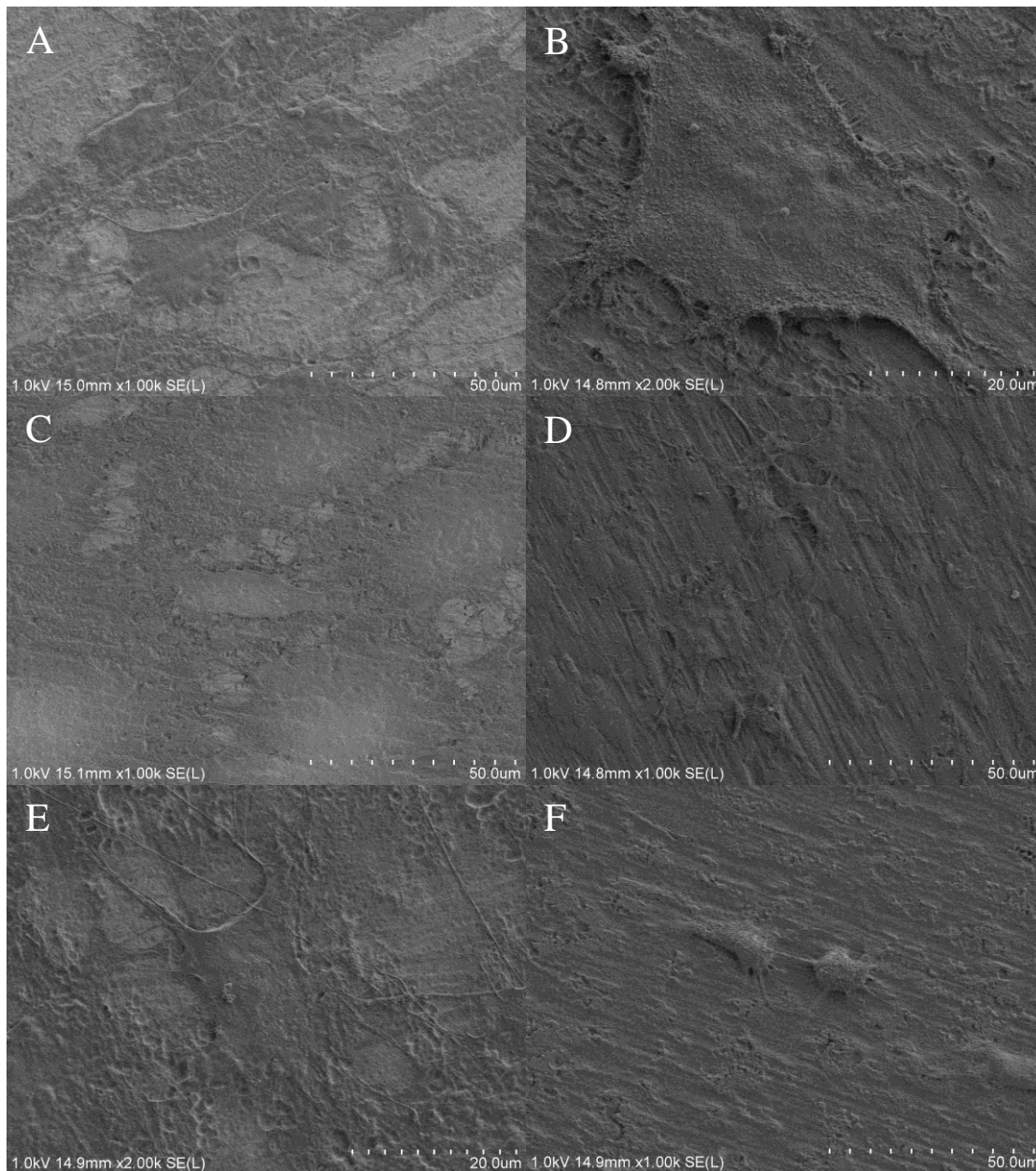
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Figure 8: SEM-FEG imaging displaying gradual adherence decrease (fewer pseudopods, rounder cells) and density decrease of L929 cells on surfaces of increasing cationic densities

421 and especially Q-PVP-PEG. A: Control; B: Q-PVP-36h; C: Q-PVP-2 days, D: Q-PVP-iodo, E:
1 422 Q-PVP-4 days, F: Q-PVP-PEG

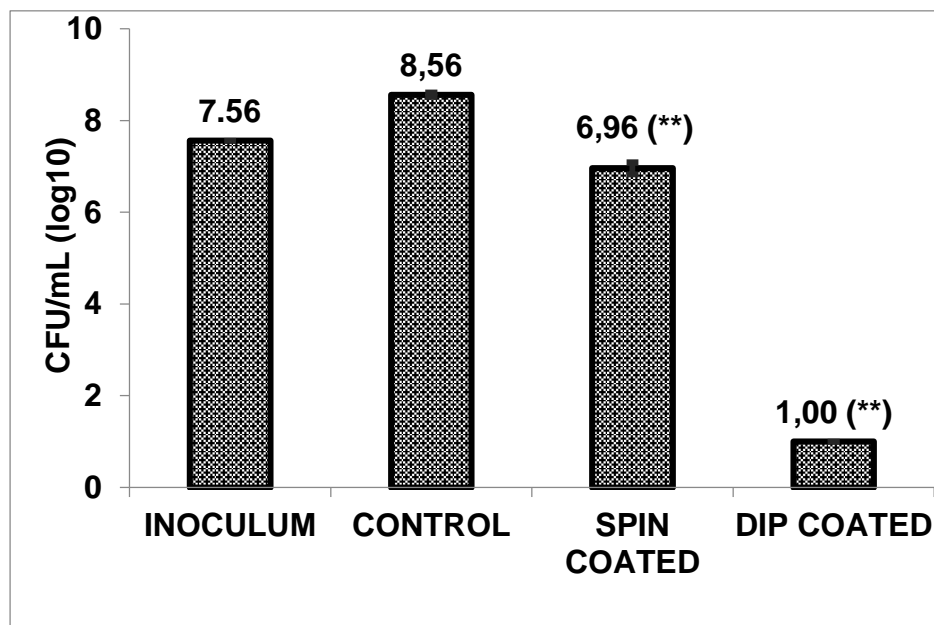
3 423 Qualitative morphological assessment showed that MC3T3 cell behavior also differed in terms
4 424 of adhesion, morphology and shape on depending on surface charge density. Surfaces of
5 425 increased charge density decreased cell adhesion and shape. Indeed, a gradual transition in the
6 426 shape of cells was observed between Q-PVP-4 days (scarce cells observed) and Q-PVP-iodo or
7 427 controls (large, well defined, flat and spread cells). Q-PVP-PEG coated surfaces displayed
8 428 abnormally shaped cells, round, poorly spread and obviously lacking adherence compared with
9 429 controls. Again, Q-PVP-PEG surfaces stood out from the rest of samples as the least suitable
10 430 for cell viability.



435 **Figure 9:** SEM-FEG imaging displaying gradual adherence decrease of MC3T3 cells on surfaces of
1 436 increases cationic densities and Q-PVP-PEG. A: Control; B: Q-PVP-iodo; C: Q-PVP-36h, D: Q-PVP-
2 437 2days, E: Q-PVP-4days, F: Q-PVP-PEG
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6) Bactericidal activity

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441 *In vitro* assays on MRSA demonstrated a fast (within one hour) and highly efficient killing
442 activity on surfaces grafted with Q-PVP-4 days, especially on dip-coated titanium (complete
443 sterilization, Figure 10). Under proliferating conditions (37°C in a rich medium, BHI), the
444 observed bacterial growth inhibition was more modest, though significant (Figure 11).
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449 **Figure 10:** *In vitro* killing (1 hour) in rich medium (BHI) at 37°C. Inoculum: n=1 represents
450 the bacterial count (CFU/mL) of the bacterial suspension initially deposited on titanium
451 substrates ; Control: n=9 ; Spin-coated: n=9 ; Dip-coated: n=9 ; All data are represented as
452 mean ± standard error. All treated plates showed statistically significant differences in
453 bacteriological count values compared with control (P<0.01).
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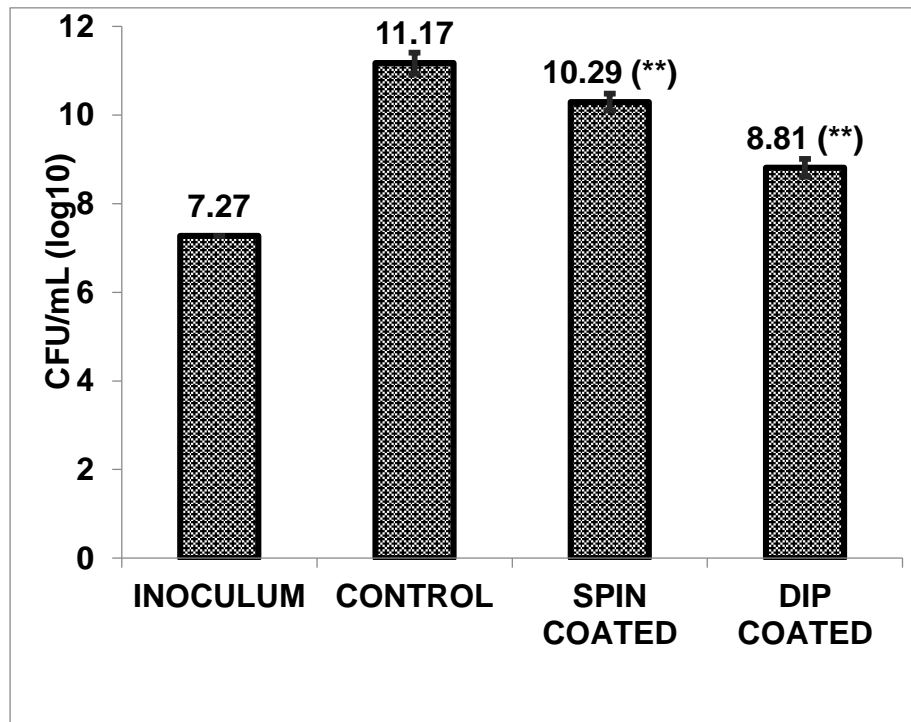


Figure 11: *In vitro* growth inhibition (24 hours) in rich medium (BHI) at 37°C. Inoculum: n=1 ; Control: n=9 ; Spin-coated: n=9 ; Dip-coated: n=9 ; All data are represented as mean \pm standard error. All treated plates showed statistically significant differences in bacteriological count values compared with control (P<0.01).

7) Antibiofilm activity

SEM-FEG imaging revealed a rapid-onset bacterial adherence reduction on a young biofilm (6 hours) on dip-coated titanium plates (Q-PVP-4 days). The effect was prolonged despite systematic medium replacement and enriching with BHI every 24 hours. At seven days, the observed biofilm appeared multi-layered and richer on control plates compared with grafted plates. On grafted plates, biofilm was scattered across the surface and made the underlying titanium support still visible on numerous areas (Figure 12).



Figure 12: MRSA biofilm growth on Q-PVP-4 days dip-coated surfaces vs control.

8) Specific effects on bacterial morphology: bacterial killing

The latter grafted surfaces exhibited signs of direct bacterial destruction by splitting or shrinkage (Figure 13A, left). Loss of volume occurred in tandem with bacterial cell wall perforation exhibited by sphericity loss (Figure 13B right).

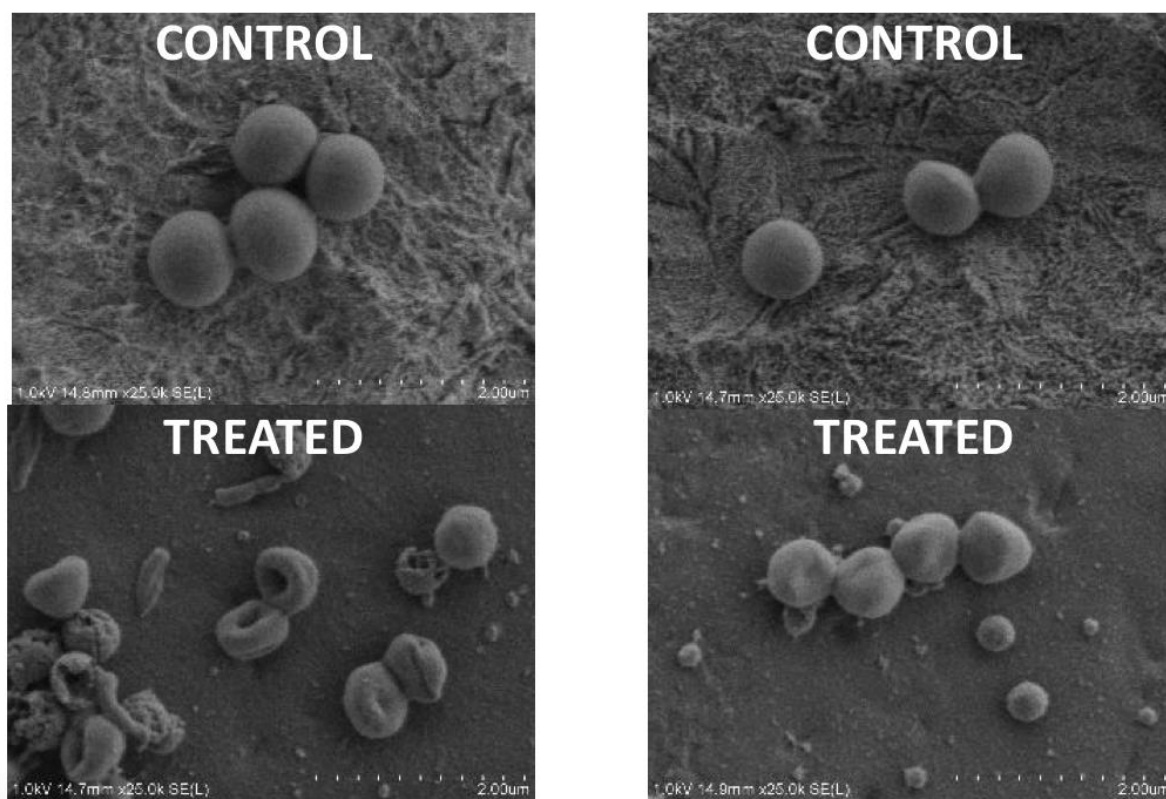
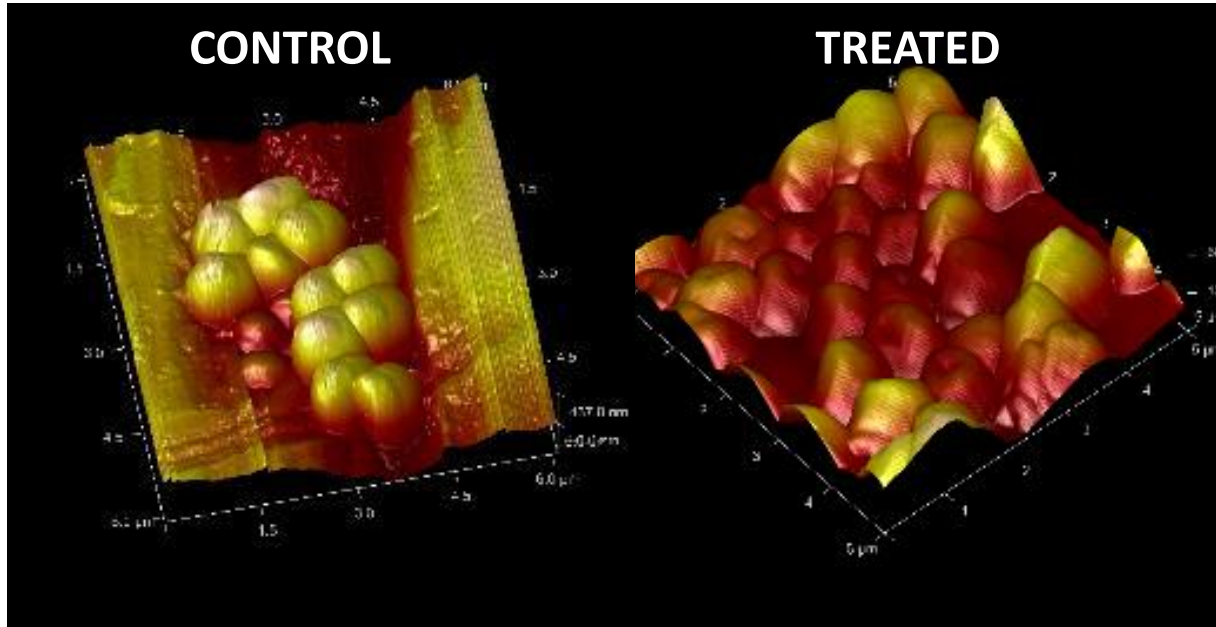


Figure 13: (A) left, (B) right: SEM-FEG imaging of MRSA bacteria on control vs treated surfaces (dip-coated Q-PVP-4 days) after 24 hours.

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1 486 These findings were also verified using 3D AFM. Bacterial perforation and volume loss was
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3 487 observed on killed bacteria after a 3 hours contact with Q-PVP-4 days dip-coated titanium
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5 488 compared with control.
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Figure 14: 3D AFM imaging of bacterial killing (MRSA) after a 3 hour-contact with dip-coated Q-PVP-4-days.

Yellow: spherical bacteria, appropriate height (live)

Red: loss of height and perforation of bacteria (dead)

DISCUSSION

Quaternary ammonium compounds have been known as an antibacterial agent in solution for almost a century (22). Their primary and main use to this date is surface disinfection (23). Their mechanism of action in solution has been already extensively described (24)(25)(26)(27). Because of their efficacy in solution, toxicity thresholds were defined, especially regarding hemolysis (19). Also, there have been efforts to mitigate the risk of cytotoxicity by associating quaternary ammonium compounds with various molecules in solution (28). However, quaternary ammonium polymers (QAP) in solution are of limited use for implantable devices as they do not permit any lasting antibacterial effect on surfaces due to rapid dispersion of the polymers. The immobilization of antibacterial polymers on implantable surfaces led to the discovery of various techniques that allowed the use of smaller polymer quantities with local

510 efficacy and ideally little to no leaching, reducing the risk of systemic toxicity (9)(16). Most of
1 511 these techniques are assimilated to either a “grafting to” or a “grafting from” strategy (10).
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3 512 Among “grafting to” anchors, silanes nowadays stand as one of the most easily mastered
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5 513 standard (29). Silane anchors can either be integrated into the polymer of interest forming a
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7 514 ready-to-graft polymer or they may be used in the setting of an intermediate step before adding
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9 515 the polymer onto the surface, the latter being the most conventional strategy (silane spacer)
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11 516 (30). In both cases, covalent grafting occurs and provides a strong attachment onto various
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13 517 surfaces displaying hydroxyl groups. In our study, we opted for a ready-to-use quaternized
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15 518 polyvinylpyridine polymer covalently grafted on pure titanium, which is a gold standard metal
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17 519 in terms of biocompatibility(31). The synthesis was carried out in an methanolic solution in a
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19 520 single step, which considerably simplified other processes for quaternized polyvinylpyridine.
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21 521 The adjunction of polyethylene glycol (PEG) into the reaction was performed because PEG is
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23 522 classically described as an anti-adhesive and bacterial inhibiting agent (32). Also, PEG-derived
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25 523 polymers such as polyethylene glycol methyl ether methacrylate (PEGMA) and hydroxyethyl
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27 524 methacrylate (HEMA have the reputation to be biocompatible and can render quaternized
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29 525 polyvinylpyridine biocompatible once incorporated as a copolymer (33). Though Allison et al.
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31 526 worked on polymers in solution, we speculated that replacing 1-bromobutane with PEG in the
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33 527 initial synthesis would provide a polymer with excellent biocompatibility when grafted on
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35 528 titanium. Alas, this had the complete opposite effect and Q-PVP-PEG displayed the lowest
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37 529 viable cells at 72h (MTT-assay), regardless of the cell line. Progressively, it became obvious
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39 530 that despite the reputation of quaternized PVP of being cytotoxic and not biocompatible, fine
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41 531 changes in quaternization rates by modulation of reaction time thus affecting surface cationic
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43 532 density, would reveal a biocompatible/antibacterial window. Therefore, candidate polymers of
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45 533 interest were selected: Q-PVP-iodo with no butyl group, Q-PVP-36h, Q-PVP-2 days and Q-
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47 534 PVP-4 days. Q-PVP-iodo increased MC3T3 cell viability at 72h, which may be of interest to
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49 535 promote bone adhesion on modified surfaces. Besides, these findings were not found between
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51 536 Q-PVP-iodo and L929. The implied mechanism remains to be proven. Furthermore, the four
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53 537 other polymer solutions displayed gradually increasing $\frac{N^+}{N}$ ratios with an inverse correlation
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55 538 between biocompatibility and antibacterial activity. All of them except Q-PVP-iodo exceeded
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57 539 the threshold for bactericidal activity described by Kugler et al. (16). However, it seemed that
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59 540 above 10^{15} charges/cm², bactericidal activity did not follow an on/off rule but most likely a
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61 541 continuous increase in efficacy. Indeed, in this study, in the most stringent conditions (i.e
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63 542 regularly enriched *in vitro* MRSA biofilm model), Q-PVP-4 days dip-coated surfaces did best.
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543 In our opinion, two main reasons account for this: 1) it had the highest achievable $\frac{N^+}{N}$ ratio in
544 our setting 2) the coating thickness was increased. Yet these findings disregard the fact that Q-
545 PVP-4 days decreased both tested cell line viabilities close to the critical viability threshold
546 (70%). Following L929 and MC3T3 MTT survival assessment, Q-PVP-2 days demonstrated
547 the best compromise in terms of preliminary biocompatibility testing (L929 and MC3T3
548 survival) and surface cationic density (systematically above 10^{15} cations/cm²). Therefore, we
549 postulate that the best compromise of viability and antibacterial activity would be using Q-
550 PVP-2 days as a ready-to-use and covalently graftable coating. Regarding coating thickness, it
551 seemed to be an important factor to modulate as it impacted leaching severity. The leaching
552 mechanism in this study depended on coating thickness and probably butyl group lengths. A
553 longer carbon-chain may have decreased hydrolysis by distilled water of the silane bond from
554 the surface. In this study, Only SLSC plates in distilled water demonstrated leaching that was
555 significant enough to drop below the biocidal effect threshold (10^{15} charges/cm²). Thicker
556 coatings may also prevent water from interacting with the titanium-silane bond. Therefore,
557 optimizing coating thickness and cationic density through reaction time modulation is expected
558 to lead to durable grafting and persistence *in vivo*, which should be confirmed in animal studies.

559
560 This is the first AFM study visually demonstrating the 3D killing effect of surfaces covalently
561 grafted with high-density QAP. The displayed effects were perforation and shrinking
562 dramatically impacting the external shape of bacteria (MRSA). In contrast, AFM studies on
563 QAP in solution raise the issue of the external stress (AFM tip) that may enhance QAP action
564 and eventually destroy bacterial cell membranes (18). In this study, high cationic density was
565 sufficient to cause bacterial cell perforation and death.

566 567 **Limitations of the study:**

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569 We have not explained why PEGylated polymers performed poorly in terms of cell viability.
570 There was a seemingly contradictory result concerning fluorescein test measurements regarding
571 SLSC with Q-PVP-4 days compared with Q-PVP-B36h. It was expected that Q-PVP-4 days
572 would display a higher absorbance, which appeared to be systematically the opposite. This
573 warrants further investigation too. Also, biocompatibility studies were performed on spin-
574 coated surfaces while biofilm studies relied on dip-coated samples, which may introduce a bias
575 artificially maintaining a high viability due to thinner surfaces.

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2 **577 CONCLUSION:**

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5 579 Partially quaternized and silanized polyvinylpyridine in solution, especially when synthesized
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7 580 in 48 hours, may represent a good compromise of biocompatibility and bactericidal activity
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9 581 against MRSA when grafted on titanium. If ultimately confirmed *in vivo*, these findings could
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11 582 pave the way for easier synthesis and grafting to render surfaces permanently biocidal and
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13 583 biocompatible.

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DISCLOSURE:

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45 648

Houssam Bouloussa, MD, MS declares that he is co-inventor of issued patent US

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US10238110B2 and owns stocks from DeBogy Molecular, Inc. (current assignee).

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