Final Draft
of the original manuscript:

Willumeit, R.; Schuster, A.; Iliev, P.; Linser, S.; Feyerabend, F.:
Phospholipids as implant coatings
In: Journal of Materials Science: Materials in Medicine (2007) Springer

DOI: 10.1007/s10856-006-0702-9

Phospholipids as implant coatings

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Keywords:

Phospholipid; implant surface;

Abstract

Bio-interfaces such as bio-membranes are of outmost importance for a variety of live processes. Among them are cell-interactions which take place in, on or through cell membranes. Therefore we propose to cover metallic surfaces with phospholipids to facilitate cell-material interaction. Four lipids, POPC, POPE, POPS and POPG, were applied to four metallic growth substrates with different surface structure, roughness and porosity. The interaction of the osteosarcoma cell line MG-63 was investigated in terms of cell adhesion and viability (MTT assay). While POPS in general had a negative influence, the most suitable combination in terms of viability per adherent MG-63 is the coating of porous Ti6Al4V material with the phospholipids POPE or POPC. The analysis of viability of mouse macrophages RAW 264.7 and their TNF-α release showed that the adhesion and viability is worst on POPS while the TNF-α release was highest. To elucidate the potential of phospholipids to prevent or support bacterial growth, the bacterial number of Gram positive and Gram negative bacteria was investigated. For lipid concentrations higher than 1 mM in solution a growth stimulating effect independent of the lipid type was detected. On a lipid coated surface the number of bacteria was reduced by 81%, 74% and 51% for POPC, POPG and POPE.

Introduction

Properties of bio-interfaces such as biological membranes are essential for directed and proper molecular life functions [1], and a designed control of biological or bio-mimetic functions along these interfaces seems feasible [2]. In this context the interplay of membrane lipids and membrane proteins is crucial [3]. The variability of these components is high, and the processes involved include alterations of biophysical properties of membrane lipids (caused by internal and external triggers), membrane fusion, inter-protein interactions, signalling pathways and cell re- and interactions.

The importance of these bio-interface related processes is based on lipid-protein interactions and much work was dedicated during the last decades to unravel the protein involvement. Lipids were so far considered as necessary but not very interesting molecules with (almost) no function except building the matrix for membrane proteins and having the right properties to enable for example membrane fusion. But evidence is accumulating that the role of membrane lipids in cellular function is underestimated. A prominent example is the much discussed 'Lipid Raft' hypothesis where specific membrane components (glycosphingolipids, sphingomyelin, cholesterol and proteins) form microdomains responsible – among others – for signal transduction [4, 5].

If lipids play a more important role in cell re- and interactions these molecules should also influence the interaction of cells with lipid covered surfaces. Therefore in this work the attempt is made to evaluate whether lipids are suitable as substrate for cell growth with the aim in the long run to establish a coating for permanent implants which increase the durability of these materials significantly.

Although biomaterials used clinically are claimed to be inert and non-toxic, they induce reactions in the body such as foreign body response [6]. It was also shown, that surface properties like chemical reactive groups, surface energy, roughness, and topography can directly influence the cellular response [7]. In order to improve the interaction between artificial materials and cells, modifications by a variety of active, 'non biological' (as compared to proteins or lipids) coatings have been introduced. They can consist of other biomaterials like alumina [8], bioactive glass [9], diamond-like carbon [10], or more biological materials like calcium phosphate or hydroxyapatite [11]. Extended protein related research over the last years showed, that the cells do not interact directly with the material, but with a passive coating of adsorbed proteins, which react with the surface oxide layer on metallic surfaces [12, 13]. Therefore, much effort is put into modifications of the interface between the materials surface and the targeted cells e.g. by means of pre-adsorbing

proteins or peptides [14, 15], or adhesion sequences like RGD [16]. The latest techniques of surface modification aim at identifying biological molecular components which play a significant role in cell-cell interaction and adhesion and to mimic this on an artificial surface with the perspective to expose adhesion points [17].

But as seen in this short summary the main focus was on the implementation of protein based coatings neglecting the possibilities lipids might pose, which in fact contribute up to 40% of the molecules in cell membranes. The variety of the phospho- and sphingolipids in membranes is high and very variable from species to species but also within one organism (Table 1).

In nature the membranes of the cells show a high stability although being resilient and highly dynamic. The biophysical properties of their lipids, especially their phase behavior and their amphipathicity, are closely connected with the functionality and integration of membrane proteins. Some of the lipids even play a direct functional role in the organism (like phosphatidylserin or phosphatidylinositol). Lipids have self-assembling properties and are already used in a variety of applications as supported lipid membranes, self-assembled monolayers [18, 19] or as non-adhesive coating for materials with blood contact [20, 21].

With this in mind we studied a lipid based bio-mimetic surface coating that resembles a cell membrane in some aspects to hide the surface of metallic samples (mirror polished Titanium and Ti6Al7Nb, porous Ti6Al4V and Vacuum Plasma Sprayed (VPS) Titanium). Four different phospholipids were used:

(1) POPC (2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine), as a representative of eukaryotic lipids;

(2) POPE (2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphoethanolamine), as a representative of a lipid abundant in pro- and eukaryotes;

(3) POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]), which is present in eukaryotic membranes and known to be a signal for apoptosis and involved in calcification [22], and

(4) POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol)) which originates mainly from bacterial membranes.

Cellular reaction on the lipid covered surfaces was evaluated for the osteosarcoma cell line MG-63, mouse macrophages RAW 264.7 and Gram negative and positive bacteria.

Materials and methods

Preparation of metal samples

Four materials were used as cell carriers (Fig. 1):

(i) Mirror like polished non-porous Titanium (10 mm diameter, 1 mm thickness, by courtesy of DOT GmbH Rostock) and Ti6Al7Nb plates (10 mm diameter, 1 – 1.3 mm thickness) were produced as follows: from a starting material in form of a round bar, discs with a diameter of 10 mm and a height of 1.5 mm were cut. The discs were ground using 30, 18 and 10 µm grinding papers, and finally polished with a 1 µm diamond paste and finished by mirror-polishing with 0.05 SiO_2-paste. A more detailed description can be found in [23].

(ii) Porous Ti6Al4V (composition in mass percent) samples (10 mm diameter, height 2 mm) were manufactured using the low-density-core (LDC) process as described by Schwartz et. al [24]. Ti6Al4V powder is filled into a specially designed can which is outgassed and then charged with pressurized Argon gas. The sealed can is hot-isostatically pressed (HIPed) and hot-worked into final shape, here by hot-rolling to produce a sheet. After annealing for 6 – 24 hours at a temperature higher than 0.6 times the melting point a porous body is obtained exhibiting 20 – 40% of unconnected porosity (in terms of cellular length scales). Typical pore diameter ranges from 10 to 200 µm. Samples were cut from the bulk material by electro-discharge machining (EDM).

(iii) Vacuum Plasma Sprayed Titanium (VPS-Ti): This material is a donation of the DOT GmbH, Rostock. It was manufactured by application of a 30 ± 5 µm porous (porosity 20 – 30 %, Ra 8 ± 3 µm) Ti layer on bulk Ti via vacuum plasma spraying.

All materials were ultrasonically cleaned for 30 minutes each in acetone, chloroform (VWR International, Darmstadt, Germany), a 2% Hellmanex-solution (Hellma GmbH & Co KG, Mühlheim, Germany) and distilled water. After cleaning, the specimens were dried at room temperature. Prior to lipid apposition and cell culture experiments the samples were steam sterilized.

Lipid films

The phospholipids POPC, POPE, POPG (purchased from Sigma-Aldrich-Chemie, Taufkirchen, Germany) and POPS (Avanti Polar Lipids, Alabaster, USA) were solved in a chloroform:methanol (80:20 for POPE, 70:30 other lipids, Merck, Darmstadt, Germany) solution to a final concentration of 1 mM for the coverage of the metallic samples. The experiments with Tissue Culture Plastic (TCP) were performed with lipids solved in

isopropanol:ethanol (50:50, 1 mM, Merck, Darmstadt, Germany). The coatings were applied (30 µL per sample) on metallic materials (mirror polished Ti, Ti6Al7Nb, VPS-Ti and porous Ti6Al4V (LDC) discs) and tissue culture plastic (TCP), evaporated and dried for at least 30 minutes at room temperature. In order to remove excess lipid the coated specimen were incubated for 2 hours in the medium used for the following tests. Then the samples were transferred to the cell experiments.

SEM characterization of lipid films on mirror polished, lipid covered Ti6Al7Nb

Native and POPC, POPE, POPS and POPG coated Ti6Al7Nb carriers were incubated for 2 hours at 37°C in phosphate buffered saline PBS. Afterwards, the probes were dehydrated at room temperature. Samples were examined using a Leo Gemini 1550 VP (LEO Elektronenmikroskopie GmbH, Oberkochen, Germany) Scanning Electron Microscope (SEM), operating with a secondary electron detector (SE1) in low voltage field emission mode at 0.7 keV.

Cell Adhesion and proliferation of osteoblast-like cells MG-63 on lipid covered specimen

Human osteosarcoma MG-63 cells were cultured in Dulbecco's modified Eagle medium Glutamax-I (DMEM; Invitrogen Corporation, Karlsruhe, Germany) with 10 % FCS, 1% penicillin and 100 µg/mL streptomycin for about ten days with medium change every other day. Cells were passaged at subconfluency (70 – 80 %) and reseeded in a density of 5 x 10^4 cells/cm^2.

For *adhesion* experiments cells in the 5^{th} passage were used. The experiments were done in a 6-fold redundant assay for mirror polished Ti and VPS-Ti discs and 2-fold redundant assays for mirror polished Ti6Al7Nb discs and porous Ti6Al4V (LDC) culture substrates. MG-63 cells were applied in a density of 5 x 10^4 cells in 30 µL medium per carrier in agarose-coated wells and allowed to adhere for 30 minutes. Thereafter, the specimens were fully covered with medium. The medium was removed after two hours, and trypsin solution (Invitrogen Corporation, Karlsbad, USA) was added in a way, that the culture substrates were completely covered. After five minutes incubation at 37°C the trypsin reaction was stopped by adding a two-fold amount of medium with 10% FBS. The cells in the supernatant were counted triplicate using a Coulter Z2 cell counter (Beckman-Coulter GmbH, Krefeld, Germany).

The *viability* measurements of MG-63 were performed for all materials and lipid coatings as follows: The experiments were done in a 6-fold redundant assay for mirror polished Ti and

VPS-Ti discs and 2-fold redundant assays for mirror polished Ti6Al7Nb discs and porous Ti6Al4V (LDC) culture substrate. 5 x 10^4 cells in 30 µL medium were applied to the carriers in agarose-coated wells to minimize adhesion to TCP. After 30 minutes to allow initial adhesion, the carriers were covered with 2 ml DMEM. The specimen were incubated at 37° C in an atmosphere of 5% CO_2 and 100% humidity for two days before MTT assays were performed.

MG-63 viability on lipid coated TCP was measured in 96-well plates (8 replicates). 1 x 10^3 cells were seeded per well and incubated for 24 and 48 hours, respectively. Thereafter, MTT-assays were performed as described below.

Macrophage experiments

The mouse macrophage cell line RAW 264.7 was used to determine immunogenic cell response to the used lipids. The cell line was cultured for about ten days with medium change every other day in DMEM low glucose (Invitrogen Corp., Karlsbad, USA) with 10 % FCS (PAA Laboratories, Linz, Austria), 1% penicillin and 100 µg/mL streptomycin (Invitrogen Corporation, Karlsbad, USA). Cells were passaged at subconfluency (70 – 80 %) and reseeded in a density of 5 x 10^4 cells/cm^2. For adhesion experiments cells in the 5^{th} passage were used.

Cell *adhesion* on mirror polished Ti and lipid covered surfaces was analyzed by the application of 1 x 10^5 cells per carrier in 30 µL medium. After 2 hours of incubation the cell adhesion protocol (see above) was performed. The assay was performed with 6 replicates per treatment.

The macrophage *viability* was analyzed on TCP, mirror polished Ti, porous Ti6Al4V (LDC) and lipid covered surfaces (6 to 8-fold replicates per experiment). In TCP experiments cells were applied to 96-well plates in a density of 1 x 10^3 cells / well and incubated for 48 hours. Then, the MTT-assay was performed as described. The viability measurements on metallic materials and lipid coatings were performed similar to the procedure described for MG-63 cells.

The *activation* of the macrophages as a reaction to the different materials and coatings was determined by the release of the inflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1) into the cell culture supernatant. The concentrations of these cytokines were determined by enzyme-linked immunosorbent assays (ELISA). For TNF-α the Quantikine Mouse TNF-α/TNFSF1A-Kit, for IL-1 the Quantikine mouse IL-1α/IL-1F1-Kit (R&D-systems, Minneapolis, USA) was used according to the manufacturers instructions.

The supernatants were obtained from samples prior to MTT-measurements. Each sample was measured in 4 independent replicates. A treatment with 1 µg/µL lipolysaccharide (LPS) served as positive control, as it is known to stimulate the cytokine production in macrophages [25].

MTT-assay for viability

Viability was analysed by the cell proliferation kit MTT (Roche Diagnostics GmbH, Mannheim, Germany). The MTT-assay is based on the cleavage of the yellow tetrazolium salt MTT (methylthiazolyldiphenyl-tetrazolium bromide) into purple formazan by metabolically active cells. In brief, cells were cultured for two days on the carriers in agarose-coated 12-well plates with 2 mL of the cell-type specific medium. 1 mL of the supernatant was discarded and 100 µL of the MTT-solution (5 mg/mL MTT in PBS) was added. After an incubation period of 5 hours the cells were lysed and the formazan crystals solubilized by adding 1 mL solubilization solution and incubation overnight in a humidified atmosphere (37° C, 5% CO_2). The solubilized formazan product was photometrically quantified using an ELISA reader (Tecan Sunrise, TECAN Deutschland GmbH, Crailsheim, Germany) at 570 nm with a reference wavelength of 655 nm.

The protocol was adjusted for the examinations of lipids coated on TCP, which were performed in 96-well plates. Here, the cells were incubated for 24 to 48 hours with 100 µL medium, afterwards 10 µL of the MTT-solution and 100 µL of solubilization solution were added.

Bacterial interaction

The Gram negative bacteria *Escherichia coli* strain K-12 (ATCC 23716) and two Gram positive bacteria *Staphylococcus carnosus* (ATCC 51365) and *Bacillus subtilis* (ATCC 6051) were used for these investigations. The bacteria were cultivated on agar plates at 37°C for 24 h. Then one colony was picked from the plate with an inoculating loop and suspended in the respective medium to grow overnight at 37°C. The bacteria were inoculated again in medium and kept growing shaking at 37°C to reach the log-phase. Within 2 to 4 hours the extinction at 600 nm wavelength was photometrically measured (Helios photometer, Thermo Spectronic, Waltham, USA) to determine the density of bacteria. It was experimentally determined that for an OD_{600} of 0.1 to 0.2 the bacteria reached the log-phase.

The agar plates used for *E.coli* were prepared by dissolving 5g Bacto Agar (BD Diagnostic Systems, Heidelberg, Germany) in 1000 mL Luria-Bertani medium (LB) – medium. This medium was produced by mixing 10 g NaCl (Merck, Darmstadt, Germany), 10 g Bacto tryptone, 5 g Yeast extract (BD Diagnostic Systems, Heidelberg, Germany) and 1000 mL double distilled water. The *S.carnosus* bacteria were cultured in Corynebacterium medium (CM) consisting of 10 g Casein peptone (BD Diagnostic Systems, Heidelberg, Germany), 5 g Yeast extract, 5 g Glucose (Merck, Darmstadt, Germany), 5 g NaCl and 1000 mL double distilled water. The respective agar plates were prepared by mixing 15 g Bacto Agar with 1000 mL CM. *B.subtilis* was grown in Nutrient medium (NM). It was prepared by dissolving 5 g peptone (BD Diagnostic Systems, Heidelberg, Germany), 3 g meat extract (Merck, Darmstadt, Germany) in 1000 mL double distilled water. The agar plates consisted of 15 g Bacto Agar dissolved in 1000 mL NM.

Influence of phospholipids on bacterial growth

A volume of 1 mL respective medium was inoculated with 100 CFU of log-phase bacteria. POPC, POPE, POPS and POPG vesicles were prepared in double distilled water and added to the bacteria in final concentrations of 10 µM (total volume 10 µL), 100 µM (total volume 100 µL) and 1mM (total volume 10 µL). As control 100µL double distilled water was added to the bacteria. The suspensions were incubated shaking at 37°C overnight and the amount of bacteria was determined photometrically in terms of OD_{600} (Helios photometer, Thermo Spectronic, Waltham, USA).

SEM characterization of bacterial adhesion on mirror polished, lipid covered Ti6Al7Nb

Native and POPE, POPC and POPG coated Ti6Al7Nb carriers were incubated with 100 CFU of log-phase bacteria in 1 mL medium and incubated over night at 37°C. Following the overnight incubation the mirror polished Ti6Al7Nb specimen were removed from the wells and washed twice with PBS. They were fixed in 2.5 % glutaraldehyde (Sigma-Aldrich Chemie, Steinheim, Germany) overnight, and counterstained in osmium tetroxide for 30 min. Afterwards, the probes were dehydrated in a graded isopropanol series – 20 %, 40 %, 60 %, 80 % and 100 %, respectively for 1 hour in each concentration. All steps were performed at room temperature. Samples were critical point dried (CPD) with a Baltec CPD 030 (Baltec, Balzers, Liechtenstein) and examined using a Leo Gemini 1550 VP (LEO Elektronenmikroskopie GmbH, Oberkochen, Germany) Scanning Electron Microscope (SEM).

Bacterial adhesion was quantified by counting each bacteria strain on four independent SEM-pictures in an area of 50 μm^2. On each image six spots were analysed summing up to 24 counted quarters. Bacterial size (n = 24) was measured with the Auto-classification method provided by digital image analysis with AnalySIS (Soft Imaging Systems GmbH, Münster, Germany), calculating the exposed area above a certain threshold.

Statistics

Statistics were performed using the SigmaStat package (Systat software GmbH, Erkrath, Germany). Standard analysis comparing more than two treatments was done by using the One-way ANOVA. Depending on the data distribution either a one-way ANOVA or an ANOVA on ranks was performed. Post hoc tests were Holm-Sidak or Dunn´s versus the control group, respectively. Statistical values are indicated at the relevant experiments.

Results

Lipid coating on different culture substrates

Low voltage field emission SEM was used to visualize the lipid layers on mirror polished Ti6Al7Nb. Depending on the lipid different morphologies and structures are visible (Fig. 2). The formation of bubbles or liposomes during the process of exfoliation of excess lipids during incubation [26] can be seen for all lipids. For the larger scan area (370 x 300 µm) the coating with POPC seems to be relatively homogenous while especially for POPE many white regions are visible. However, upon higher magnification it appears that also these areas are still lipid coated. In general a closed lipid layer on the growth substrate down to the sub-micrometer scale is obtained.

Lipid influence on MG-63 grown on different culture substrates

The MG-63 viability on lipid coated TCP was measured after 24 and 48 hours. Fig. 3 shows a decrease in viability in relation to the control for the examined lipids (One-way ANOVA on ranks, Dunn´s post-hoc-test; $H = 35{,}903$, $n = 40$, $p<0{,}001$). The highest viability was found for POPC and POPE covered TCP. Interestingly on POPG, which is bacteria derived, the MG-63 cells show a sufficient viability. The strongest reduction in viability was found for POPS.

By normalizing the data to the control and comparing the time dependent development of MG-63 viability, POPG led to a further decrease in viability during the second day (from 53% to 32%). POPC also induced a decrease, which was not as pronounced (from 61% to 51%). The viability of MG-63 on POPE was not time dependent (from 66% to 63%), for POPS even a slight increase (from 22% to 30%) was observed for longer incubation times.

The previous measurements showed that for very smooth TCP surfaces the lipids in general reduced the viability of the MG-63. In the next step it was tested how the osteosarcoma cell line MG-63 interacted with lipid covered mirror polished titanium and Ti6Al7Nb surface as well as on rough VPS-Ti and porous Ti6Al4V (LDC) culture substrates. The initial cell number was $5x10^4$. After 2 hours the adhesion was measured (Fig. 4). On TCP as positive control 82.5% of the cells adhered.

The initial adhesion on the lipid coated mirror polished materials is in general higher (Ti6Al7Nb) or comparable (Ti) to the native metallic material. Strikingly for those substrates the POPC coverage was most important. For POPE, POPS and POPG less cells were adherent. This systematic decrease was not found for rough or porous culture substrates.

Here the general tendency of cell attachment was more influenced by the roughness of the substrate than the applied lipid, although a weakly positive effect of POPE was found.

The determination of the MG-63 viability revealed that, in comparison to the cells grown on native material (Fig. 4: Absorption = 1), especially on the porous Ti6Al4V (LDC) material a significant increase of cell viability was detected for POPE ($Q=2.996$, $n=12$, $p<0.05$). For the other lipids no significant differences were measured, although an increase was observed also for POPC and, to a lesser extent, for POPG. On the VPS-Ti the viability was slightly, but significantly reduced (POPC: $Q=5.274$; POPE: $Q=2.677$; POPS: $Q=6.698$; POPG: $Q=6.979$; $p<0.05$, all lipids $n=12$). On the mirror polished culture substrates for the lipids POPC ($Q=6.285$, $p<0.05$, $n=12$) and POPG ($Q=6.690$, $p<0.05$, $n=12$) a proliferation stimulating effect was found.

If the viability is related to the initial adherent cell number (Fig. 5) the materials with a rough surface are considerably better suited. A strong influence of the lipid type is observable especially for the mirror polished surfaces. Interestingly the bacterial derived lipid POPG seems to have a strong impact on MG-63 for all metal surfaces, a quite unexpected result since this lipid is considered to be not abundant in eukaryotic cells.

Influence of phospholipids on macrophage viability

The influence of the phospholipids on the viability of RAW 264.7 was evaluated by an MTT assay after 48 hours on lipid covered *TCP* in order to separate the influence of the metal morphology from the lipid influence. As it can be seen in Figure 6, the application of the phospholipid POPC did not reduce the cell viability in comparison to the TCP control; a slight promotion of proliferation was visible. The other lipids reduced the MTT-values significantly (One-way ANOVA, Holm-Sidak´s post hoc test versus control; POPE: $t=5{,}049$; POPS: $t=14{,}381$; POPG: $8{,}152$; $p<0{,}05$, $n=38$).

Mirror polished Ti as a representative for very smooth surfaces and porous Ti6Al4V (LDC material) were used to evaluated the influence of the lipid coverage on viability of RAW 264.7 and the production of TNF-α and IL-1.

The viability measurements showed in general a significant decrease in cell viability for all lipid coatings, as shown in Figure 7. The most negative effect was observed for POPS ($Q=7.31$, $p<0.05$; One-way ANOVA, Dunn´s post hoc test versus control), followed by POPG ($Q=5.082$, $p<0.05$). POPC and POPE still led to a significant decrease of viable macrophages, but the effect was lower (POPC: $Q=3.78$; POPE on mirror polished Ti: $Q=3.424$; $p<0.05$). Only in the case of porous, POPE covered Ti6Al4V (LDC) an

improvement of RAW 264.7 viability was found. If this is then compared to the percentage of adherent cells (%-values in Fig. 7) the positive effect for POPE is even more pronounced.

The accompanying measurements of released TNF-α (Fig. 8) to the cell culture supernatant revealed in all cases detectable concentrations. In general, the highest values were observed for POPS and POPG. A significant increase of TNF-α release on mirror-polished Ti was observed for the POPS-coated specimen ($t=4.986$, $n=4$, $p<0.05$). In relation to the positive control (LPS), which exhibited a TNF-α concentration of 2488 ± 41 pg/mL the lipid induced TNF-α values are very low.

For IL-1 out of 58 samples in total only 20 exhibited a measurable O.D., which was for all samples below the minimum standard of 4.6875 pg/mL. As the minimum detectable dose is in the range of 2.5 pg/mL, these values were regarded as non-detectable. The treatment with LPS again led to very high values (521 ± 21 pg/mL).

Bacteria interaction with phospholipids

Bacterial infections are one of the major reasons for implant removal. Therefore the interaction of bacteria with lipid vesicles and lipid covered mirror polished Ti6Al7Nb discs was tested and compared with the bacterial interaction or attachment to a native surface. As test systems Gram negative *E. coli* (K-12 (ATCC 23716)) and Gram positive *S. carnosus* (ATCC 51365) and *B. subtilis* (ATCC 6051) bacteria were chosen.

To evaluate the influence of phospholipids on bacterial growth, different lipid concentrations were applied to the bacterial growth medium (Fig. 9). Lipid concentrations typical for lipid covered samples, which is 15 µM if 30 µL of a 1 mM lipid solution would be dissolved completely in 1 mL growth medium, did not induce a considerable effect. For high concentrations (1 mM) a noteworthy improvement of bacterial growth was found upon addition of POPC and POPG, while this effect was not as pronounced for POPE and POPS. Gram positive bacteria showed a stronger reaction (1.1 – 1.3 fold more absorption) on the lipid vesicles than *E. coli* bacteria.

The direct analysis of *E. coli* adhesion on mirror polished lipid covered Ti6Al7Nb surfaces showed in general a reduction of bacterial number in comparison with the uncovered mirror polished Ti6Al7Nb surface (17.5 ± 4.1 bacteria/50 µm^2), a positive fact in terms of possible infections (Fig. 10). The strongest reduction was found for a POPC coating (3.4 ± 2.7 bacteria/50 µm^2), followed by POPG and POPE (4.6 ± 2.1 and 8.6 ± 4.5 bacteria/50 µm^2, respectively). The bacteria are homogeneously distributed on the native surface while they tend to aggregate on the lipid covered surfaces. This is the explanation for the severe

variation in standard deviation on lipid covered surfaces: it happened from time to time that on the randomly chosen 24 counting areas no bacteria were found while in others clusters accumulated.

Morphological differences of the bacteria to the ones adhered to native material were found for the POPC and POPG-coating (Fig. 10). On POPC covered surfaces the bacteria exhibit a cell surface with protrusions while on POPG covered surfaces the bacteria are in general by 35 % smaller (1.17 ± 0.37 μm^2) compared to their size on the native surface (1.77 ± 0.24 μm^2).

Discussion

The idea to mimic the inert surface of erythrocytes by covering polymeric haemocompatible materials with phospholipid head groups (phosphatidylcholine) is not new [27]. But contrary to these developments aiming at the decrease of cell attachment on polymers, the aim of this study was to evaluate if lipids are suitable as surface coating for metallic implant materials to promote cell adhesion and proliferation.

Even more sophisticated are the attempts to use complete cell membranes as substrate for cell cultivation [28] following the hypothesis that all necessary proteins will be available for cell-surface-interaction. We show in this study that already the appropriate choice of phospholipids is sufficient to promote cell adhesion and proliferation. It should be noted that at this stage we do not address whether this positive influence is correlated with the direct cell-phospholipid-interaction or due to a modification in the adherent protein layer if growth medium is used.

The lipids were chosen such that they resemble the most abundant lipids in eukaryotic membranes (POPC, POPE and POPS). Lipids with a phosphatidylglycerol headgroup are usually not found in eukaryotic cells though it is possible that in specific cell types a very small amount is included in the cell membrane (Table 1). Therefore the bacterial phospholipid POPG was considered to be a negative control in this study.

The coating of the surfaces was performed in a very simple way by evaporating the solvent of the lipids. As previous studies showed this led to multilamellar bilayer stacks completely covering the growth substrate but loosely bound to the surface [26]. To reduce the exfoliation of excess lipid during the cell experiments all samples were pre-incubated without cells and the supernatant was removed prior to cell seeding.

The stability of the lipid layers is a crucial but not easily answered issue. A measurement of the stability with standard methods (for example peeling tests, AFM) is bound to fail because we are dealing with a liquid crystalline system which will either rearrange itself as a reaction to external forces or give way to single molecules. Still, in a first approach the homogeneity of the coating can be analysed by microscopy.

SEM analysis revealed that the lipid coating was still intact after 2 hours of incubation in PBS but not very homogenous. Despite the optical impression of many uncoated regions (white areas), upon higher magnification still lipid layers are visible. We can distinguish between areas which are relatively thick coated (dark) and those which are less thick coated (grey or white). Regions which appeared to be without coating were not found for a size scale down to micrometers. Since it is not possible to measure single lipid bilayers with

SEM due to limited contrast, we can not calculate the average percentage of coated area. However, the existence of uncoated nano-size areas can not be excluded by these measurements. One might speculate that under such circumstances the cells could still be in contact with the uncoated metallic growth specimen – or rather adherent proteins from the growth medium. These questions are currently under investigation.

Surface morphology is different for the four lipids. POPC covers the mirror polished Ti6Al7Nb growth substrate homogenously while POPE forms irregular structures. This can be due to the small but measurable negative charge of this zwitterionic lipid [29]. Upon closer look we can identify that those structures are regions with thick lipid layers and very thin coated areas. Although the coating by these two lipids is not directly comparable, the effect on MG-63 adhesion – as discussed below – is positive in both cases indicating that the thickness of the lipid layer does not seem to be important. The two negatively charged lipids POPS and POPG show a similar coating pattern indicating that charge effects do indeed play a significant role in the self assembly of the lipids.

Due to the simple preparation of the lipid coating we can not exclude that also during cell incubation some of the adherent cells were removed during medium exchange. This effect is integrated in the standard deviation and in most cases the reason for the lower cell number compared to the native material and the high error bars in some of the measurements. Covalently linked lipids or the application of defined mono/bilayers will overcome this drawback.

Independent of the cell type a significant influence of the lipid coverage was found. For the osteosarcoma cell line MG-63 the highest viability was found for POPC and POPE surfaces if only the lipid influence was investigated (TCP culture substrate). Considering a time dependent effect, only POPE was sufficiently promoting MG-63 viability. A small effect was found also for POPG and the viability is mostly reduced by POPS. This finding agrees with the assumption that only limited cell growth will take place on lipids which are an apoptotic signal (POPS) or of non-eukaryotic origin (POPG). They are in contradiction to studies of HeLa and NIH3T3 cells where cell adhesion and growth exclusively occurred on Silica supported phosphatidylserine bilayers [30].

With the addition of culture substrate morphology (mirror polished, rough or porous), a more diverse picture was established. Again, MG-63 cells adhered best on POPC covered mirror polished Ti6Al7Nb and Ti culture substrates. This is a surprising result because many studies (including our own with primary chondrocytes and stem cells) have proven that the choline headgroup (as in POPC) prevents cell adhesion. At this point we can only speculate

that the osteosarcoma cell line MG-63 shows a different behaviour than primary cells due to their immortality and cancerous origin. For rough surfaces the coating with POPE was slightly favourable. This can be explained by the fact that this lipid is an important compound of the cell membrane and its small but measurable negative charge [29]. Furthermore the specific shape of this lipid (small headgroup in comparison to the space consuming acyl-chains) might cause a better interaction with proteins, either from the adherent cells or the growth medium.

Analysing the viability of the cells on mirror polished samples, beside POPC also POPG turned out to be a suitable surface coating. This finding is very unusual. From the procaryotic origin of POPG, the surface morphology and charge of the POPG coating, which is similar to POPS, one would expect a comparable negative influence on MG-63 viability. The opposite observation indicates that the cells do sense the actual chemical composition rather than only the layer thickness, surface roughness or charge. One reason might be a synergistic effect between lipid structure and adherent proteins. On rough surfaces this effect was not as pronounced but still visible.

The best results were obtained for the combination of a porous surface with POPE or POPC which confirms our initial assumption that already a lipid layer - a very simple model of a complex membrane - can influence cellular reactions.

The correlation of the number of adherent cells with the viability revealed even more clearly that beside the stimulating lipids POPC and POPE also the bacterial derived lipid POPG is favoured by the MG-63 cells. This is an unexpected result because this lipid should not promote eukaryotic cell interaction. From the literature nothing is known about the exact lipid composition of MG-63 cells. But if we deduce what we know from other skeletal cells like chondrocytes or osteoblasts ([31]), it seems possible that a very small amount of POPG is present also in the MG-63 cell membrane and an inclusion in the cell metabolism might be possible. In addition one can speculate that MG-63 has a mechanism to incorporate the lipid if the cells are attached to the lipid coated surface since it has the same phase behaviour (liquid crystalline) and charge as POPS. Studies with labelled POPG might reveal this.

If we consider that no direct lipid-cell-interaction takes place but that adherent proteins from the growth medium are of some importance, one can hypothesize that these proteins can arrange in a biological active way within the lipid bilayers (which is valid most likely for all lipids) which could be supported in the case of POPG by charge effects. Further studies with defined protein adsorption will be performed to clarify this point. Already at this stage

we can conclude from the negative effect of POPS that a combination of direct lipid-cell and protein-mediated cell interaction is the case. Overall the coating of metallic surfaces with phospholipids has a measurable positive effect on MG-63 adhesion and proliferation.

The reduction or avoidance of an inflammatory reaction is highly relevant for implant integration. Two important aspects of this reaction were investigated in this paper: the relationship between lipid coating and lipid-induced activation of macrophages and the bacterial invasion.

The number of adherent mouse macrophages RAW 264.7 was highest when the phospholipid POPC was used while their number dropped to 17-27% for the other lipids which is comparable to the native material. One reason might be that macrophages prefer a relatively smooth lipid coating as it is produced by POPC. This lipid is known to form very well ordered lipid bilayer stacks and did not show the massive bubble formation as the other lipids. In terms of viability all lipids have a negative influence as compared to the negative mirror polished Ti specimen. It is more pronounced on the thicker POPC coating (high initial adhesion and low viability) but also detectable for the other lipids (low initial adhesion and 17-30% lower viability as compared to the native metal). One reason might be the removal of cells during the incubation process together with the loosely bound lipids.

More interesting is the activation of macrophages, here analyzed by the release of TNF-α. For POPC and POPE a response similar to the uncovered material was detected. POPG induces some activation, but not as much as expected if POPG is recognized as a foreign molecule. We see this in agreement with early studies showing that this lipid is abundant in minor amounts also in mammalian cell membranes [32]. A recent study has shown a cytotoxic effect leading to RAW 264.7 apoptosis for dioleoylphosphatidylglycerol (DOPG) used for drug delivery liposomes. The authors conclude, that this is mainly due to the oxidation of the unsaturated acyl chains [33]. However, for POPG this effect should not be as pronounced, because it has only one unsaturated acyl chain.

For POPS a countertendency was found: While the viability went down the production of TNF-α went up. This is in agreement with the literature since the presentation of POPS on the outer membrane leaflet of erythrocytes trigger macrophages to phagocyte blood cell or apoptotic cells in general [34-39]. The observed TNF-α concentrations and the LPS-stimulated IL-1 production are in good agreement with other studies [40, 41].

In summary these results show that macrophages are not significantly activated by the lipids POPE and POPC and that their adhesion and proliferation is supported. In this respect lipid covered implant surfaces seem to be feasible.

The interaction of bacteria with lipid covered surfaces is another important aspect. We showed that the growth of Gram positive and negative bacteria is stimulated if the lipid concentration is high enough. This effect is more pronounced for Gram positive strains than compared to *E. coli*, which might be caused by the missing outer membrane. All bacteria show a slight preference for POPG, which is natural because this lipid is involved in the physiology of the bacteria [42] and an incorporation of PG lipids in the bacterial membranes is most favourable as it was shown for the Gram negative bacteria *Haemophilus parainfluenzae* [43]. PE lipids, which are also abundant in bacterial cell membranes, are only half as fast incorporated which is reflected in the weaker stimulation of the bacteria in our experiments.

A comparable influence on the bacterial growth was found for POPC (stimulating as POPG) and POPS (comparable to POPE) which is very unusual because these lipids are not – to our best knowledge – abundant in the membranes of the bacteria tested here. Some bacterial strains can incorporate lipids with PC (for example some Methylotrophs) or PS headgroups (20% in *Bordetella pertussis*) [44]. Therefore a small chance exists that these lipids are used for the proliferation of the bacteria because of their high availability in our experiments. It is more likely that other aspects of lipid-bacteria interaction have to be considered. We have to take into account that bacteria – if they adhere to host cells – should either have specific receptors for host cell proteins or their lipids. That the latter could be the case was shown for *Streptococcus pneumoniae* adherence to type II pneumocytes which was hindered in a competitive reaction due to the strong interaction with DMPC liposomes [45].

The bacterial stimulation was only achieved for lipid concentrations approximately 100-fold larger than what can be re-dissolved from the lipid covered metallic surface. Therefore no stimulating effect is expected if an implant is lipid covered.

The previously discussed results were obtained for dissolved lipids at high concentrations. To evaluate how the bacterial adhesion to surfaces is influenced, the number of *E. coli* bacteria on a lipid covered mirror polished Ti6Al7Nb was measured. All lipids showed a significant reduction of *E. coli* invasion in comparison with the native mirror polished Ti6Al7Nb. The strongest repulsion was found for POPC which is caused by the general tendency to omit bacterial adhesion [46]. This sounds like a contradiction to the previously described stimulating effect of POPC especially on Gram positive bacteria and the possible explanation by lipid specific adhesion molecules. But it can be explained by the fact that for the lipids attached to the surface a pre-incubation with protein containing growth medium

occurred. These proteins also interact with the lipid layer and might create a ‘new’ surface were only small amounts of lipids are reachable for the bacteria. In addition it is not known if the Gram negative *E. coli* bacteria possess proteins specific for PC headgroups, and if they do, if these proteins depend on a certain curvature of the host’s lipid bilayer which is not present for flat lipid coated surfaces.

The bacterial originated POPG had the second strongest effect on bacterial repulsion which might be due to electrostatic interaction because the lipid covered metallic and the bacterial surfaces are of the same negative charge. The few bacteria adherent on the POPG coated surface were significantly smaller than *E. coli* on the native TI6Al7Nb. This seems to be an inconsistency to the above mentioned positive effect of POPG on bacterial proliferation but taking into account that in the first case free unilamellar lipid vesicles can interact with the bacteria, the repulsive effect might be overcome by the kinetic energy of the vesicle, a more favorable membrane curvature of the vesicle or an active, protein supported uptake of lipid molecules from the vesicles. For those bacteria adherent to the POPG covered surface it might be that the specific lipids receptors were blocked on the surface because it was not possible to remove the lipid from the uncurved bilayer on the metallic substrate resulting in a change of bacterial morphology.

For POPE, still about 50% of the *E. coli* bacteria can attach to the surface after 24 hours incubation. As it was shown for EPEC, the enteropathogenic *E. coli* bacterium [47] a strong interaction between the bacteria and PE-lipids takes place. It could be possible that also the non pathogenic strain used in this study might develop a weak preference for POPE.

In summary we can state that, despite the existing interaction between phospholipids and bacteria which occur only at high concentration of dissolved vesicles, a significant reduction of the bacterial number on a phospholipid covered mirror polished metallic surface is achieved.

Conclusions

Interaction at or in bio-interfaces are complicated processes with many – mostly – unknown parameters. Each cell type has its own metabolism, each organism its own cell membrane protein and lipid composition. Little is known about the manifold possibilities of how proteins and lipids or even cells and lipids can interact. If a reasonable explanation for findings related to one cell type or lipid is found it must not necessarily be possible to transfer this knowledge to another type of cells or lipid. The study of the influence of lipids on cell attachment and viability has to consider several aspects: which lipids are present to which amount in the cells? Do the lipids interact with proteins and how? Do the biophysical properties of the lipids influence cell interaction? How are they changed if the lipids (either in the coating or in the cell) are in contact with proteins? This list can be prolonged but what is clear from this study is that phospholipids do influence the cell adhesion and metabolism of eukaryotic and prokaryotic cells. If it will be possible to coat a metallic implant surface permanently with phospholipids it will lead – depending on the lipid – to a positive cell-material interaction. In summary we conclude that the phospholipids POPE and POPC in combination with a suitable metallic implant surface are the most promising combination for forthcoming implant modifications.

Additional studies taking into account covalently linked lipids, the influence of the possible protein interaction with the lipid layers and the analysis of the differentiation behavior of primary cells instead of cell lines will further elucidate the potential of lipid covered implant surfaces.

Acknowledgements

Our special thanks are devoted to Jessica Rutz, GKSS Research Center, Institute for Materials Research, who contributed significantly to the laboratory experiments, and Michael Schossig, GKSS Research Center, Institute for Polymer Research, who did a wonderful job to visualize the bacteria by SEM. We gratefully acknowledge the donation of RAW 264.7 cells by Dr. A. Gasser, University Hospital Eppendorf, Hamburg, Germany and wish to thank Dr. J. Schnieders, Phillips University Marburg, Germany, for the donation of the osteosarcoma cell line. We thank PD Dr. J. Andrä, Research Center Borstel, Germany, who kindly contributed the *E.coli* strain. Prof. H. Clemens, Montanuniversität Leoben, Austria, was so generous to support us with the porous Ti-6Al-4V material. The VPS-Ti and mirror polished Ti material was donated by DOT GmbH, Rostock, Germany. The work is financially supported by the country of Mecklenburg-Vorpommern and the Helmholtz Association.

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Figure captions

Figure 1:

Examples of the samples used in this paper. Since the mirror polished pure Titanium and Ti6Al7Nb samples optically look the same only one representative disc is shown.

Figure 2:

Visualisation of lipid coating on mirror polished Ti6Al7Nb by low voltage field emission SEM (charge contrast). Starting from a relatively large scan area close ups are presented. The white rectangular indicates the scan area which is magnified from left to right. Leo Gemini 1550 VP, detector SE1, 0.7 keV.

Figure 3:

Influence of lipid coatings on TCP on the viability of MG-63 after 24 and 48 hours. Left: MTT-absorption. Asterisks denominate significant differences. Right: MTT-values plotted as percent of the control value.

Figure 4:

Influence of lipid coatings on metal surfaces on the adhesion and viability (MTT Assay) of MG-63 cells. *Upper Row:* Left: Adhesion measurements on mirror polished Ti6Al7Nb and Ti discs. Right: Comparison of the MG-63 adhesion on VPS-Ti and porous Ti6Al4V (LDC) culture substrates. The numbers indicate how many percent of the initially seeded cells (50000) were adherent after 2 hours incubation. *Lower Row:* Left: MTT measurements on mirror polished Ti6Al7Nb and Ti discs. Right: Comparison of the MG-63 viability on VPS-Ti and porous Ti6Al4V (LDC). All values are normalized to the absorption values for the native metal surface which was set to 1.

Figure 5:

Ratio of MTT absorption in relation to initial cell adhesion. The data were calculated in the following way: The adherent cells number and the MTT absorption values were normalized to the native metal value. Then the ratio normalized absorption / normalized adherent cell number was calculated.

Figure 6:

Influence of lipid coatings on TCP on the viability of macrophages as measured by MTT assay. Asterisks denominate significant differences.

Figure 7:

Influence of lipid coatings on metal substrates on the viability of macrophages as determined by MTT assay. Asterisks denominate significant differences. Values are normalized to absorption values of native metal surfaces (=1). The percentage indicates the adherent cells on mirror polished lipid covered Ti with respect of 2 h incubation. The adhesion on the native metal surface was 18%.

Figure 8:

TNF-α-release measured in the medium supernatant of RAW 264.7 macrophages on native and lipid coated metal substrates. Asterisks denominate significant differences compared to the release on native surfaces.

Figure 9:

Influence of lipids in the bacterial growth medium.

The absorption is normalized to the OD_{600} of the control + 100 µL doubledestilled water (control + 100 µL doubledestilled water: *E. coli*: OD_{600} = 0.977 +/- 0.011 for POPC and POPG and OD_{600} = 1.134 +/- 0.021 for POPE and POPS; *S. carnosus*: OD_{600} = 0.668 +/- 0.002 for POPC and POPE and OD_{600} = 0.743 +/- 0.002 for POPG and POPS; *B. subtilis*: OD_{600} = 0.669 +/- 0.013 for POPC and POPE and OD_{600} = 0.801 +/- 0.008 for POPG and POPS).

Figure 10:

Adhesion of *E. coli* on native mirror polished Ti6Al7Nb and with lipid coating. The inserted small pictures show the according bacterial morphology. The lipid coverage reduced the bacterial number by 80.6% for POPC, while POPG and POPE decreased the amount of bacteria by 73.7 and 50.9%.

Figure

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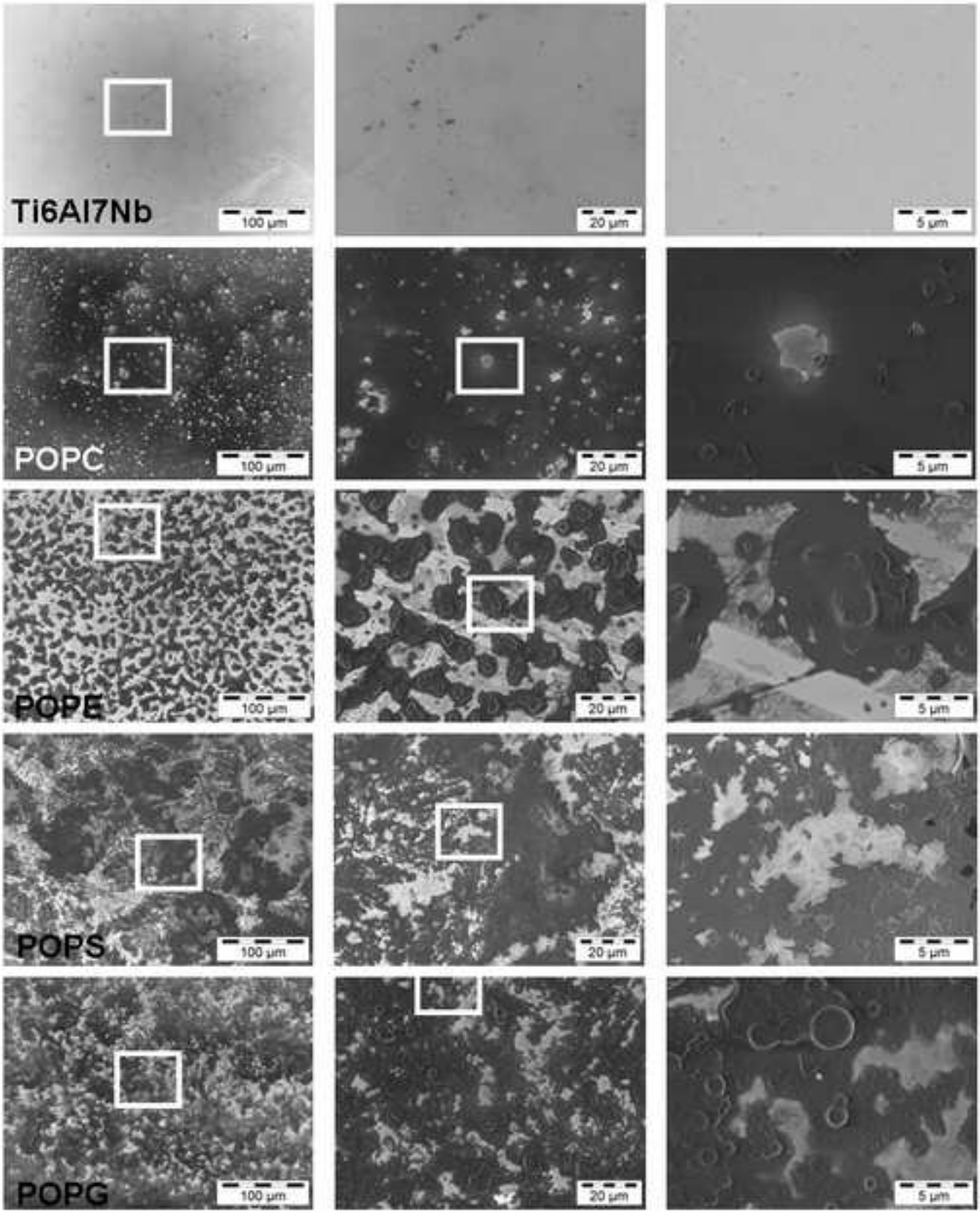

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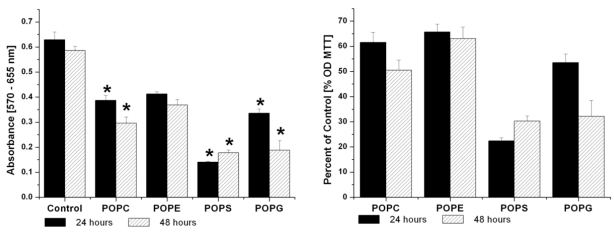

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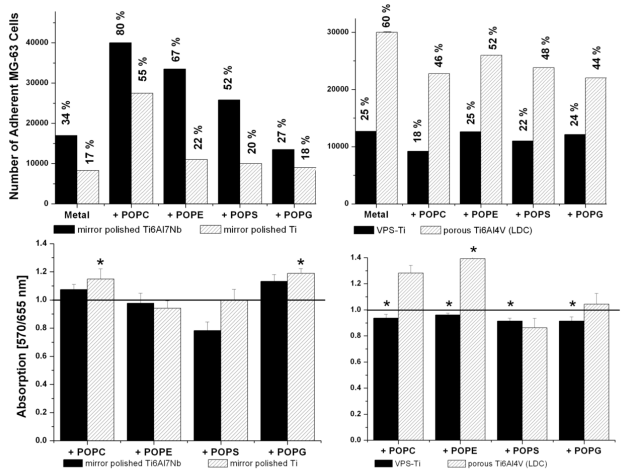

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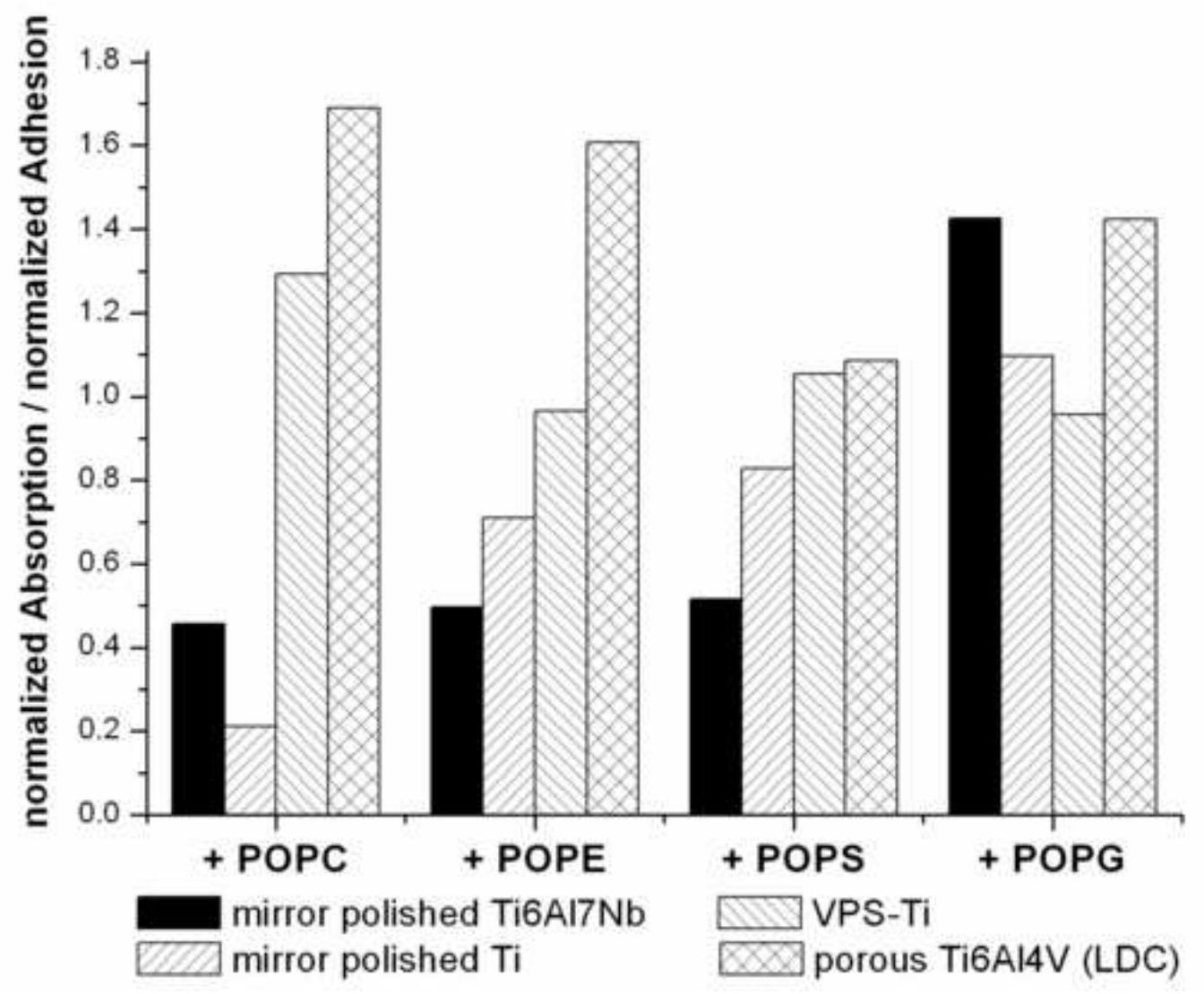

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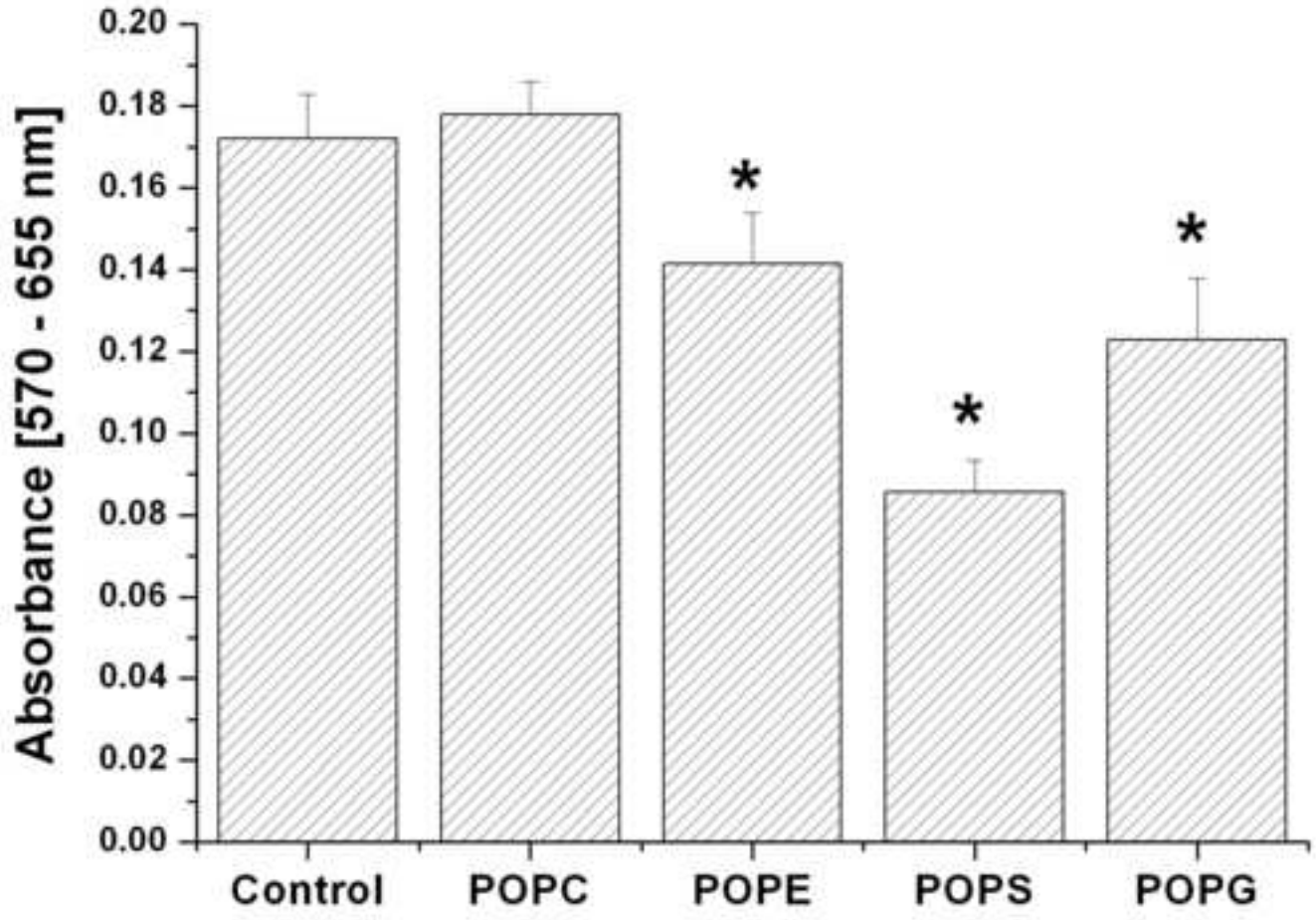

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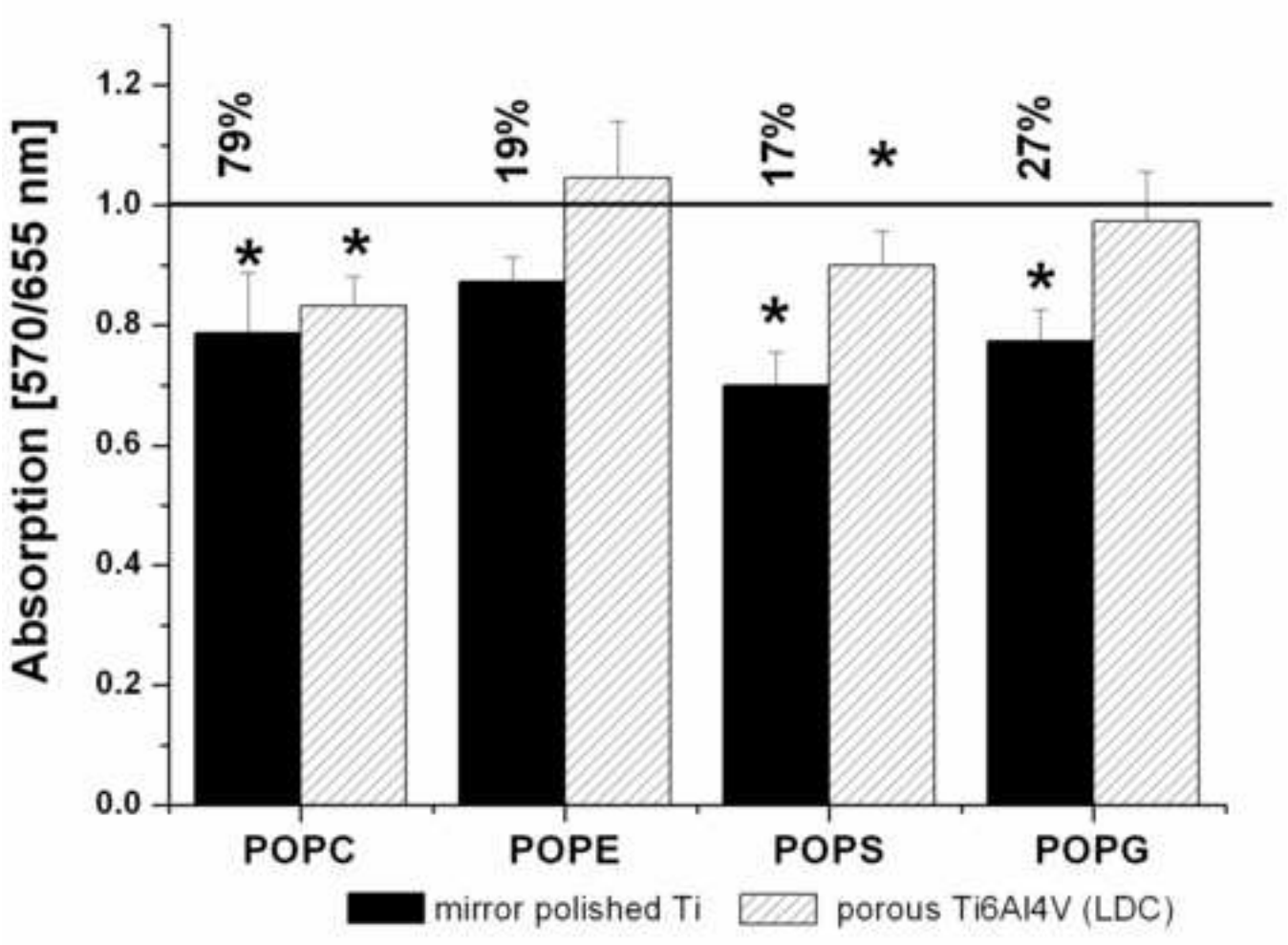

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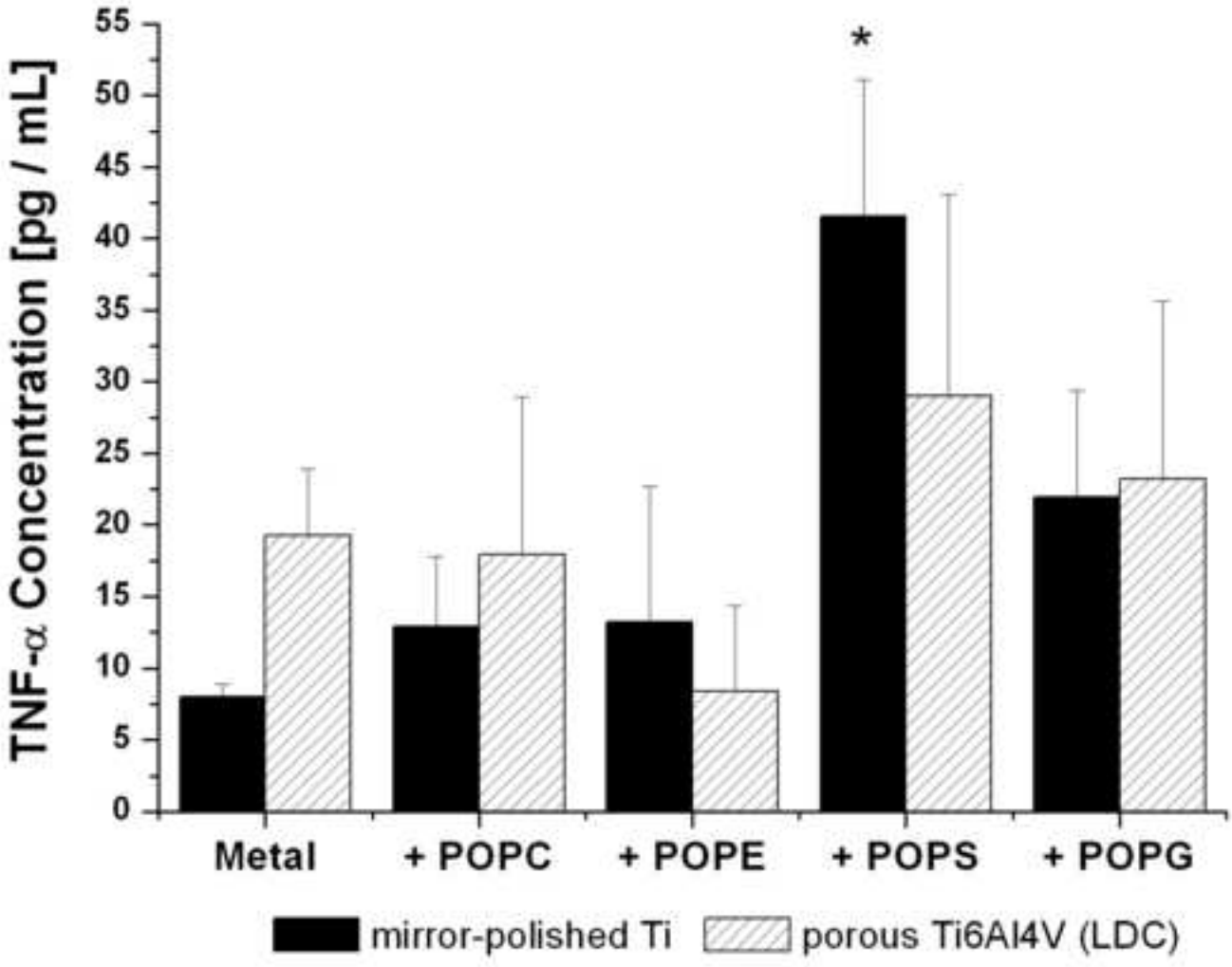

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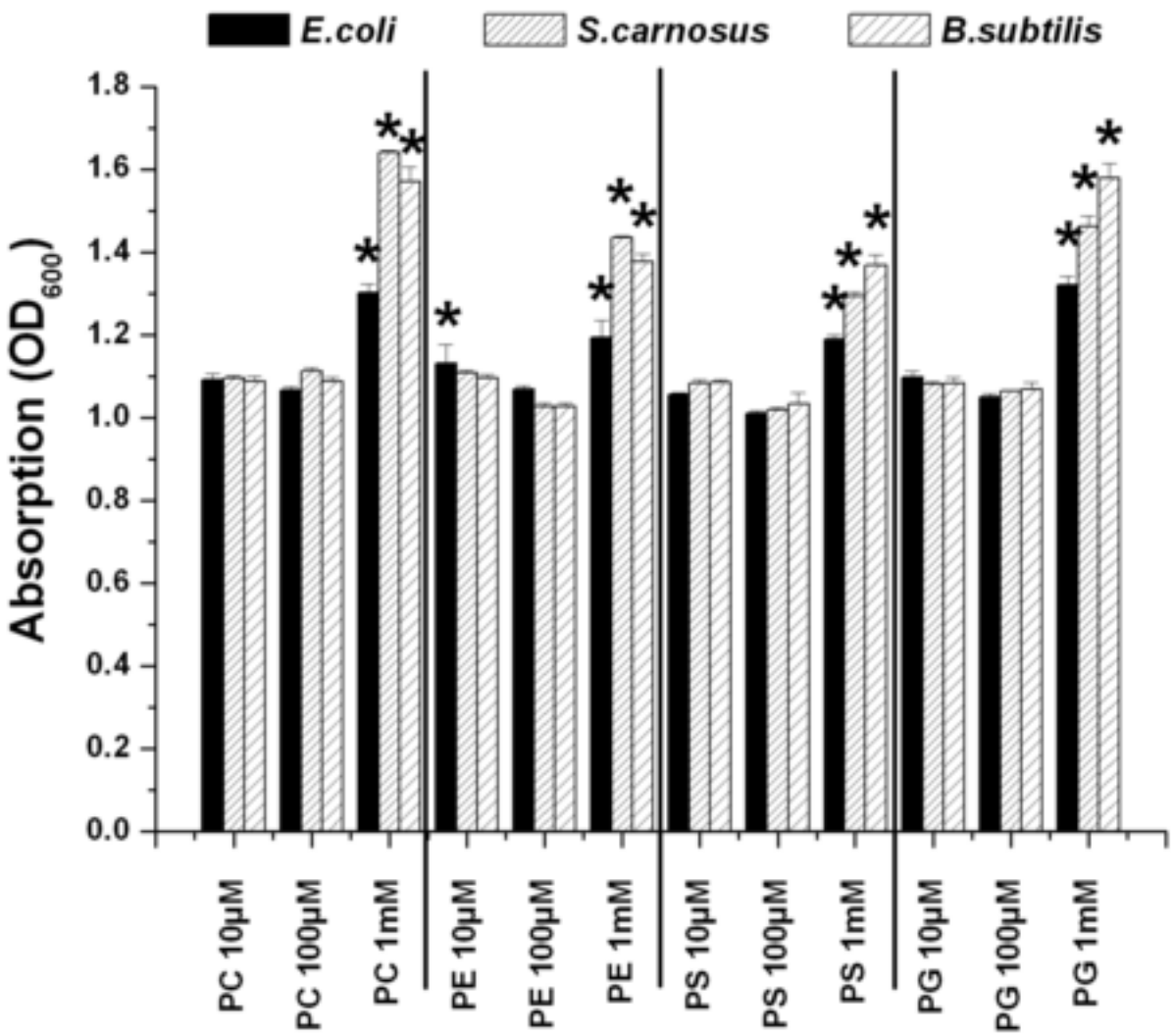

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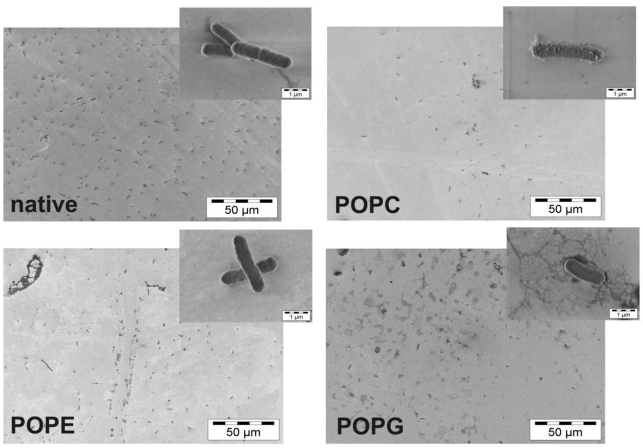

Tables

Table 1: The phospholipid composition of bacteria or eukaryotic cells [18, 19]

IM = Inner Membrane; PG = Phosphatidyl-glycerole, PE = Phosphatidyl-ethanolamine, CL = Cardiolipin, PC = Phosphatidyl-choline, SM = Sphingomyelin, PS = Phosphatidyl-serine, *Isolated from tissue [20]

	PG	PE	CL	PC	SM	PS	others
Gram negative							
E.coli IM	6	82	12	0	0	0	0
Gram positive							
B. subtilis	29	10	47	0	0	0	0
S. aureus	57	0	43	0	0	0	0
Erythrocyte	0	18	0	20	18	7	37
Mitochondria	0	29	14	38	0	0	19
Cartilage (resting)*	1	15	0	61	6	4	13
Bone (cancellous)*	1	18	0	52	11	6	13

Table 2: Statistical values for the differences between the lipid coatings. A One-way Anova on ranks with Dunn´s post hoc test was performed. Given are the Difference of Ranks and the Q-value as describing factors and the significance level p. PS = POPS, PG = POPG, PC = POPC, PE = POPE, C = control = TCP.

Comparison		Diff of Ranks	Q	P<0,05
PS vs C	**24 hours**	**32,000**	**5,475**	**Yes**
	48 hours	**29,625**	**5,068**	**Yes**
PG vs C	**24 hours**	**23,750**	**4,063**	**Yes**
	48 hours	**26,375**	**4,512**	**Yes**
PC vs C	**24 hours**	**15,625**	**2,673**	**Yes**
	48 hours	**16,000**	**2,737**	**Yes**
PE vs C	**24 hours**	**1,476**	**1,476**	**No**
	48 hours	**8,000**	**1,369**	**No**