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Covalent immobilization of liposomes on plasma functionalized metallic surfaces

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ABSTRACT

A method was developed to functionalize biomedical metals with liposomes. The novelty of the method includes the plasma-functionalization of the metal surface with proper chemical groups to be used as anchor sites for the covalent immobilization of the liposomes. Stainless steel (SS-316) disks were processed in radiofrequency glow discharges fed with vapors of acrylic acid to coat them with thin adherent films characterized by surface carboxylic groups, where liposomes were covalently bound through the formation of amide bonds. For this, liposomes decorated with polyethylene glycol molecules bearing terminal amine-groups were prepared. After ensuring that the liposomes remain intact, under the conditions applying for immobilization; different attachment conditions were evaluated (incubation time, concentration of liposome dispersion) for optimization of the technique. Immobilization of calcein-entrapping liposomes was evaluated by monitoring the percent of calcein attached on the surfaces. Best results were obtained when liposome dispersions with 5 mg/ml (liposomal lipid) concentration were incubated on each disk for 24 h at 37 °C. The method is proposed for developing drug-eluting biomedical materials or devices by using liposomes that have appropriate membrane compositions and are loaded with drugs or other bioactive agents.

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1. Introduction

Balloon angioplasty was introduced in 1979 and is currently the main therapy in cardiology. From 1990, metallic scaffolds or stents were implanted at the lesion site for prevention of restenosis. Nevertheless the injury caused by stent implantation results in neointimal proliferation and in-stent restenosis. To overcome this problem, drug eluting stents, which can be considered as local drug delivery systems, are under extensive research [1]. Recently the use of liposomes as drug containers for the development of controlled rate drug eluting surfaces or biomedical devices (e.g., stents or vascular/ureteral grafts) was proposed [2–5]. Indeed it was proven that by using different types of liposomes, the release of drugs encapsulated or incorporated into them could be adequately controlled in order to achieve the required dose of drug (or bioactive substance), resulting in optimized performance of the device under consideration. As an example, dexamethasone can be released form stents [3,5] in order to control the inflammation induced during the insertion procedure [6–8]; also, heparin can be released in active form in order to improve the haemocompatibility of stent materials [4,5].

Nevertheless, all previous studies were performed on polymer coated stents which are certainly not ideal biomaterials for vascular grafts, due to the questionable biocompatibility of the polymers (especially when implanted in veins or arteries injured to a certain percent during the implantation) [9,10], and/or due to problems related with the physiology of the insertion sites (when used as ureteral grafts) [11]. In any case, it would be highly advantageous if liposomes were used as drug eluting systems on plain metal surfaces, for their superior mechanical properties with respect to other stent-forming materials [12,13].

In order to immobilize liposomes on metals, which are rather inert to this purpose, their surface should be suitably functionalized with proper "anchor" chemical groups to which liposomes could possibly be tied, preferably with covalent bonds, for remaining stable after in vivo implantation. Herein, for proof-of-principle study, we decided to immobilize –NH_2 (surface) functionalized liposomes on stainless steel (SS) substrates through amidic bonds on plasma-deposited thin coatings, surface dense of –COOH groups, from radio frequency (RF) glow discharges.

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Surface modification methods, including cold plasmas, have been often used to adapt surface composition and properties of materials to the best interactions with proteins, cells and living tissues in biomaterials, prosthesis and biomedical devices [14–17]. Cold plasma processes can alter the surface properties of materials while keeping intact their bulk properties. Plasma enhanced chemical vapor deposition (PE-CVD), plasma etching (ablation) and plasma treatments (grafting of chemical groups) are the surface modification processes triggered by cold plasmas. Chemical composition, hydrophilic and polar character, morphology and charge of material surfaces can be easily adjusted by properly tuning experimental plasma parameters such as input power, flow rate, composition of feed gas, and others [14,15,18]. By feeding the discharge with proper gas/vapors, e.g., with NH_3 to graft N-containing functional groups onto polymers, or with acrylic acid (AA) or other suitable mixtures to deposit coatings characterized by a certain density of –COOH groups [19–23], it is possible to functionalize surfaces of biomedical interest, where the chemical groups could be possibly used for optimal surface interactions of the modified material with protein and cells [24] or in further conventional surface modification reactions for the direct immobilization of biomolecules [25]. This approach allows to develop new biomedical materials characterized by drug-eluting capability, or by the ability to imitate, to some extent, the proper environment for cell adhesion and growth, as in the extracellular matrix of living tissues.

For this research, SS-316 surfaces (1.13 cm^2) were plasma processed in RF glow discharges fed with vapors of acrylic acid to deposit adherent stable plasma deposited acrylic acid (pdAA) coatings characterized at their surface by carboxylic functionalities to be utilized in conventional wet immobilization procedures [26] of liposomes with incorporated amino-terminated polyethylene glycol [PEG] (MW 2000) conjugated lipid, in order to have reactive amino-groups on their surfaces.

Appropriate types of liposomes, to be used as control (e.g., with no reactive group on the terminal PEG), were also synthesized and used under identical conditions with the functionalized liposomes, in order to test the extent of non-specific binding. Attachment to pdAA-coated surfaces was achieved by incubating liposomes and substrates together, after having activated the –COOH groups of the pdAA coating. Calcein, a hydrophilic fluorescent dye, was encapsulated in the liposomes, for quantitative evaluation of their immobilization by measuring the fluorescent intensity emitted from the processed surfaces after disruption of the liposome membrane (of the attached vesicles) with a surfactant-containing buffer.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC), phosphatidylglycerol (PG) and 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Lipoid Gmbh (Germany). The chemical purity of phospholipids was verified by TLC. 1,2 Distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)-2000] (amino-PEG lipid) and 1,2 distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (methoxy-PEG lipid), were purchased from Avanti Polar lipids (USA). Calcein, cholesterol (99%) (Chol), and all other chemicals (salts used for buffer preparation, reagents for lipid concentration determination and surfactants for liposome disruption) were of analytical grade from Sigma–Aldrich and were purchased from Chemilab (Greece). N-Hydroxy succinimide (NHS) and diisopropylcarbodiimide (DIC) were from Merck (Germany).

A Shimatzu UV-1205 spectrophotometer was used for measurement of liposomal lipid concentration, and all measurements of fluorescence intensity (FI) were performed with a Shimatzu RF-1501fluorescence spectrophotometer. A Julabo SW-20C shaking incubator was used for incubation of samples when needed. When needed sample centrifugation was carried out in a Heraeus Biofuge 28RS.

2.2. pdAA coatings

SS-316 polished disks (1.13 cm^2, 1 mm thick) were used as substrates for pdAA deposition and liposome immobilization. Disks were plasma-coated in two different plasma reactors with two similar PE-CVD processes able of depositing stable coatings with pre-determined –COOH surface density.

The first deposition system (Reactor A), described in detail in Ref. [27], is a SS parallel plate reactor. The upper electrode (22 cm dia), ground-shielded, is connected to the RF (27.12 MHz) power generator via a matching network; the bottom electrode (19 cm dia, 2.5 cm gap) is grounded, works as substrate holder and can be heated up to 500 °C. Prior the deposition, the reactor is pumped down to a base vacuum of 1×10^{-5} mbar by using a turbomolecular pump (Alcatel ATP100), while during the process gases and vapors were pumped out by using a roots pumping system (Alcatel 2033C rotary pump and Alcatel 151B roots pump) equipped with a suitable multitrap (MV Products 355040). Liquid AA (Sigma–Aldrich 99% purity) was heated at 40 °C and its vapors let in the reactor with He (N5.5, BOC) or He in mixtures with H_2 (N6.0, BOC) through 0.1 cm dia holes in the upper showerhead electrode, to allow uniform distribution of the gas and vapors in the discharge. Pressure was monitored through a capacitance manometer (MKS 6B17) installed in the exhaust of the reactor and He and H_2 gas flow were independently adjusted by using mass flow controllers (Brooks Instruments 5850TR). Eight (8) samples of pdAA-coated SS substrates were prepared at each run.

The second reactor (Reactor B), described previously [23], consists of a tubular glass chamber (68 cm long, 9 cm dia) equipped with two parallel internal symmetric SS electrodes (19 cm long, 7 cm wide, 3 cm gap). The upper electrode, ground-shielded, is connected to an RF (13.56 MHz) generator through a manual matching network; the lower electrode, grounded, served as sample holder. The reactor is evacuated with a rotary pump equipped with a SS liquid nitrogen trap. AA vapors mixed with Ar (Air Liquide, France) were fed in the discharge through a lateral port of the reactor from a SS reservoir equipped with a needle valve. The pressure was monitored with a MKS baratron. AA (99%, Sigma–Aldrich) was degassed through three freeze–thaw cycles and used without any further purification in both reactors. Several (5–10) pdAA-coated SS disks were prepared in each run.

2.3. Characterization of plasma treated surfaces

The stability of pdAA films was checked after immersion in dichloromethane (DCM), in DCM/DMF (dimethylformamide) mixtures (9:1, v/v), and in d.d (distilled and deionized) water, solvents used during activation of the COOH groups and liposome immobilization. Static water contact angle (WCA, KSV Instrument – model CAM200) measurements were performed on pdAA-coated SS substrates before and after immersion in the liquids for 3, 24 and 48 h. Samples were inspected also with an optical microscope (OM, Metaloplan, Leinz) to check for delamination. The surface chemical composition of the coatings was measured by means of X-ray photoelectron spectroscopy (XPS) with a Theta Probe Thermo VG Scientific Instrument (monochromatic AlKα radiation 1486.6 eV; 300 W; 200 μm X-ray spot) at 52° take-off angle, corresponding to about 10 nm of sampling depth. Samples were neutralized with a flood gun (Mod. 822-06 FG; 1 eV; 10^{-7} mbar).

According to the relevant literature, C1s spectra of pdAA films were best-fitted with four peak components [19–23] corresponding to C-atoms with zero, one, two and three carbon–oxygen bonds: C0 (C–H, C–C; BE = 285.0 eV, reference), C1 (C–OH, C–O–C; BE = 286.6 ± 0.2 eV), C2 (O–C–O, C=O; BE = 288.2 ± 0.2 eV) and C3 (COOH, COOR; BE = 289.1 ± 0.2 eV). Since XPS cannot distinguish carboxyl from ester groups, that can be found at the same binding energy (BE) [28] derivatization with Toluidine Blue O (TBO) dye was used [23,29], to probe the presence of –COOH groups on pdAA surfaces. A calibration curve was produced by measuring the optical density at 633 nm of TBO solutions of known concentration with a UV/VIS 6505 G/ACC spectrophotometer. For this test pdAA coatings were deposited on PTFE substrates, then rinsed in 20 ml of a 5×10^{-4} M TBO water solution (pH 10, adjusted with NaOH). Separate experiments have shown that TBO is not adsorbed on bare PTFE. The formation of ionic complexes between the –COOH pdAA surface groups and the cationic dye was allowed to proceed for 5 h at 30 °C. Substrates were then rinsed with plenty of 0.01 M NaOH to remove all unbound TBO. Complexed TBO was then desorbed from pdAA with 3.5 ml of 50% (v/v) acetic acid solution (1 min, vortex). The surface density of –COOH groups on pdAA was calculated from the optical density of the desorbed dye solution at 633 nm, assuming that 1 mol of TBO complexes with 1 mol of –COOH group on pdAA.

2.4. Preparation and characterization of liposomes

2.4.1. Preparation of liposomes

Functionalized (F) and control (C) liposomes were prepared with the following lipid compositions:

(i) PC or DSPC/Chol/amino-PEG lipid at 20:10:0.3 mol/mol/mol [F liposomes];
(ii) PC or DSPC/Chol/methoxy-PEG lipid at 20:10:0.3 mol/mol/mol [C liposomes];
(iii) PC or DSPC/PG/Chol/amino-PEG lipid at 18:2:10:0.3 mol/mol/mol [F liposomes];
(iv) PC or DSPC/PG/Chol/methoxy-PEG lipid at 18:2:10:0.3 mol/mol/mol [C liposomes].

In order to prepare small unilamellar liposomes (SUV), multilamellar vesicles (MLV) were initially prepared by the thin film hydration method [30]. In brief, a proper weight of lipid (or lipids) was dissolved in a chloroform/methanol (2:1, v/v) mixture, and subsequently evaporated under vacuum until a thin lipid film was formed. The lipid film was hydrated with the appropriate volume of Tris buffer (pH 7.4) containing NaCl (0.9% (w/v) final concentration), or a 100 mM solution of calcein which was adjusted to be isotonic (in the same buffer), at 25 °C in the case of PC liposomes, and at 60 °C in the case of DSPC liposomes. After complete lipid hydration, for formation of SUV liposomes, the vesicle dispersion was placed under the microtip of a probe sonicator for two 10 min cycles, or until the liposome dispersion was completely clear. Finally, liposome dispersions were left in peace for annealing structural defects, at a temperature above the transition temperature of the lipid, for 1–2 h. In all cases liposomes were incubated with solutions of similar osmolarity (adjusted with NaCl), in order to avoid disruption due to osmotic pressure differences. Separation of liposomes from non-encapsulated molecules (calcein) was achieved by gel filtration on a column (30 cm × 1 cm) packed with Sephadex G-50 (course) gel.

2.4.2. Characterization of liposomes

Liposome dispersions were characterized for size distribution, zeta-potential and calcein encapsulation, and retention. Lipid concentration of liposomes was measured with the Stewart colorimetric assay [31], in which phospholipids form a colored complex with ammonium ferrothiocyanate (OD 485 nm) that is extracted with chloroform. After measurement, the lipid concentrations of the liposome dispersions were adjusted at the desired value, in order to carry out liposome stability (integrity) studies or to immobilize the liposomes on the pdAA coated SS disks.

Size distribution (mean diameter and polydispersity index [PI]) and zeta-potential of some of the liposome dispersions were measured by dynamic light scattering (DLS) and laser Doppler electrophoresis (LDE), respectively, on a nanosizer (Nano-ZS, Nanoseries, Malvern Instruments, UK), which enable the mass distribution of particle size as well as the electrophoretic mobility to be obtained. Measurements were performed at 25 °C with a fixed angle of 173°. Sizes quoted are the *z*-average mean (dz) for the liposomal hydrodynamic diameter (nm). Zeta-potential (mV) was calculated by the instrument (from the electrophoretic mobility measurement).

Calcein was encapsulated in the vesicles at a quenched concentration (100 mM). Calcein encapsulation efficiency in liposomes (calcein/liposomal lipid) is calculated after measuring the lipid concentration (as mentioned above) and the corresponding concentration of encapsulated calcein. This is done after disruption of liposomes with Triton X-100 (1% (v/v) final concentration) and intensive vortex agitation.

After purification of liposomes from non-entrapped dye, the percent calcein latency is measured in order to be sure that separation of free calcein from liposome-entrapped is complete [32]. Liposomes were re-purified until the calcein latency was higher than 95%; for calculation of latency a sample from the liposome dispersion (20 μl) is diluted with 4 ml buffer, pH 7.40, and the fluorescence intensity (FI) is measured (EM 470 nm, EX 520 nm), before and after vesicle disruption, by addition of Triton X-100 to a final concentration of 1% (v/v). The percent of calcein latency (% latency) is calculated from Eq. (1), where F_{BT} and F_{AT} are the calcein FIs before and after addition of Triton X-100, respectively.

$$\%\ \text{Latency} = \frac{1.1(F_{AT} - F_{BT})}{1.1F_{AT}} \times 100 \quad (1)$$

The integrity of calcein encapsulating liposomes during incubation at the conditions used for liposome immobilization was also evaluated [32], in order to be sure that the calcein/lipid ratio calculated initially for the liposome dispersion is not altered during the immobilization procedure. For this, the various types of liposomes were incubated under mild agitation at 37 °C, and calcein latency was measured at fixed time points, as explained above. Additionally, the integrity of some liposome types was measured when liposome dispersions were in contact with the SS surfaces, in order to be sure that this contact does not induce vesicle de-stabilization.

2.5. Liposome immobilization procedure

Liposomes were immobilized on pdAA coated SS-surfaces through the formation of amidic bonds [33], as presented in Fig. 1. The pdAA carboxylic groups were initially activated by carbodiimide in the presence of N-hydroxy-succinimide (NHS or HOSu), in an organic solution [34]. For this an excess of NHS/DIC (N,N′-diisopropylcarbodiimide) (1.10:1.00, mole:mole), dissolved in a DCM/DMF (9:1, v/v) mixture, was incubated with the plasma-functionalized surfaces for 20 min; at room temp. The activation step was performed twice. The reaction mixture was subsequently removed and the surfaces were gently washed with excess of DCM/DMF (9:1, v/v) followed by phosphate buffer, pH 7.40. After the activation step, the liposomes (F or C) were incubated with the activated pdAA surfaces, in 8-plate cell culture containers (for easy handling), for 3, 24 h or 48 h at 37 °C, under mild agitation. At least three different liposomal lipid concentrations of the liposome dispersion were tested. Non-specific immobilization of vesicles was

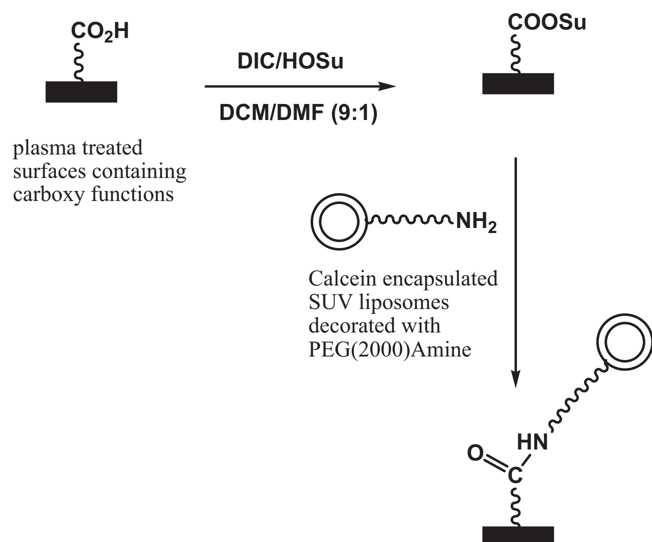

Fig. 1. Covalent immobilization of liposomes on plasma treated metallic surfaces via a two step amide bond formation method. DIC, diisopropylcarbodiimide; HOSu or NHS; N-hydroxy-succinimide; DCM, dichloromethane; DMF, dimethylformamide.

evaluated by using control liposomes (C) under the same conditions as the corresponding functionalized ones (F). Additionally, the need for prior-activation of the carboxylic groups was evaluated by using also surfaces which were not subjected to the activation step.

After liposome incubation, the surfaces were carefully washed with 3 ml of PBS (phosphate buffer saline; gentle agitation) 3 times. Then 0.4 ml of triton X-100 (10%) and 2.1 ml of PBS buffer were added and the surfaces were subjected to vigorous agitation in order to disrupt the surface attached liposomes and release the encapsulated dye. The quantity of calcein bound to each surface was calculated by measuring the fluorescence intensity of the liquid (EM-470 nm, EX-520 nm) in each container. The percent of vesicle binding (lipid) to surfaces was determined by taking the calcein/lipid ratio (of the corresponding liposome preparation) into account.

3. Results and discussion

3.1. Plasma treated surfaces

It has been previously [22] shown that pdAA coatings deposited in low monomer fragmentation conditions (e.g., at low plasma power or in pulsed plasma regime) exhibit high surface density of carboxylic groups, low density of other polar groups, low cross-linking degree and very high hydrophilic character (WCA < 20° some cases) but, at the same time, they are not stable in water due to either delamination from the substrate or/and leaching of polar moieties. PdAA coatings deposited at higher plasma power, i.e., in high monomer fragmentation conditions, maintain instead their surface composition (lower density of –COOH groups, lower wettability, etc.) unaltered after immersion in water for 50 h. Stable pdAA coatings are thus characterized by relatively low surface density of –COOH groups (<10% with respect to the total number of surface carbon atoms) still suitable though, for improving directly cell adhesion [24], and for immobilizing RGD peptides through the formation of amide bonds [35].

During the preparation of pdAA samples, several tests were performed to optimize composition and structure of pdAA coatings to their best stability in water and other solvents utilized for liposome immobilization in both reactors. It was attempted to increase the

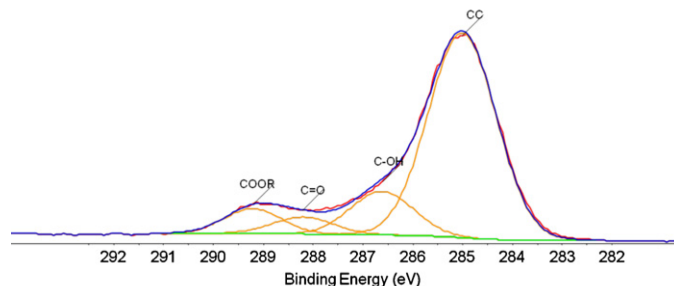

Fig. 2. C1s signal of a pdAA coating deposited at 27.12 MHz (0.6 W/cm^2, pressure 0.1 mbar, pure AAc, substrate temp. 250 °C, deposition time 20 min) best-fitted with C0–C3 peak components: C0 (C–H, C–C) 72.9%; C1 (C–OH, C–O–C) 14.3%; C2 (O–C–O, C=O) 5.3%; C3 (COOH, COOR) 7.5%. C/O ratio 3.4. WCA 47 ± 2°.

pdAA surface density of –COOH groups above the 10% threshold. XPS, WCA and optical microscope observations confirmed, though, that low power conditions and pulsed regimes lead to coatings unstable in liquids. Increasing RF power and/or temperature, lead to pdAA coatings stable in water and DCM, but with limited surface density of –COOH groups (<8% with respect to all surface carbon atoms, according to XPS analysis), which were found in any case suitable for immobilizing liposomes. We do not present here all data relative to the effect of plasma parameters in the deposition of pdAA coatings, some of which have been previously published [21,24].

The immobilization of liposomes was achieved on 400–500 nm (Reactor A), and 70–150 nm (Reactor B) thick pdAA coatings characterized by a C1s XPS spectrum very similar to that shown in Fig. 2. The presence of the C3 peak component and the TBO titration data attest the presence of –COOH groups on the pdAA surface. No changes in the C1s spectrum were detected after immersion/rinsing in bi-distilled water and in DCM for 24 h, and no signals (Fe2p, Cr2p) from the SS substrate underneath, attesting for the stability of the coatings in the solvents used for the immobilization reactions [22,36]. Since XPS analyses cannot distinguish carboxyl from ester moieties for their similar binding energy, the TBO test was performed, sensible only to carboxylic groups. pdAA surfaces utilized in the liposome immobilization procedures, all characterized by a C3 peak component of 5–8% importance in the XPS C1s spectrum, became colored in blue after the test, attesting for the presence of the –COOH groups. A surface density of about $5.0 \pm 0.2 \times 10^{-9}$ mol/cm^2 of –COOH groups was measured on the pdAA surfaces prepared in both reactors utilized in this work for liposomes immobilization.

3.2. Liposome physicochemical properties and stability

3.2.1. Vesicle size distribution and zeta potential

As seen in Table 1, the liposome mean diameter was between 70 and 90 nm, in all cases; while all the liposome dispersions had a very low polydispersity index (PI) between 0.11 and 0.22, which proves that all dispersions used had very narrow vesicle size distribution. Liposomes that incorporate PEG-lipids (F and C types) are slightly larger than vesicles with the same lipid composition but no PEG-lipid in their membrane, in accordance to previous observations [37,38], due to the contribution of the PEG layer on their outer surface.

Regarding surface charge, it is observed that incorporation of PG, a negatively charged lipid, in the vesicle lipid membrane, provides a minor negative charge to the vesicles; liposomes are not charged (zeta-potential values around 0) when they are composed solely by zwitter-ionic lipids (PC or DSPC). When the PG-incorporating negatively charged liposomes are pegylated (by including PEG-lipids in their membrane) their surface charge is abolished (zeta-potential

Table 1
Physicochemical properties of the liposome used. Each value is the mean of at least 3 different preparations.

Liposome composition	Mean diameter (nm)	Polydispersity index (PDI)	Zeta-potential (mV)
DSPC:Chol 2:1	70.98 ± 0.91	0.154 ± 0.013	–
DSPC:PG:Chol 9:1:5	91.6 ± 1.9	0.160 ± 0.023	−8.3 ± 1.4
DSPC:Chol 2:1 – F	80.1 ± 2.4	0.220 ± 0.012	0.1 ± 1.9
DSPC:PG:Chol 9:1:5 – F	91.1 ± 1.5	0.204 ± 0.025	−2.25 ± 0.49
DSPC:Chol 2:1 – C	86.91 ± 0.10	0.112 ± 0.021	0.5 ± 2.3

becomes practically zero) providing proof that the vesicles are coated with PEG, as reported previously [37,38].

Calcein encapsulation in the vesicles is reported as calcein/lipid (mole:mole) ratio, and ranges between 0.05 and 0.09, in the various liposome preparations used for immobilization (Table 1). These values were used for estimation of vesicle immobilization on the SS surfaces (after measuring the amount of calcein attached to them). It should be mentioned here that the initial latency of calcein in all the vesicle dispersion prepared (after they were purified from non-entrapped calcein) was always higher than 94.8%.

3.2.2. Liposome integrity

The integrity of liposomes was investigated during a 48 h incubation period, at 37 °C and under mild agitation (10–20 rpm). The retention of a hydrophilic and low molecular weight dye, calcein, was used as a measure of the vesicle integrity. As seen in Fig. 3, all liposomes prepared were very stable for the full time-period investigated, since the calcein released was always below 5% of the initial amount contained. Since the incorporation of PG in the liposome bilayer did not improve the integrity, it was decided to use the plain DSPC/Chol 2:1 mol/mol, liposomes for the immobilization studies.

In order to study if the presence of the SS surfaces in the proximity of the vesicle dispersions had any effect on their integrity, calcein retention in some vesicle types was additionally studied when the liposome dispersions were placed in contact with surfaces. As seen in Fig. 4, for all the liposome-types studied accordingly, there was no significant difference observed in terms of vesicle integrity, when the dispersions were studied in test tubes or in contact with the surfaces (SS-316), at least during the evaluated time periods. Thereby, according to these results, it is anticipated that these vesicles remain stable during the immobilization procedure, and that the estimation of vesicle immobilization from the amount of calcein immobilized on them, is accurate and reliable.

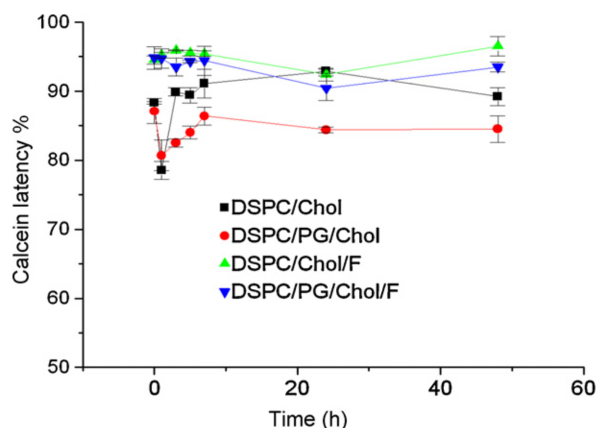

Fig. 3. Calcein retention (latency %) in various types of small unilamellar liposomes during incubation in presence of buffer at 37 °C. Liposomes were incubated for a 48 h period under mild agitation (40 rpm) in a thermostated shaking waterbath, and at fixed time points, samples were drawn and calcein latency was calculated (as described in detail in Section 2.1). The sample key is presented in the figure insert. Each value is the mean of three different experiments and bars represent standard deviation of the mean.

3.3. Liposome immobilization on surfaces

The amount of liposomal lipid immobilized on the pdAA surfaces is presented in Fig. 5, for F (wide patterned bars) and C liposomes (narrow solid blue bars) and for the different liposome/surface incubation protocols used (lipid concentrations [L] of 2.5, 5.0 and 10.0 mg/ml, and incubation periods of 3, 24 and 48 h). It is important to emphasize that no lipid was immobilized on the surfaces without prior activation of the –COOH groups of the pdAA coatings (the amount of calcein immobilized was null).

As seen in Fig. 4, the best incubation period (i.e., resulting in immobilization of the highest amount of lipid) is 24 h; while the best lipid concentration for incubation with the substrate deposited-films is 5 mg/ml. This is true for all sample surfaces used, originating from Reactor A or Reactor B, and under these specific conditions similar amounts of lipids were immobilized via the amidic bond (differences are statistically insignificant at $p = 0.01$).

In terms of the incubation period used, it is demonstrated by the results (Fig. 5 and Table 2) that a 3 h incubation period is not sufficient for vesicle immobilization via the covalent bond; however the non-specific binding (of C liposomes) is faster, compared to the specific (of F liposomes), as indicated by the lower specific/non-specific ratio calculated (in the case of 3 h incubations). When liposomes are incubated with pdAA surfaces for 24 h, the amounts of lipid immobilized are the highest measured; while at prolonged incubation periods (48 h) a significant reduction in the amount of

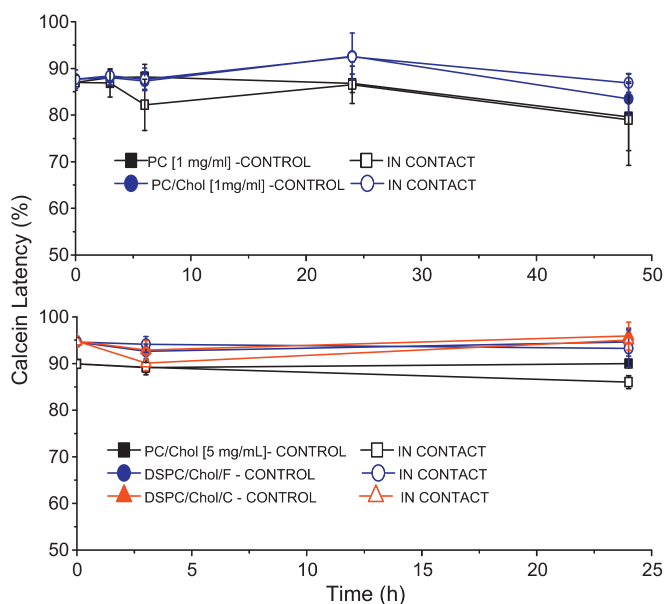

Fig. 4. Calcein retention (latency %) in various types of small unilamellar liposomes during incubation in presence of buffer at 37 °C, when the liposome dispersions are placed in contact with SS-316 surfaces (in contact) or in test tubes (control). Liposomes were incubated for a 24 or 48 h period under mild agitation (40 rpm) in a thermostated shaking waterbath at 37 °C. At fixed time points, samples were withdrawn and calcein latency was calculated (as described in detail in Section 2.1). The sample key is presented in the figure insert. Each value is the mean of three different experiments and bars represent standard deviation (SD) of the mean.

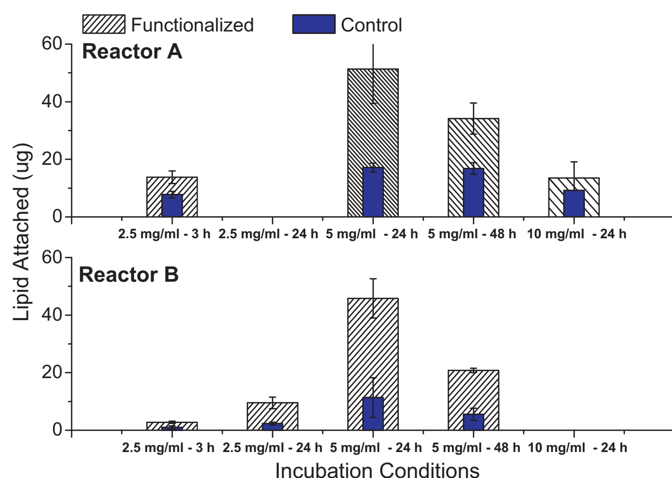

Fig. 5. Amount of lipid immobilized in the form of intact vesicles on plasma treated SS-316 surfaces, for two plasma treatment reactors utilized (A and B). DSPC/Chol/amino-PEG lipid at 20:10:0.3 mol/mol/mol [F liposomes or funtionalized], or DSPC/Chol/methoxy-PEG lipid at 20:10:0.3 mol/mol/mol [C liposomes or control] encapsulating calcein (100 mM) were used, at different lipid concentrations (2.5, 5 or 10 mg/ml, as seen in the *x*-axes of the graphs) and liposomes were incubated with the surfaces after the carboxy-groups of the surfaces were activated (as explained in detail under Section 2). Each value is the mean of 4–6 experiments and the SD of the mean is represented as bars.

lipid immobilized is observed. If we assume that pdAA coatings are not affected by the incubation conditions (in line with the stability studies carried out), the later reduction should be due to release of some of the encapsulated calcein from the liposomes. The demonstrated high integrity of the liposomes when they are in contact with surfaces (Fig. 4B), overrules this possibility. Nevertheless, there is still a question whether the integrity of the liposomes that are covalently bound to the pdAA surface is affected (jeopardized) by the many non-bound liposomes free to move in the aqueous medium (and perhaps bounce on and mechanically disturb the immobilized ones). If this is happening, any prolongation of the incubation period after the binding is completed, would indeed result in reduced final binding values. An even more pronounced reduction in the immobilization of functionalized liposomes was observed when the concentration of the liposome dispersion incubated with the films was doubled (10 mg/ml) (Fig. 4A). This later observation can also be explained on the basis of the suggested theory.

It is worth reporting that (data not shown) under all conditions evaluated, the amount of functionalized and control liposomes immobilized was found always slightly higher on pdAA surfaces from Reactor A. When the non-specific lipid binding is expressed as a percent of the corresponding specific binding measured under the same incubation conditions, this percent ranges from 33 to 72% in the case of the films deposited on substrates in Reactor A, while for the films deposited in Reactor B, it is always 25–26%. In fact, when the amounts of lipids immobilized non-specifically (control liposomes) are subtracted from the corresponding lipid amounts immobilized via covalent binding (functionalized liposomes) the values for both types of films are practically the same. This is mainly the result of the similar surface densities of –COOH groups ($\sim 5.0 \pm 0.2 \times 10^{-9}$ mol/cm^2) for the samples deposited in both reactors. Moreover, in Table 2 are presented the specific/non-specific binding ratios which were calculated for each surface type and condition investigated. It is clear that the specific covalent binding is favored for samples prepared in Reactor B, indicating that except of the density of –COOH groups in the surface there are also other film properties as surface morphology affect the immobilization procedure. In fact, the samples prepared in Reactor A are thicker compared to Reactor B due to the higher deposition time. This in turn results to rougher films with film height differences of about 100 nm, which can promote the physical absorption and the non-specifically binding of liposomes. Thus, except of the chemical composition, the surface morphology needs also to be taken into account for the optimization of the immobilization procedure.

Table 2
Specific/non-specific binding of liposomes (DSPC/Chol-F and DSPC/Chol-C) on plasma treated surfaces, using different plasma treatment methodologies and immobilization procedures. Each result is the mean of at least 3 different experiments. Deviations were always lower than 10%.

Plasma reactor	Immobilization conditions		Specific/non-specific binding F/C
	Lipid conc. (mg/ml)	Incubation duration (h)	
Reactor A	2.5	3	1.78
	5.0	24	3.00
		48	2.03
	10.0	24	1.46
Reactor B	2.5	3	2.81
		24	4.07
	5.0 mg/ml	24	4.02
		48	3.74

4. Conclusions

It has been demonstrated herein that liposomes can be immobilized in intact form, by a stable covalent bond on plasma treated surfaces. Important factors that determine the covalent attachment of functionalized liposomes on surfaces via amidic bonds, are: (i) the availability of –COOH– groups on the surfaces and (ii) the ability of these groups to be activated. Furthermore, it is also very important that the surface is stable under the experimental conditions applying during the specific procedure, and of course, on a later step, under the in vivo conditions that will apply in the area of implantation, when this methodology is used for preparation of drug-eluting materials or devices.

Under the best conditions for liposome immobilization on the deposited films utilized herein (24 h incubation with a 5 mg/ml liposome dispersion), it is calculated that 58.4 ± 8.6 μg of lipid can be immobilized per cm^2 of surface. This first investigation provides proof of principle about the feasibility of the proposed methodology. Several steps related with the surface coating methodology (increase of functional group density on the surfaces) as well as liposome design (different amount of anchoring units per vesicle, etc.) could result in further improvement of the amounts of liposomes attached to surfaces.

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