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Haemocompatibility improvement of metallic surfaces by covalent immobilization of heparin–liposomes

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ABSTRACT

Stainless steel surfaces were processed by means of plasma enhanced chemical vapor deposition (PE-CVD) fed with acrylic acid vapors in order to functionalize them with carboxyl groups, which were subsequently activated for covalent immobilization of heparin-loaded (HEP) NH_2 group-functionalized (Fun) nanoliposomes (NLs). Empty Fun or HEP non-functionalized (control) NLs were used as controls. NLs were characterized for mean diameter, surface charge and heparin encapsulation/release. Different lipid compositions were used for NL construction; PC/Chol (2:1 mol/mol) or PC/Chol (4:1 mol/mol) (fluid type vesicles) [which allow gradual release of heparin] and DSPC/Chol (2:1 mol/mol) (rigid type vesicles). Surface haemocompatibility was tested by measuring blood clotting time. Platelet adhesion on surfaces was evaluated morphologically by SEM and CLSM. The haemocompatibility of plasma-processed surfaces was improved (compared to untreated surfaces); Fun-HEP NL-coated surfaces demonstrated highest coagulation times. For short surface/blood incubation periods, surfaces coated with Fun-HEP NLs consisting of PC/Chol (2:1) had higher coagulation times (compared to DSPC/Chol NLs) due to faster release of heparin. Heparin release rate from the various NL types and surface platelet adhesion results were in agreement with the corresponding blood coagulation times. Concluding, covalent immobilization of drug entrapping NLs on plasma processed surfaces is a potential method for preparation of controlled-rate drug-eluting metallic stents or devices.

1. Introduction

The introduction of drug eluting stents (DES) to replace bare metallic stents (BMS) in the last decade has revolutionized the treatment of symptomatic coronary artery diseases (CAD) (Gershlick, 2002; Kivela and Hartikainen, 2006; Brown et al., 2009). Nevertheless, several approaches for regulation of the properties of DES are still under extensive investigation in order to improve their performance and/or minimize any problems encountered during their use (Tsimikas, 2006; Kedia and Lee, 2007; Venkatraman and Boey, 2007; Kraitzer et al., 2008; Bavry and Bhatt, 2008; Raber and Windecker, 2011).

In the past, liposomal formulations of dexamethasone were developed and used for coating arterial or ureteral polymer-covered-stents (by physical adsorption of liposomes on the polymer cover of the stents) (Kallinteri et al., 2002). By this method, controlled release of drug molecules at the specific site of stent placement could be realized, by modulating the liposomal drug loads, or using liposome types with different drug release kinetics (modulating the vesicle size or lipid-membrane composition) (Antimisiaris et al., 2000; Tsotas et al., 2007). In accordance with the hypothesis mentioned above, stents which were coated with heparin-loaded liposomes demonstrated superior blood compatibility, compared to uncoated ones (Antimisiaris et al., 2006; Koromila et al., 2006). However, all of the studies mentioned above were performed using polymer (PFE or PTFE) covered stents which are not ideal biomaterials when used as vascular grafts, due to

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the questionable biocompatibility of the polymers, especially when implanted in veins or arteries that have been injured to a certain extent during the implantation procedure; or due to problems related with the physiology of the insertion sites (i.e. when used as ureteral grafts) (Liatsikos et al., 2007; Pendyala et al., 2008; Hanawa, 2009). In addition, coating with liposome on the previous stent materials was accomplished by physical adsorption and not by covalent immobilization, which would be more stable.

Thereby, it would be highly advantageous if the technology described above (use of liposomes for construction of drug eluting biomaterials) could be applied on plain metallic surfaces, which are superior in terms of mechanical properties compared to all other types of materials proposed for stent manufacturing (Hanawa, 2009) and furthermore if the coating could be done covalently.

Cold (non-equilibrium) plasma processes allow to modify the surface of materials with coatings, grafted groups, or through the ablation of surface layers. The historical development of plasma techniques is reported by D'Agostino et al. (2005). Plasma deposition and grafting processes are widely used in the biomedical field for functionalizing biomaterials with proper chemical groups, e.g., for improving cell adhesion on materials and/or for immobilizing biomolecules. Recently, immobilization of liposomes on stainless steel surfaces was achieved by plasma coating with pdAA (plasma deposited acrylic acid); appropriate functional groups (—COOH) were deposited on the surfaces and were subsequently used as "handles" for covalent attachment of liposomes which were decorated with NH_2-groups (on the distal end of their PEG layer) (Mourtas et al., 2011).

The proposed platform technology, could find many applications for delivery of different types of bioactive agents from stents or other biomedical devices. In fact, stents or other types of surfaces could be initially treated for implantation of handles and at a second step liposomes could be attached to them (providing possibilities to individualize therapy). Furthermore, liposome technology provides a mean to immobilize large amounts of different drugs or active substances (encapsulated in the same or different vesicles) within a fully biocompatible carrier, and achieve optimized release rates for each substance (according to the specific mode of action and therapeutic requirement), by modulation of liposome characteristics (membrane integrity, lipid composition, surface properties, etc.) (Antimisiaris, 2011).

Herein, we investigate the validity of using plasma processes followed by covalent liposome attachment on surfaces for preparation of drug eluting surfaces (with controllable elution rates), by using heparin as a model active substance and evaluating the improvement of the surface blood compatibility. To this end, different types of nanosized-liposomes (NLs) were used for immobilization on stainless steel surfaces: (i) heparin-loaded (HEP) NLs for covalent immobilization on the plasma-treated surfaces [functionalized NLs (Fun)]; (ii) HEP-NLs without reactive groups on their surface, as controls (Con) for evaluation of the effect of non-specific binding; (iii) Empty NLs of both types [Fun and Con]. Furthermore, NLs with different lipid membrane compositions were constructed; in order to investigate the potential to modulate the release rate (of the active substance) from the coated surfaces.

2. Materials and methods

Phosphatidylcholine (PC), and 1,2 distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Lipoid Gmbh (Germany). The chemical purity of the 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. Heparin, cholesterol (99%) (Chol) and all other materials (as 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-hydroxysuccinimide (HOSu) and N,N′-diisopropylcarbodiimide (DIC) were from Merck (Germany). Human platelet rich (PRP) and platelet-poor (PPP) plasma were produced from human blood provided from the University Hospital (Rio, Patras, Greece). Blood was collected in CTAD (citrate/theophyline, dipyridamole) 9:1 (v/v) (Diagnostica Stago, France) tubes and centrifuged for 15 min at 1500 and 6000 rpm in order to obtain PRP and PPP, respectively. The determination of kaolin-activated partial thromboplastin time (aPTT) was performed using the CK Prest kit from Diagnostica Stago (France), and coagulation time was measured using an ST4 coagulometer, Diagnostica Stago (France). Ethanol and glutaraldehyde used for SEM sample preparation were purchased by Applichem, Germany and Sigma–Aldrich (Chemilab, Greece), respectively. FITC conjugated mouse monoclonal antibody (MAb) against platelet glycoprotein CD62P, which was used for identification of platelet activation, was purchased from Abcam, UK.

A Shimatzu UV-1205 spectrophotometer was used for measurement of the lipid concentration of NL formulations. A Julabo SW-20C shaking incubator was used for incubation of samples, and a Heraeus Biofuge 28RS centrifuge for centrifugation, when needed.

2.1. Plasma treatment of surfaces

2.1.1. 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 with PE-CVD processes able of depositing stable coatings with pre-determined —COOH surface density. Plasma parameters affect the deposition of pdAA coatings as previously published (Palumbo et al., 1999; Detomaso et al., 2005a,b; Pistillo et al., 2007). The reactor, consisted of a tubular glass chamber (68 cm long, 9 cm diameter) equipped with two parallel internal symmetric SS electrodes (19 cm long, 7 cm wide, 3 cm gap) (Pistillo et al., 2007). 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 was 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. PdAA films, (thickness of 100 ± 20 nm), were employed to immobilize liposomes. The amount of carboxyl groups was $5.0 \pm 0.2 \times 10^{-9}$ mol/cm^2 as determined by the Toluidine Blue-O test (Pistillo et al., 2007).

2.2. Preparation and characterization of liposomes

Different liposome preparation techniques were initially utilized in order to select the one that permits highest heparin loading; for this, DRV (dried-reconstituted vesicles), extruded-DRV (extruded through polycarbonate membranes with known pore diameter), and SUV (small unilamellar vesicle) liposomes were constructed. Functionalized (Fun) and control (Con) liposomes, having the following lipid compositions were prepared: (i) PC or DSPC/Chol/Amino-PEG lipid at 20:10:0.3 or 4:1:0.05 mol/mol/mol [Fun]; (ii) PC or DSPC/Chol/Methoxy-PEG lipid at 20:10:0.3 or 4:1:0.05 mol/mol/mol [Con].

For all types of liposomes the thin film hydration method was initially applied (Mourtas et al., 2011). In brief, the lipid or lipids (13 μmol) were dissolved in a chloroform methanol (2:1, v/v) solution and the appropriate amount of each lipid was placed in a 50 ml round-bottomed flask. The lipid mixture was dried by connection to a rotary evaporator, until a thin film of the lipids was deposited on the flask walls. Residual organic solvents were removed by flashing with nitrogen for 5 min, and the films were hydrated by adding 1 ml of heparin solution (concentration 10,000 ppm in buffer) for the SUV or 1 ml buffer (for DRV liposomes), at a temperature above the transition temperature of the lipid used in each case. After this: (a) for SUV liposomes, the liposome dispersion was sonicated for two 10 min cycles by a probe-sonicator (Sonics and Materials, USA), or until the liposome dispersion became completely clear. (b) For DRV liposomes, a previously published method was used (Antimisiaris, 2010). In brief, Empty SUV's were prepared, as described above. Following this, 1 ml of SUV dispersion was mixed with 1 ml heparin solution (10,000 ppm) and the mixture was freeze dried and then rehydrated in a controlled manner. (c) For extruded-DRV's, the DRV dispersions were filtered through a 400 nm pore polycarbonate membrane initially (for at least 15 times), followed by a 100 nm diameter pore membrane (for at least 15 times), using a hand operated LIPO-SO-FAST apparatus (Avestin, Canada).

After preparation, all liposome dispersions were left to stand for at least 1 h at a temperature above the lipid Tm, in order for any structural defect to anneal. Separation of DRV liposome encapsulated heparin from free heparin was accomplished by centrifugation at 12,000 rpm for 40 min (two or three times), while for SUV and extruded-DRVs, this was achieved by molecular weight exclusion chromatography on a Sepharose 2B-CL column (1 cm × 30 cm), eluted with buffer. A typical separation of liposomal and free heparin can be seen in Supplementary Data.

2.3. Liposome characterization and stability

2.3.1. Liposome size distribution and zeta-potential measurements

The size distribution (mean diameter and polydispersity index [PI]) and ζ-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 enables 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).

2.3.2. Retention of heparin in liposomes

For the determination of encapsulation efficiency and stability of heparin-encapsulating liposomes, the phospholipid content of the liposomal dispersions was determined by the Stewart assay (Stewart, 1980).

Vesicle membrane integrity was studied by measuring liposome encapsulated heparin release during incubation in buffer or in PPP at 37 °C, under mild agitation (40 rpm). For incubation in buffer, the heparin released from the vesicles at each time point (3 or 24 h), was separated from the liposomes by molecular weight exclusion chromatography (Sepharose 2B-CL [1 cm × 30 cm] column) and the heparin retained in the liposome fraction was measured as described below. In plasma, the heparin released from the vesicles was measured directly (in presence of the liposomal heparin) with the method described below.

Heparin concentration in NLs dispersed in buffer was measured by a colorimetric method as adjusted before (Antimisiaris et al., 2006; Koromila et al., 2006) for measurements in presence of lipid and Triton X-100 (used for liposome disruption). This method is based on the development of a complex between heparin (anion) and dimethyl methylene blue (cation) with maximum OD at 525 nm. The measurement is carried out rapidly (max 15 s) after mixing the heparin solution with the dye, because the complex is very unstable. The dye solution is prepared by solubilizing 16 mg of dye in 1 l of distilled water that contains 2.37 g NaCl and 3.04 g glycine, and pH is adjusted to 3.0 with HCl. For the standard curve, heparin solutions with known concentrations between 20 and 400 ppm (in TBS buffer pH 7.4) were prepared, and 25 μl (from each solution) were mixed with 75 μl buffer and 25 μl of the dye solution. After gentle mixing, OD-515 nm was measured.

For determination of heparin in PPP, a kaolin-activated partial thromboplastin time (aPTT) measuring kit was used. The kit consists of: Reagent 1 (R1): cefalin (platelet substitute) from rabbit brain and Reagent 2 (R2), a 5 mg/ml dispersion of kaolin. In brief, reagent R2 is transferred to reagent R1 and mixed until the mixture becomes homogenous. A typical calibration curve of heparin in plasma is presented in Supplementary Data. Control measurements were carried out in absence and presence of liposome-encapsulated heparin, in order to assure that the presence of HEP-liposomes does not affect the measurements.

2.4. Coating of plasma-processed SS-surfaces with liposomes

The surfaces were coated by heparin-encapsulating liposomes (or control liposome types, depending on the experiment) by the technique developed previously (Mourtas et al., 2011) for covalent immobilization of liposomes on plasma-processed SS surfaces. In brief, the -COOH groups were activated using excess of HOSu (N-hydroxysuccinimide)/DIC (N,N′-diisopropylcarbodiimide) (1.1:1 molar ratio)) in DCM/DMF (9:1) for 20 min at 25 °C. The activation step was performed twice, the reaction mixture was removed and the surfaces were gently washed using excess of DCM/DMF (9:1). After this, the activated surfaces were placed into 12-well cell culture plates that have appropriate dimensions to allow easy manipulation of surfaces during the incubation procedure (with NLs). The appropriate NL dispersion (Con or Fun, HEP or Empty) was added on the activated surfaces, at a lipid concentration of 5 mg/ml, for 24 h at 37 °C, and under mild agitation (conditions which were previously demonstrated (Mourtas et al., 2011) to result in highest immobilization of liposomal lipid).

Following the NL immobilization procedure, surfaces were carefully washed three times with 3 ml of phosphate buffer saline (PBS) (iso-osmotic with the contents of the liposomes) under gentle agitation. After this, the surfaces were used in the blood compatibility experiments immediately or after keeping at 5 °C, until use.

2.5. Activation of the coagulation system—plasma recalcification (activated partial thromboplastin) time

Blood-coagulation system activation was evaluated under static conditions in the case of reference and NL-coated surfaces by measuring activated plasma re-calcification time (αPTT). Reference and NL-coated surfaces were placed in a twelve well static chamber and were incubated with 2 ml PPP. After 15, 30 and 60 min, as well as 8 h, 24 h or 72 h contact time, PPP was collected and the activated Partial Thromboplastin Time (αPTT) was determined by the plasma recalcification technique (Rhodes and Williams, 1994). Additionally, PPP (without material contact) was used as control. The control value was set as 100%, and all other measurements were normalized accordingly.

Table 1
Physicochemical properties and encapsulation efficiency of HEP Fun-NLs.

Liposome type	Mean diameter (nm)	ζ-Potential (mV)	Hep/lipid (g/mol)
DRV	10,150 ± 1194	−7.29 ± 0.57	13.62 ± 0.37
Extrusion big	271 ± 12	−4.01 ± 0.53	10.60 ± 0.14
Extrusion small	149.3 ± 2.6	−3.3 ± 1.3	8.60 ± 0.44
SUV (F)	72.75 ± 0.72	−1.76 ± 0.44	3.75 ± 0.27

NLs were consisting of DSPC/Chol/Amino-PEG lipid (20:10:0.3 mol/mol/mol) and were prepared by different techniques, as described in detail under Section 2.

Table 2
Physicochemical properties and encapsulation efficiency of extruded-DRV NLs.

Liposome type	HEP/Lipid (g/mol)	Mean diameter (nm)	PI	ζ-Potential (mV)
Fun-HEP	10.32 ± 0.11	109.11 ± 0.54	0.220	−6.11 ± 0.50
Fun-HEP (PC/Chol/Amino-PEG lipid)	9.98 ± 0.25	103.5 ± 1.2	0.185	–
Con-HEP	10.23 ± 0.18	115.92 ± 0.43	0.191	−5.91 ± 0.92
Fun-Empty	–	99.73 ± 0.71	0.293	−5.61 ± 0.45

NLs (except Fun-HEP*), consisted of DSPC/Chol/Amino-PEG lipid [Fun] or Methoxy-PEG lipid [Con] (4:1:0.05 mol/mol/mol) and were loaded with heparin [HEP] or not [Empty].

2.6. Mophological studies

The morphology of platelets deposited (platelet adhesion, activation and spreading) on the different surfaces was investigated after PRP/surface contact for 1 h or 72 h. Plain SS surfaces, or plasma-coated ones, or plasma coated surfaces on which Empty-NLs or HEP-NLs were immobilized (Fun or Con), were evaluated. After surface and PRP contact the surfaces were washed 3 times with saline and conditioned appropriately for visualization.

For scanning electron microscopy (SEM) a JEOL Scanning Electron Microscope at 20 kV (JSM 6300, JEOL, USA) was used and specimens were fixed in 1.5% glutaraldehyde solution, dehydrated in graded ethanol series and covered with a 20 nm layer of gold using a sputter coater.

In confocal scanning laser microscopy (CSLM) studies, platelet activation was evaluated by determining the expression of platelet glycoprotein CD62P. CD62P is a 140 kDa glycoprotein located in alpha and dense granules of platelets. Activation of platelets results in mobilization of CD62P from storage granules to the cell surface while resting platelets show no surface staining for the molecule. CD62P expression was visualized using FITC conjugated mouse monoclonal antibodies to CD62P (Abcam, UK) and observing the surfaces using a Nikon C1 Confocal microscope (Nikon, Japan) equipped with an epifluorescent microscope (Nikon TE 2000 Eclipse, Nikon, Japan).

2.7. Statistical analysis

All results are expressed as mean ± SD from at least three independent experiments. The significance of variability between results from various groups was determined by one-way analysis of variance. When appearing in the graphs, (*) denotes significance at $p = 0.05$ and (**) at $p = 0.01$.

3. Results

3.1. Physicochemical properties of heparin-loaded liposomes

The physicochemical characteristics of HEP-liposomes consisting of DSPC/Chol/Amino-PEG lipid (20:10:0.3 mol/mol/mol) and prepared by various techniques are presented in Table 1. As seen, DRV liposomes have the highest heparin loading, however their mean diameter is over 10 μm. As mean vesicle size decreases after extrusion through 400 nm pore membranes (Extrusion Big) and then through 100 nm pore membranes (Extrusion Small), so does heparin loading; however the vesicles extruded through 100 nm pore membranes have 2.3 times higher heparin loading compared to SUV liposomes. Thereby it was decided to use the extruded-DRV technique for preparation of HEP-NLs in the following experiments.

When DSPC was replaced by PC, or when Methoxy-PEG lipid was used in place of Amino-PEG lipid there was no change in heparin loading, however when the Chol amount included in NL composition was lowered to half (from 2:1 to 4:1 [mol/mol] lipid/chol), there was a slight increase in heparin loading, as seen in Table 2 (from 8.6 to 10.3 g heparin/mol lipid).

During incubation in buffer the retention of heparin in extruded-DRV NLs (at a final lipid concentration of 1 mg/ml) was found to be high (above 90% of initially loaded amount) during a 24 h incubation period for all the lipid compositions evaluated (Fig. 1), with the exception of plain PC liposomes (with no PEG lipid) in their composition, which released 40% of initially loaded heparin after 24 h.

Concerning the integrity of the NLs during incubation in blood, it was decided to evaluate only Chol-containing liposomes, which are known to have increased integrity during incubation in presence of serum proteins (Antimisiaris, 2011). As seen in Fig. 2, NLs composed of PC/Chol/Methoxy-PEG lipid (4:1:0.05) release higher heparin amounts compared to those composed of DSPC/Chol/Methoxy-PEG lipid (20:10:0.3). Indeed, the first type of NLs release heparin linearly with time after the first day of incubation, reaching approx. 12% release after 3 days and 18% after 5 days, while at the same 5-day period the second type of liposomes release only about 6% of the initially entrapped heparin. Both of the NL types studied were

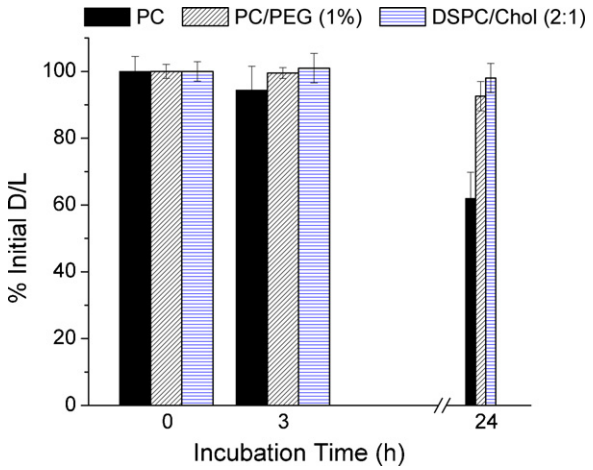

Fig. 1. Retention of heparin in HEP extruded-DRV NLs following incubation in PBS buffer pH 7.40. Lipid compositions of liposomes tested: PC, PC/Methoxy-PEG lipid (30:0.3 mol/mol) (shown as PC-PEG (1%)) or DSPC:Chol (2:1 mol/mol).

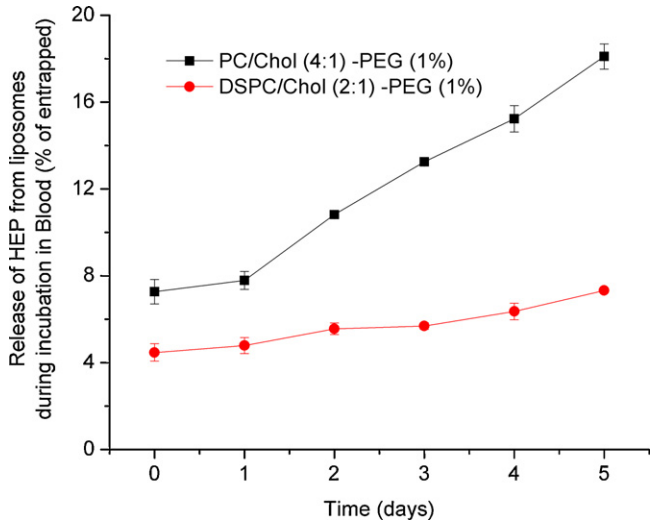

Fig. 2. Heparin release kinetics from Con-HEP NLs consisting of PC/Chol/Methoxy-PEG lipid (40:10:0.5 mol/mol/mol) [seen as PC/Chol (4:1)-PEG (1%)] and DSPC/Chol/Methoxy-PEG lipid (20:10:0.3 mol/mol/mol) [seen as DSPC/Chol (2:1)-PEG (1%)] during incubation in presence of blood at 37 °C (calculated as described in detail in Sections 2 and 2.3.2). Each experiment was repeated at least 3 times and the standard deviation of the mean is presented as errors bars.

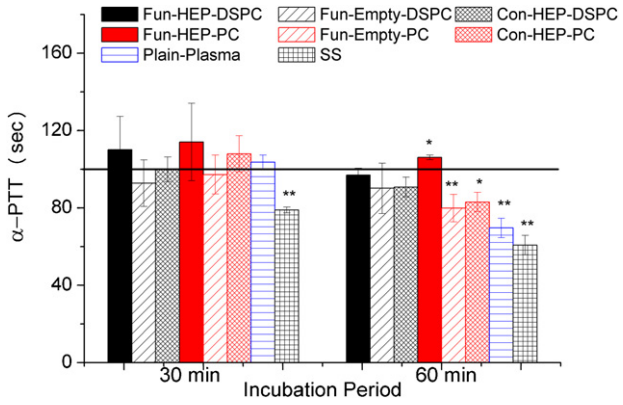

Fig. 3. Blood coagulation time after contact with various types of surfaces (plain SS, Plasma processed, or coated with HEP or Empty NLs) for 30 or 60 min periods. The plasma-processed surfaces were either studied as such (plain plasma) or after immobilization of Empty or heparin-loaded (HEP) liposomes. The liposomes used were PC/Chol (2:1 mol/mol) [PC] or DSPC/Chol (2:1 mol/mol), containing 1 mol% of Methoxy-PEG lipid (Con) or Amino-PEG lipid (Fun), for non-specific or covalent (specific) NL binding on surfaces, respectively. Coagulation of reference blood (without contacting any material) was set as 100, and all other values were adjusted accordingly for easy comparison between experiments. Each experiment was repeated at least 3 times and the standard deviation of the mean is presented as errors bars. The sample key appears in the graph insert.

seen to release a low percent of heparin, ranging from 4 to 8% of the initially encapsulated amount, at time zero, something similar to a "burst effect", which is most possibly due to rapid release of the heparin amount which is absorbed on the liposome surface. This seems to be readily released when the liposomes are diluted in the incubation media. In fact this amount would be probably released from the surfaces on which NLs are immobilized, during the washing protocol applied immediately after the coating procedure (see Section 2.3 above), and should not be taken into account in any relevant calculations or comparisons.

The above presented results, concerning the release rate of heparin from the HEP-NLs should be taken into account in order to explain the effects of NL-coating on the haemocompatibility of surfaces.

3.2. Activation of the coagulation system

In the first (screening) experiment carried out, blood coagulation time was measured after it was brought in contact for 30 or 60 min with various types of surfaces (plain SS, plasma-processed, or plasma-processed and coated with various types of NLs) (Fig. 3). The values were normalized to the reference value (coagulation time of the blood on its own) which was set as 100 (appears as a horizontal line in the figure for direct comparison with sample values). As seen, the 30 min period of material/blood contact was not sufficient to give any significant modulation of blood coagulation time due to the high variability of the measurements realized in most cases, with the exception of the SS surface (non-plasma-processed) which decreased blood coagulation time by 25 percent ($p < 0.01$). However, after 60 min of material/blood contact, almost all of the surfaces studied were found to result in significant reductions of blood coagulation time (indicating poor haemocompatibility) with the exception of the two surfaces on which HEP-NLs were (covalently) immobilized. The surface with DSPC/Chol NLs gave a coagulation time very close to the reference value (non-significant difference), while the one with PC/Chol NLs increased the blood coagulation time by 6% ($p < 0.05$). Thereby, it is indicated that covalent immobilization of HEP-NLs on the SS surface results in increased haemocompatibility. Additionally, the results presented in Fig. 3 (for 60-min material/blood contact time) demonstrate that: (i) contact with the non-processed SS surface decreased the blood coagulation time by 35%; (ii) Plasma-processing results in slight (but not significant) increase of surface haemocompatibility (compared to non-treated surface); (iii) all liposome-coated surfaces (Con or Fun) have significantly increased haemocompatibility compared to non-plasma processed SS surfaces.

In view of the results of the haemocompatibility screening experiment presented above, a second study was designed using longer material/blood contact periods and HEP-NLs which were composed of PC/Chol (4:1) (which were demonstrated to release heparin faster compared to the NL-types used in the first-screening experiment [Fig. 2]). As seen in Fig. 4, the surface which was coated with Fun-HEP NLs demonstrated a significant increase of blood coagulation time, after 3 days (72 h) of incubation with blood. Indeed, the blood coagulation time was increased more than

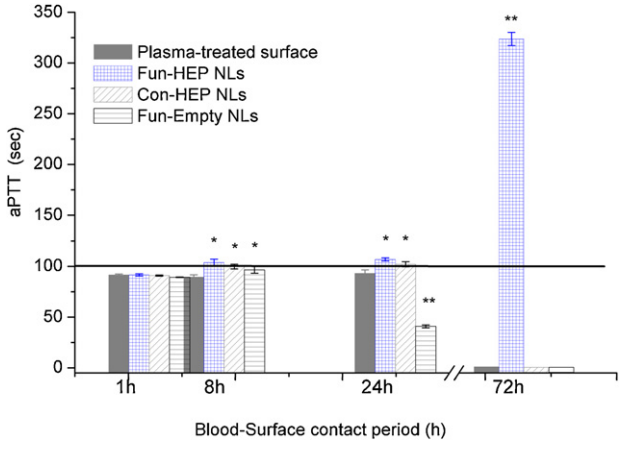

Fig. 4. Blood coagulation time (normalized to reference blood values) after contact with various types of surfaces for varying time periods. Surfaces were coated with Empty or heparin-loaded (HEP) liposomes, as presented in the graph insert. In all cases the liposomes used were PC/Chol (4:1 mol/mol) containing 1 mol% of mPEG lipid (Con) or Amino-PEG lipid (Fun) for non-specific or covalent (specific) NL binding on surfaces, respectively. Each value is the mean, from at least 4 treated surfaces and error bars are standard deviations. Coagulation of reference blood (without contacting any material) was set as 100 (s), and all other values were adjusted accordingly for easy comparison.

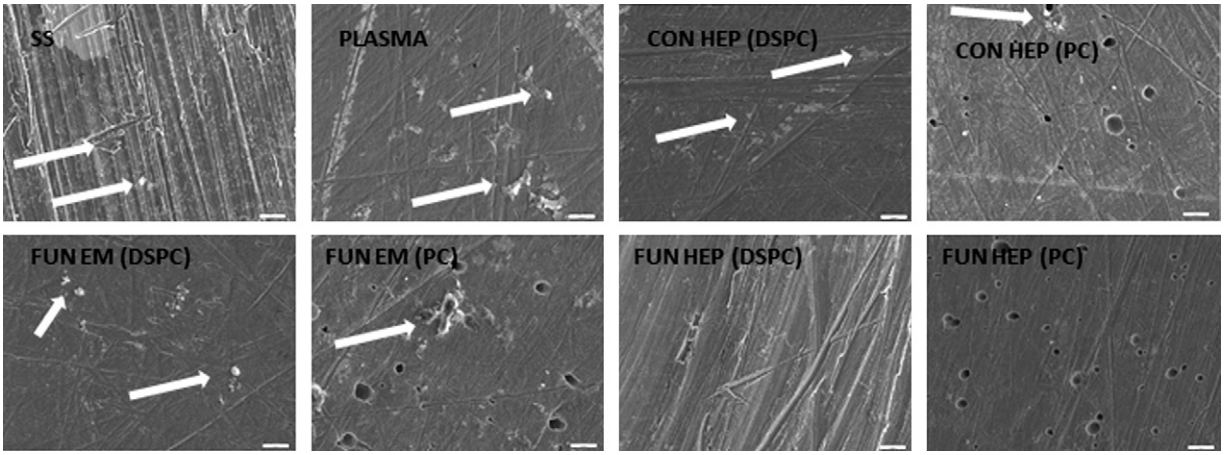

Fig. 5. SEM morphological evaluation of platelet adhesion on surfaces after 1 h contact with PRP. The surfaces were incubated with PRP, before (plain surface, SS) or after processing for immobilization of heparin-loaded (HEP) liposomes (PLASMA), as presented in the graph labels. The liposomes used were PC/Chol (2:1 mol/mol) [PC] or DSPC, containing 1 mol% of mPEG lipid (CON) or Amino-PEG lipid (FUN), for non-specific or covalent (specific) immobilization of the liposomes on the surface, respectively. The micrograph bar corresponds to 20 μm. Adhered platelets (indicated by arrows) appear as either white circular spots or are spread-out on the surface (indication that activation has been initiated).

3 times (compared to plain blood value). In all the other cases examined, the blood was already clotted during the 72 h period of material/blood contact. This result correlates nicely with the rate of heparin release from the specific NL type used (Fig. 2), which starts to release heparin after 24 h of incubation with blood and releases approximately 6 percent of the loaded HEP, after 3 d (or 72 h) of incubation.

4. Morphological studies

The morphology of the various types of surfaces after they were incubated with blood was studied using SEM, in order to detect surface-attached platelets and evaluate their morphology. Adhered platelets may appear on the surfaces if the surfaces are not haemocompatible, since platelet adherence is the first step for blood clotting. The adhered platelets appear as either white circular spots or as spread-out areas on the surface. In the last case the "spreading out" of the platelets indicates that platelet activation has been initiated. As seen in Fig. 5, in which representative micrographs from all the experiments carried out are presented, in almost all of the surfaces visualized by SEM, several platelets have been deposited on them (pointed-out by arrows). In some cases, as for example in the SS or PLASMA samples, it is clear that most of the adhered platelets are also activated. Only on the surfaces which were specifically coated with HEP-NLs, there were no visible adhered platelets.

Similar pictures were also realized, when the surfaces were treated with FITC-conjugated mouse monoclonal antibodies to CD62P in order to detect CD62P glycoprotein expression on the surfaces with a higher sensitivity. As mentioned in the methods section, the expression of CD62P indicates the presence of activated platelets. After treatment with the FITC-tagged MAb, activated platelets were observed in all the surfaces (Fig. 6). Nevertheless, the number of fluorescent spots on the Fun-HEP NL-coated surfaces was significantly reduced, compared to all other surface-types evaluated and especially on the Fun-HEP PC NLs, almost no fluorescent spots were visible. As mentioned above, within the time period of blood-surface incubation used in this study (1 h) only a minimum amount of heparin would be released from the HEP-NLs explaining why the surface haemocompatibility was just slightly improved.

In Fig. 7, a morphological repetition of the second blood coagulation time experiment (presented in Fig. 4) was carried out, using PC/Chol (4:1 mol/mol) NLs to immobilize on the SS-surfaces, and a

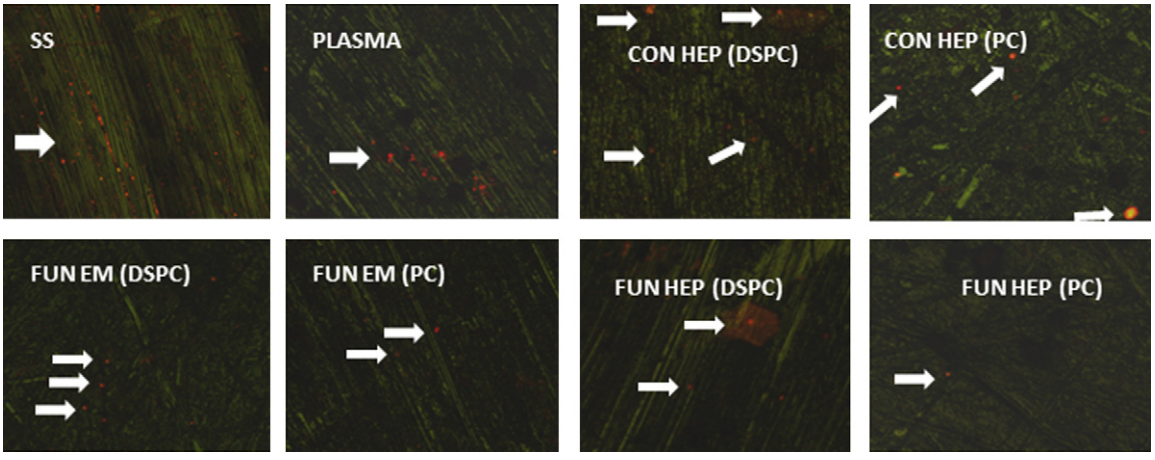

Fig. 6. Confocal laser scanning microscopy (CLSM) of stainless steel surfaces (SS), that have been incubated in presence of PRP for 1 h, before (SS) or after processing with plasma (PLASMA), and (in some cases) additional immobilization of Empty (EM) or heparin-loaded (HEP) liposomes, as presented in the graph insert. The liposomes used were PC/Chol (2:1 mol/mol) [PC] or DSPC, containing 1 mol% of mPEG lipid (CON) or Amino-PEG lipid (FUN), for non-specific or covalent (specific) immobilization of the liposomes on the surface, respectively. Red spots (some emphasized by arrows) correspond to activated platelets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

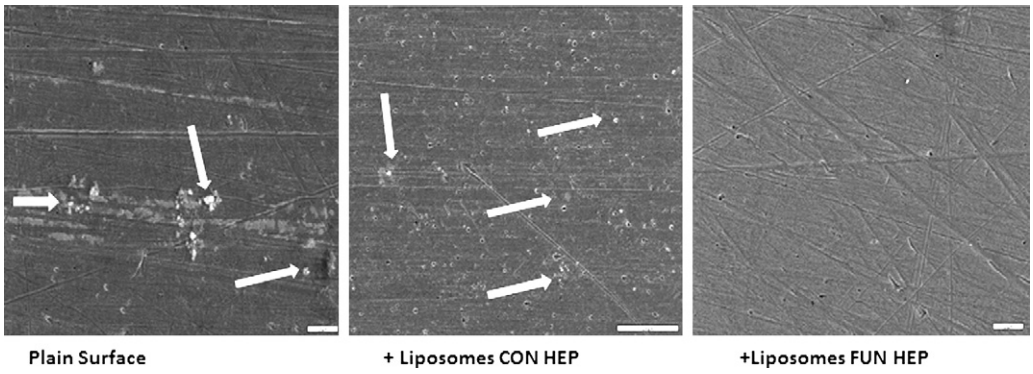

Fig. 7. SEM morphological evaluation of surfaces after contact with blood. The surfaces were incubated with PRP for 72 h, before (plain surface) or after processing for immobilization of heparin-loaded (HEP) liposomes, as presented in the graph labels. In all cases the liposomes used were PC/Chol (4:1 mol/mol) containing 1 mol% of mPEG lipid (CON) or Amino-PEG lipid (FUN), for non-specific or covalent (specific) binding of liposomes on the surface, respectively. The micrograph bar corresponds to 20 μm. Adhered platelets (indicated by arrows) appear as either white circular spots or spots which are spread-out on the surface (after activation).

longer blood/surface contact period (72 h). As seen from the representative micrographs of the three different types of surfaces evaluated in this experiment, the difference in haemocompatibility (expressed in this case by the number and geometrical characteristics of surface-adhered platelets) between the surfaces is clear, suggesting that the haemocompatibility of SS surfaces can be significantly improved by covalent immobilization of heparin releasing NLs, achieved via plasma processing.

5. Discussion

Stent thrombosis with BMS or DES may result in death or acute myocardial infarction (Ong et al., 2005). Platelet activation and thrombus formation in addition to the risk of artery occlusion can also trigger In-Stent Restenosis (ISR) (Richter et al., 1999). Thereby, it is critical to avoid thrombus deposition following the implantation of stents. To this end, several approaches have tried to generate modified stent-surfaces, by physical or chemical grafting of hydrophilic polymers, such as heparin, hydrophobic heparin or phosphorylcholine (Whelan et al., 2000; Mehran et al., 2003; Lee et al., 2007) with limited success.

Recently, dual-drug eluting stents (DDES) (Huang et al., 2009, 2010), which release two agents concurrently, usually one antiproliferative and one anti-thrombotic drug, are under investigation, with promising initial results. In line with the later technology, in this study we evaluate the potential of preparing drug eluting devices by covalently immobilizing drug-loaded-liposomes on biomaterial surfaces. This platform technology could be applied for immobilization of different active substances (by using different or the same vesicles) the release kinetics of which could be modulated (by modulating the physiochemical properties of the liposomal carriers) according to therapeutic needs.

Using heparin as a model active substance, heparin-loaded-NLs were constructed and covalently immobilized on plasma-processed surfaces. For this, anchoring -COOH groups were initially deposited on SS-316 surfaces (by pdAA) and used as "handles" to react with the (complementary) NH_2 groups on the surface of functionalized NLs, as recently demonstrated (Mourtas et al., 2011). As proven by the experiments carried out, when the HEP NLs immobilized on the surfaces release heparin during the material/blood contact period, the heamocompatibility of the surface is significantly increased (compared to plain SS or plasma processed surfaces). Following a first screening study, in which short material/blood contact periods were evaluated, a second study was conducted using a prolonged contact period (3 days), during which a sufficient amount of heparin was released from the NLs, to result in a therapeutic heparin concentration. Indeed, it is estimated that approx. 60 μg of lipid (58.4 ± 8.6) will be immobilized per cm^2 of surface, meaning that a 6 percent release of heparin (percent released after 72 h according to results presented in Fig. 3 [after subtraction of the burst release]) from the NLs, corresponds to a concentration of approx. 0.01 units heparin per ml of blood. This calculation explains the substantial increase in blood coagulation time in the case of Fun-HEP NL-coated surface (Fig. 4) as well as the fact that no platelets were observed on this specific type of surface following its incubation in blood (Fig. 7). In fact, the haemocompatibility increase or antithrombotic effect of each surface was demonstrated to be well correlated with the heparin release rate from the specific NL-type immobilized onto it.

Thereby, it is proven that by controlling the drug (or active substance) release from the surface (by immobilizing different liposome types) maximum therapeutic outcome can be achieved, and additionally that the proposed methodology may be used as a platform technology for construction of controlled release dual drug (or drug and other active substances) eluting metallic stents or other types of (metallic) medical devices with improved haemocompatibility and performance, by using different types of liposomes for each drug (depending on the specific application). Finally, this approach may also be useful for construction of stents to treat various types of cancer-related stenosis (Moon et al., 2011).

Compared to other technologies currently marketed or under investigation for construction of drug-eluting or dual-substance-eluting surfaces (or devices) (Kennedy et al., 2011), the currently proposed platform technology has the advantage of using liposomes which are completely biocompatible active substance carriers. As proven by the current results, the duration of drug release can be controlled/extended by using different liposome types (slow and fast drug-releasing vesicles can be mixed, if required). Furthermore, since the proposed liposomal-drug immobilization technique is a two-step process, it is envisioned that plasma-processed stainless steel surfaces or stents can be initially constructed and at a second step (in a clinical setup) liposomal drugs can be selected for immobilization, depending on the specific therapeutic need (providing thus possibilities for individualized therapy).

6. Conclusion

The proposed technology may be considered as a platform technology, with different types of potential applications in therapeutics and biomaterials.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2012.04.057.

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