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Heparin incorporating liposomes as a delivery system of heparin from PET-covered metallic stents: Effect on haemocompatibility

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Abstract

We investigate the possibility of coating polymer-covered stents with heparin-encapsulating liposomes for improving their haemocompatibility. Thin-film hydration (for multilamellar vesicles, MLV), and the dehydration–rehydration vesicle (DRV) methods are used for preparation of low-molecular weight heparin (LMWH)-encapsulating liposomes with varying lipid compositions. Liposomes are characterized for LMWH encapsulation and retention. For measurement of LMWH, a chromogenic technique is adjusted. For evaluation of heparin release from vesicles in platelet poor plasma (PPP) coagulation time is measured in presence of liposomal samples. Results reveal that LMWH encapsulation in liposomes is higher in DRV, however compositions with high encapsulation are leaky during buffer incubation. Most liposomes release LMWH slowly during plasma incubation (retention after 24 h ranges between 74% and 95%). Concerning the haemocompatibility of polyethylene terephthlate-covered stents after coating with LMWH-encapsulating liposomes, there is a marked increase (higher for DRV-coated stents compared to MLV) in plasma recalcification time compared to the control (plain blood) and reference (non-coated stent), which increases with blood–material contact time. This is probably due to LMWH release, demonstrating that encapsulated LMWH retains its biological functionality. Interestingly, the DRV-coated stents retained a high plasma recalcification time and a large number of liposomes on the stents (as proven by SEM studies) even after extensive washing (high shear conditions), proving that this method may be functional under high flow applying in vivo conditions.
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1. Introduction

Several problems are related with the placement of stents as a mean to prevent restenosis following percutaneous transluminal coronary angioplasty (PTCA) [1–7], some of which are attributed to the biocompatibility of the materials used for preparation of stents [5–8] and/or the fact that stents have been demonstrated to be thrombogenic and lead to erythromatous emboli or induce neointimal proliferation, as recently reviewed [9]. A proposed method to correct stent related complications non-invasively, is the local delivery of therapeutic agents [7–11]. Different drugs have been delivered on stents, after being either dispersed or encapsulated in polymeric materials. However, the polymers used to cover stents may cause additional problems, as the intimal thickening observed with low-molecular weight PLLA-coated stents [8].

Previously, we demonstrated the applicability of liposomal formulations of dexamethasone as a coating system for polymer (polyethylene terephthlate (PET) or PTFE)-covered stents, in order to achieve sustained release of drug at the site of interest [10,11]. By modifying liposome type and size we may incorporate different amounts of drug in liposomes, while modulation of liposome membrane rigidity may result in different release kinetics of the encapsulated drug. This way, the release rate of active substances at the desired site can be adjusted for maximum therapeutic benefit.

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Herein we use heparin to improve stent haemocompatibility. Heparin, as an anticoagulant can prevent the thrombus formation and embolization observed after blood contact with the surface of polymer-coated stents, and improve the stent haemocompatibility [6,12]. Surface coating of liposomes with heparin has been shown to increase their in vivo stability and blood compatibility [13], while heparin-modified stent grafts have superior haemocompatibility (compared to unmodified ones) [6]. Therefore, we investigate here the possibility of coating polymer (PET)-covered metallic stents, with liposomal heparin.

The preparation of heparin-encapsulating liposomes has been studied previously [14], however the effect of lipid composition and liposome preparation method on the encapsulation efficiency and retention of low-molecular weight heparin (LMWH) in vesicles, was not studied. Thereby, we prepared different types of heparin-encapsulating liposome, measured the encapsulation efficiency of LMWH and evaluated their stability, in buffer and plasma, as well as their ability to be used for coating stents. Finally, we evaluated the blood compatibility of liposome-coated stents in comparison with reference stents (non-coated), before and after extensive washing of the coated stents under high shear conditions, in order to see if this approach may be functional under in vivo applying flow conditions. Complement activation and morphological studies by SEM were also performed.

2. Materials and methods

2.1. Materials

Vascular endoprosthesis—PET-covered stents were used (Boston Scientific Vascular MA, USA). Human platelet poor plasma (PPP) produced from human blood, was provided from volunteers at the University Hospital (Rio, Patras, Greece). Blood was collected in CTAD (citrate/theophyline, dipyridamole) 9:1 v/v (Diagnostica Stago, France) tubes and centrifuged (5000 rpm, 15 min) to obtain PPP. Reagents for the determination of activated partial thromboplastin time (aPTT) (Kaolin-aPTT) were obtained from Diagnostica Stago, France.

Phospholipids [PC (phosphatidylcholine), DPPC (di-palmitoyl-PC), DSPC (di-stearoyl-PC) and PG (phosphatidylglycerol)] were purchased from Lipid Products (Nutfield, UK) and Avanti Lipids (USA).

2.2. Liposome compositions used and preparation techniques

Different types of heparin encapsulating liposomes were prepared; dehydrated-rehydrated vesicles (DRV) and multilamellar vesicles (MLV) using the following lipid compositions: PC:Chol (1:1), PC:Chol (2:1), PC:PG:Chol (9:1:5), DSPC, DSPC:Chol (1:1), DSPC:Chol (2:1), DSPC:PG:Chol (9:1:5), DPPC:Chol (1:1), DPPC:Chol (2:1) and DPPC:PG:Chol (9:1:5).

For both types of liposomes the thin film hydration method was initially used. Lipids (13 μmol) were solvated in a chloroform/methanol (2:1) solution and then evaporated until a thin film was developed. Residual organic solvents were removed by nitrogen, and the film was hydrated with 1 ml of heparin solution (10 000 ppm) in TBS (Tris buffered saline) buffer, pH 7.40 (for the MLV liposomes) or 1 ml of TBS buffer (for DRV liposomes). After this: (i) For MLVs the liposome dispersion was sonicated for 15 min in a bath type sonicator. (ii) For DRV liposomes [15], sonicated unilamellar vesicles (SUV) were prepared by probe sonication and 1 ml of SUV dispersion was mixed with 1 ml of heparin solution (10 000 ppm). The mixture was freeze-dried and then rehydrated.

In all cases, annealing of structural defects followed, by incubation for 1 h at a temperature above the lipid Tm, and separation of liposome encapsulated heparin from free heparin was accomplished by centrifugation at 20 000 rpm.

2.3. Liposome characterization and stability

2.3.1. Liposome sizing

With 20 ml of filtered TBS buffer, pH 7.40, 50 μl of the liposome dispersions were diluted, and sized immediately by photon correlation spectroscopy (Malvern Instruments, Model 4700C). Measurements were made at 25 °C with a fixed angle of 90° and sizes quoted are the *z*-average mean (d*z*) for the liposomal hydrodynamic diameter (nm).

2.3.2. Retention of heparin in liposomes during incubation in buffer or plasma

For calculation of LMWH encapsulation efficiency and stability of liposomes, the phospholipid content of the vesicles prepared was always determined by the Stewart assay [16].

Heparin concentration in liposomes was measured after adjusting a colorimetric method [17] (for measurement 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 the cation of dimethyl methylene blue. Dye solution is prepared by dissolving 16 mg in 1 l of distilled water with 2.37 g NaCl and 3.04 g glycine and adjusting pH to 3.0 with HCl. For the standard curve, heparin solutions between 5 and 100 ppm (in TBS buffer pH 7.4) were measured (OD 525 nm). The applicability of this method to measure heparin encapsulated in liposomes was proved by the linearity of calibration curves prepared in presence of empty liposomes and detergent Triton X-100 ($R^2 = 0.9996$).

For the determination of heparin in presence of PPP, a kit (CKPREST Diagnostica Stago, France) for determination of the Kaolin-aPTT, was used. The kit consists of reagent 1 (kefalin [platelet substitute] from rabbit brain) and reagent 2 (pH adjusted dispersion of Kaolin 5 mg/ml containing sodium azide as preservative). Reagent 2 is transferred to reagent 1 and mixed until the mixture becomes homogenous.

Vesicle membrane integrity was studied by measuring liposome encapsulated heparin release during incubation in buffer or PPP at 37 °C, under mild agitation. In the case of buffer incubation, the released heparin is separated from liposomes by centrifugation, and then heparin is measured as described above.

2.4. Stent coating and retention of liposomes on stents

Stents were coated with heparin-encapsulating liposomes as previously in our lab [10,11]. In brief, a 1 cm long stent piece was placed in a test tube (1 cm diameter) together with heparin-encapsulating liposomes, enough to cover the stent, which is subsequently evaporated under vacuum at 40 °C by gentle rotation, until the test tube is dry. After this the yield of coating is evaluated by measuring the lipid and heparin present on the stent, and comparing them with initial amounts used. In all cases, loses were equal to the amounts remaining in the test tube. Retention of lipid and heparin was measured after emerging the stents in a home made recirculating system under flow conditions [10,11]. Two different flow rates were evaluated: 2.4 and 5.0 ml/min.

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

The activation of blood-coagulation system was evaluated for reference (non-coated stent) and liposome-coated stents by measuring plasma recalcification time (aPTT). In both cases, stents were put as part of the capillary perfusion system described previously [18,19] and whole anticoagulated (CTAD-Diagnostica Stago, France) blood taken from healthy

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