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IN FOCUS: NANOMEDICINE - ARTICLE

# Development of a Platform of Antibody-Presenting Liposomes

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**Abstract** Antibody-presenting liposomes present high interest as drug delivery systems. The association of antibodies to liposomes is usually realized by covalent coupling of IgGs or their antigen-binding fragments to lipid polar head groups by means of hetero-bifunctional cross-linkers. We present here an original platform of IgG-presenting liposomes which is based on a fusion protein between Annexin-A5 (Anx5) and the IgG-binding ZZ repeat derived from *Staphylococcus aureus* protein A. The Anx5ZZ fusion protein acts as a bi-functional adaptor that anchors IgGs to liposomes in a non covalent and highly versatile manner. The interactions between IgGs, Anx5ZZ and liposomes were characterized by PAGE, dynamic light scattering and fluorescence quenching assays, establishing that binding of Anx5ZZ to IgGs and of Anx5ZZ–IgG complexes to liposomes is complete with stoichiometric amounts of each species. We found that the sequence of assembly is important and that Anx5ZZ–IgG complexes need to be formed first in solution and then adsorbed to liposomes in order to avoid aggregation. The targeting capacity of Anx5ZZ–IgG-functionalized liposomes was demonstrated by electron microscopy on an ex vivo model system of atherosclerotic plaques. This study shows that the Anx5ZZ adaptor constitutes an efficient platform for functionalizing liposomes with IgGs. This platform may present potential applications in molecular imaging and drug delivery.

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## 1 Introduction

Drug delivery systems are nanoparticles made of lipid and/or polymer assemblies, carrying a compound to a site of interest in the body [1, 2]. A major class of drug delivery systems is made by liposomes, which consist of hollow containers limited by a lipid bilayer [3, 4]. Liposomes are biocompatible and able to transport a wide range of compounds, either in their hydrophilic aqueous compartment or in their lipophilic limiting membrane [5, 6]. In order to allow efficient drug delivery in vivo, liposomes must present long circulation time and accumulate in desired tissues or organs. This is achieved with liposomes of small size, around 100-nm diameter, coated with an inert layer of hydrophilic and biocompatible polymers, usually made of poly-ethylene-glycol (PEG) moieties [7–9]. The addressing of liposomes to a desired site is realized either by passive targeting, that is by accumulation in areas with enhanced permeable vasculature, via the so-called enhanced permeability and retention effect [10], or by active targeting via the attachment of a homing device to the liposome [11].

Several types of cell recognition molecules have already been coupled to liposomes to confer them the property of active targeting, from small molecules like folic acid to large macromolecules like antibodies [12]. Antibodies, mostly IgGs or their fragments, have received special

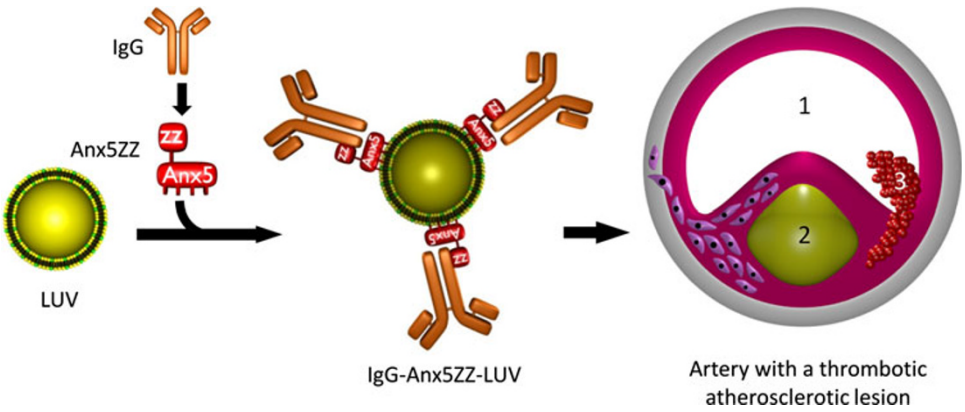


**Fig. 1** Scheme of surface functionalization of liposomes (LUV) with Anx5ZZ–IgG complexes, and targeting of the atherosclerotic plaque (*1* vessel lumen, *2* plaque, *3* thrombus)

interest because they can recognize and bind virtually any molecular target with high specificity and high affinity. The association of IgGs to liposomes is usually realized by chemical conjugation via hetero-bifunctional crosslinkers on the polar head of lipid molecules modified with a PEGylated reactive group [13]. This strategy of covalent coupling presents however several drawbacks, principally the lack of control of the site of protein modification which involves commonly their most accessible and/or most reactive protein amino groups, so that their molecular recognition properties may be altered due to chemical modification and/or non-optimal orientation [14]. The only method which ensures a favorable IgG orientation and preserves their binding domains involves IgG anchoring via carbohydrate residues located in their Fc domain to lipid molecules exposing hydrazide groups; this strategy presents however a low efficiency [15]. Furthermore, chemical modification of proteins requires often conditions which may be detrimental for protein integrity, such as low or high pH or the use of oxidants. Additionally, extensive purification steps are needed to exchange media or remove un-conjugated elements, which explains why protein chemistry requires large amounts of material and is time-consuming.

The overall aim of this study was to explore an original strategy for linking IgGs to liposomes in an oriented manner, in an entirely non covalent, yet specific, approach. An anchoring platform for IgGs is used, which consists of a fusion protein between Annexin-A5 (Anx5) and the IgG-binding ZZ repeat from *Staphylococcus aureus* protein A [16–18]. Anx5 binds membranes containing negatively charged phospholipids with extremely high affinity, as evidenced by $K_D$ values of $10^{-12}$ M or lower for phosphatidylserine (PS) containing liposomes at mM $Ca^{2+}$ concentrations [19]. The ZZ domain, which is derived from the B domain of *S. aureus* protein A, binds strongly to the Fc domain of IgGs. The Anx5ZZ fusion protein acts therefore as a bi-specific adaptor that can link IgGs to liposomes, in a simple plug-and-use fashion (Fig. 1). The proposed strategy presents several potential advantages: (1) the Anx5ZZ adaptor protein was constructed with the ZZ domain exposed on the face of Anx5 opposite to its membrane-binding face, so that Anx5ZZ proteins combine properties of membrane binding and IgG binding [20]; (2) this construction ensures an optimal orientation of IgG molecules [20, 21] so that their antigen-binding domains are exposed towards their target epitopes; (3) this system is in principle versatile, allowing the functionalization of liposomes with IgG subtypes that are recognized by protein A.

In the present study, the construction of liposomes functionalized with complexes between Anx5ZZ and IgGs is described and these assemblies are characterized using various biochemical, physico-chemical and structural methods. We demonstrate the capacity of targeting of Anx5ZZ-based immunoliposomes, ex vivo, in a model system of atherosclerotic plaques.

## 2 Experimental

### 2.1 Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (DOPE-Rh) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were of ultrapure grade. Water was purified with a RiOs system (Millipore, St Quentin en Yvelines, France).

### 2.2 Buffer Preparation

A HEPES buffered saline (HBS) solution made of 10 mM HEPES, 150 mM NaCl, 2 mM $NaN_3$ was prepared in ultrapure water. The pH was set at 7.4, except when otherwise stated.

### 2.3 Liposome Preparation

Liposomes were prepared by lipid film hydration [22] followed by extrusion, as briefly described here. Lipids were dissolved in chloroform, mixed in desired amounts and the solvent was evaporated to dryness using a rotary-evaporator during 20 min. Lipids were hydrated in HBS, resulting in multilamellar vesicles (MLVs) and homogenized by three cycles of freeze-thawing and subsequent vortexing. Large unilamellar vesicles (LUVs) were obtained by hand extrusion of the resulting lipid dispersion, passing the mixture twenty times through 0.1 μm polycarbonate filters with a LiposoFast extruder (Avestin, Sodexim, France). LUVs were composed of DOPC/DOPS (70/30, mole ratio) or DOPC/DOPS/DOPE-Rh (69/30/1 mol ratio) for fluorescence quenching experiments. Phospholipid concentration was determined by phosphate analysis according to Rouser [23].

Magnetoliposomes made of DOPC/DOPS (70/30, mole ratio) and containing an average of 40 iron oxide particles per liposome were prepared as described in Garnier et al. [24]. Briefly, a lipid film made of DOPC/DOPS (70/30, mole ratio) was hydrated with a suspension of citrate-coated iron oxide particles containing 2.4 mol iron/L (equivalent to $1.9 \times 10^{19}$ part/L). Liposomes were extruded successively through 200 and 100 nm filters. Non-entrapped maghemite particles were removed in two steps, first by 0.4 M NaCl induced particle precipitation and second by centrifugation at 4,500*g* for 3 min. The supernatant was submitted to size exclusion chromatography, using a 1.5 × 30 cm Sephacryl S-1000 column (Amersham Bioscience, Buckinghamshire, UK) pre-saturated with lipids by passing 10 mL of a 10 mg/mL lipid solution in HBS, in order to prevent non specific binding and loss of magnetoliposomes on the column. Magnetoliposomes were eluted with HBS supplemented with 10 mM trisodium citrate. Fractions containing the magnetoliposomes were pooled and concentrated by magnetic chromatography, by application onto a MACS Column (LS Column, Miltenyi Biotech, Bergisch Gladbach) rendered magnetic by application of a magnet (Supermagnete, Gottmadingen, Germany). The retained liposomes were washed with 10 mL HBS supplemented with 10 mM trisodium citrate and retrieved in 0.5–1 mL after removal of the magnet and elution with HBS supplemented with 10 mM trisodium citrate. The number of iron oxide nanoparticles per liposome was deduced from the determination of the lipid and iron contents.

### 2.4 Preparation of Annexin Proteins

The Anx5ZZ fusion protein was produced by fusing rat Anx5 with the ZZ domain of protein A from *S. aureus* as described in Wolny et al. [21]. The Anx5ZZ-SH protein was produced by fusing Anx5-SH, produced as described in Bérat et al. [25], to a ZZ domain, as described for Anx5ZZ. To obtain Anx5ZZ-SH proteins, Anx5ZZ-S–S-ZZAnx5 dimers were reduced by incubation with 10 mM DTT in HBS at pH 6.3 for 30 min. Reduced Anx5ZZ-SH proteins were purified on a HiTrap desalting column (GE-Healthcare) equilibrated with HBS at pH 6.3.

### 2.5 Covalent Coupling of Fluorescein to Anx5ZZ-SH

A selected amount of Fluorescein-maleimide dissolved in HBS at pH 6.3 was mixed with freshly prepared Anx5ZZ-SH at an Anx5ZZ-SH/Fluorescein-maleimide molar ratio of 1/7 and incubated for at least 4 h at room temperature. The excess fluorophore was eliminated by purification on a HiTrap desalting column. The concentration of Anx5ZZ-Fluorescein (Anx5ZZ-Fluo) was determined by measuring the absorbance at 280 nm, using known Anx5ZZ concentrations as standard.

### 2.6 Binding of Anx5 Derivatives to Liposomes

Proteins derived from Anx5, namely Anx5ZZ or Anx5ZZ-Fluo, complexed or not with IgGs, were incubated for 30 min with DOPC/DOPS (70/30, mole ratio) liposomes in HBS supplemented with 2 mM $CaCl_2$.

### 2.7 Characterization of Non-Covalent Binding of IgGs to Anx5ZZ, by Non-Denaturing PAGE

A non-denaturing PAGE assay was used to characterize the binding of IgGs to Anx5ZZ. An IgG available in large quantity, namely anti-BSA mAb (Biogenesis, Mill Creek, USA), was used as reference. A fixed amount (2 μg) of Anx5ZZ was incubated with varying amounts of anti-BSA mAb, corresponding to (Anx5ZZ/IgG) molar ratios ranging from 1/0.5 to 1/3, for 30 min, in a total volume around 20 μL. The whole sample volume was deposited on a non denaturing polyacrylamide gel, using a 10% resolving gel. The time of incubation used for the formation of complexes between IgGs and Anx5ZZ corresponds to conditions of saturation, as determined by complementary experiments of Quartz Crystal Microbalance with Dissipation monitoring (data not shown).

### 2.8 Characterization of Anx5ZZ Binding to Liposomes, by Non-Denaturing PAGE

The binding of Anx5ZZ to liposomes was characterized by non-denaturing PAGE. A fixed amount (2.85 μg) of Anx5ZZ was incubated for 30 min with varying amounts (1–66.7 μg phospholipids) of DOPC/DOPS (70/30, mole

ratio) 50 nm LUVs, in HBS containing 2 mM $CaCl_2$, in a total volume around 20 μL. The whole sample volume was deposited on a non-denaturing polyacrylamide gel, using a 10% resolving gel. The gel and the electrophoresis buffer were supplemented with 2 mM $CaCl_2$, which is required for binding of Anx5ZZ proteins to liposomes. The time of incubation used for the formation of complexes between Anx5ZZ and liposomes corresponds to conditions of saturation, as determined by complementary experiments of Quartz Crystal Microbalance with Dissipation monitoring (data not shown).

### 2.9 Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were performed using an ALV laser goniometer equipped with a 22 mW HeNe linearly-polarized laser operating at a wavelength of 632.8 nm and an ALV-5000/EPP multiple $\tau$ digital correlator with 125 ns initial sampling time. Liposome suspensions were maintained at a temperature of $25.0 \pm 0.1$°C in all experiments. The counting time was 300 s for each sample. Samples contained 200 μg phospholipids in a total volume of 900 μL, pH 7.4. All measurements were performed at three scattering angles (50°, 90°, 120°). The presented results correspond to 90° scattering. Data were analyzed using ALV Correlator Control software.

### 2.10 Binding of Anx5ZZ and Anx5ZZ–IgG to Liposomes, by Fluorescence Quenching

For fluorescence experiments, 30 μg of rhodamine-labelled liposomes (DOPC/DOPS/DOPE-Rh, 69/30/1, mole ratio) were mixed with 1.125 μg Anx5ZZ-Fluo, with or without 2 mM $CaCl_2$, in a total volume of 400 μL HBS.

All measurements were realized with a Perkin Ellmer LS55 (Burckinghamshire, UK) at $\lambda$exc = 490 nm. The emitted intensity was recorded between 500 and 650 nm with excitation slit of 2.5 nm and emission slit of 6.5 nm.

### 2.11 Rabbit Model of Atherosclerosis

Animal experiments were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and were accredited by the local ethical committee. Adult male New Zealand rabbits (NZW), weighing between 2.5 and 3.0 kg, were obtained from Charles Rivers Laboratories (St Germain sur l'Arbresle, France). Rabbits were submitted for 6–8 months to a fat atherogenic diet including 0.3% (w/w) cholesterol. To promote the development of complicated plaques, rabbits were subjected to an inflammatory surgical injury under anesthesia (intramuscular injection of 20 mg/kg ketamin and 2 mg/kg xylazin) 1–2 months after beginning the diet. De-endothelialization of the thoracic and abdominal aortic areas was mechanically induced three times by inflation and retraction of a 4F Fogarty balloon catheter (Edwards Lifesciences, Maurepas, France). Rabbit anesthesia was maintained by mask inhalation of 0.25 to 0.35% isoflurane, and preventive anti-thrombotic treatment was given with heparin sodium solution (1,000 IU/mL) (Heparin Choay®, Sanofi Synthelabo, Paris, France). Analgesia was performed in the presence of 100 mg of aspirin (Injectable Aspegic®, Sanofi Synthelabo).

### 2.12 Ex vivo Targeting of Thrombus with XIIF9-Functionalized Magnetoliposomes

For labelling activated platelets from atherosclerotic plaques, we used the murine monoclonal antibody (mAb) XIIF9 directed against the $\beta$3 subunit of the $\alpha$IIb$\beta$3 integrin [26].

Magnetoliposomes made of DOPC/DOPS (70/30, mole ratio) were functionalized with complexes between Anx5ZZ and XIIF9 mAbs. The complexes between Anx5ZZ and XIIF9 mAbs were formed by mixing 7.5 μg Anx5ZZ to 44.9 μg mAb, incubated for 30 min, then the mixture was added to 200 μg magnetoliposomes and further incubated for 30 min. These conditions correspond to 120 mAbs per liposome. The times of incubation used for the formation of complexes correspond to conditions of saturation, as determined by complementary experiments of Quartz Crystal Microbalance with Dissipation monitoring (data not shown).

For ex vivo labelling experiments, balloon-injured aortas from hypercholesterolemic rabbits were extracted from the aortic arch to the iliac bifurcation, washed and fractioned. A 1-cm artery portion was incubated for 60 min in HBS supplemented with 2 mM $CaCl_2$ with 200 μL of a 1 mg lipid/mL solution of XIIF9-functionnalized magnetoliposomes. Afterwards, the artery was washed with the same buffer in order to remove unbound liposomes, and the sample was processed for ultramicrotomy according to standard procedures. Briefly, 2–3 mm long pieces of artery were fixed for 2 h in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.2 M cacodylate buffer (pH 7.4). The samples were post-fixed for 1 h at 4°C with 1% osmium tetroxide in the same buffer and were dehydrated with ethanol before embedding in Epon–Araldite. Thin sections (65 nm thickness) were stained successively with 5% uranyl acetate and 1% lead citrate.

### 2.13 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was performed with a TECNAI CM120 (FEI) microscope operating at

120 kV under low electron dose conditions. Images were recorded with a USC1000 slow scan CCD camera (Gatan).

## 3 Results

### 3.1 Binding of IgGs to Anx5ZZ

To characterize the formation of complexes between Anx5ZZ and IgGs, a non denaturing PAGE assay was used. Anx5ZZ and IgG proteins have different molecular weights (about 50,000 Da for Anx5ZZ and 150,000 Da for IgGs), so that they are easily distinguished on polyacrylamide gels (Fig. 2, lanes 1–2).

Figure 2 presents a typical experiment of complex formation between Anx5ZZ and a reference anti-BSA IgG. When a fixed amount of Anx5ZZ was mixed with increasing amounts of IgGs, the amount of Anx5ZZ decreased, indicating the formation of complexes between Anx5ZZ and IgG (Fig. 2, lanes 3–8). The entire amount of Anx5ZZ was complexed for an (Anx5ZZ/IgG) molar ratio of about 1/2. The value of two IgGs per Anx5ZZ was expected as each Z domain of the ZZ tag is able to bind one IgG molecule.

### 3.2 Binding of Anx5ZZ to PS-Liposomes

The binding of Anx5ZZ to PS-exposing liposomes was monitored by PAGE in non denaturing conditions. Figure 3 presents the results of an experiment in which a fixed amount of Anx5ZZ (2.85 μg) was incubated, in the presence of 2 mM $Ca^{2+}$, with varying amounts of DOPC/DOPS (70:30, mole ratio) liposomes (1–66.7 μg lipids). The intensity of the Anx5ZZ band decreased when the amount of liposomes increased, as expected because Anx5ZZ binds to liposomes and, due to their large size, liposomes are retained at the top of the gel. The Anx5ZZ band disappeared totally for a total amount of liposomes

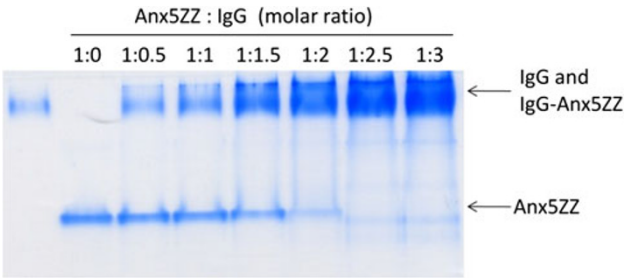


**Fig. 2** Quantitative analysis of the formation of complexes between Anx5ZZ and IgGs, by non denaturing PAGE. *First lane* 1 μg pure anti-BSA IgG. *Following lanes* a fixed amount of 2 μg Anx5ZZ was incubated for 30 min with varying amounts of IgG (2–18 μgs). The amounts of IgG are expressed as the Anx5ZZ/IgG molar ratio

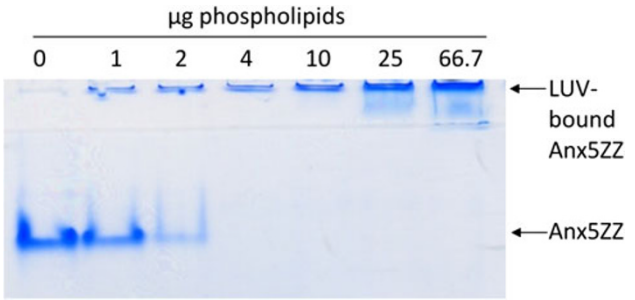


**Fig. 3** Quantitative analysis of the binding of Anx5ZZ to liposomes, by PAGE. A fixed amount of 2.85 μg Anx5ZZ was incubated 30 min with varying amounts of DOPC/DOPS liposomes (1–66.7 μg phospholipids)

corresponding to 100% theoretical membrane coverage, considering a projected molecular area of Anx5ZZ of 30 $nm^2$ [24]. This result indicates that complete binding of Anx5ZZ is achieved when the amount of liposomes corresponds to a membrane area larger than 100% theoretical coverage.

### 3.3 Functionalization of Liposomes with IgGs Via the Anx5ZZ Adaptor

Two strategies were envisioned for functionalizing liposomes with IgGs, via the Anx5ZZ adaptor protein. One strategy consisted in binding Anx5ZZ to liposomes and then adding IgGs. In the second strategy, complexes between Anx5ZZ and IgGs were formed in solution, prior to liposome addition. These strategies were evaluated by DLS, which provides information on sample dispersity.

Liposomes presented a single peak by DLS, indicating that liposome samples consisted of well dispersed vesicles with a radius of 54 nm (Fig. 4a), as expected. After addition of Anx5ZZ (60 Anx5ZZ/liposome, corresponding to 6% surface coverage), the peak profile was unchanged, with a radius of 57 nm (data not shown), indicating that Anx5ZZ binding does not affect vesicle dispersity. When IgGs were added to the LUV–Anx5ZZ suspensions, with an (Anx5ZZ/IgG) molar ratio of 1/2, a broad peak was observed by DLS, ranging from 500 nm to 7 μm, with an average radius of 1.5 μm (Fig. 4b). This result indicates that the binding of IgGs to Anx5ZZ induced aggregation of the Anx5ZZ-liposomes, certainly because IgG molecules are dimeric and can bind two Anx5ZZ.

On the other hand, when Anx5ZZ–IgG complexes were first formed in solution, at an (Anx5ZZ/IgG) molar ratio of 1/2, and then bound to liposomes, the obtained DLS peak was narrow, with a radius of 67 nm (Fig. 4c). The difference of radius observed, namely 54 and 67 nm, confirms the binding of Anx5ZZ complexes to liposomes, while the DLS signal indicates that samples remained monodisperse.

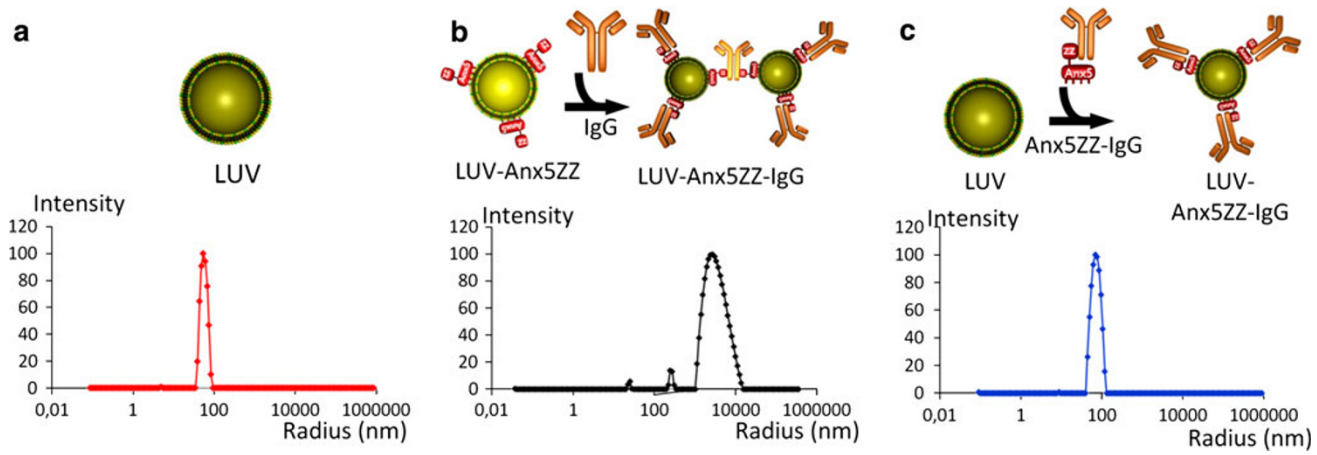


**Fig. 4** Dispersity of liposome suspensions, by DLS. Size distribution profiles determined by DLS at 90° for **a** (DOPC:DOPS) liposomes (70:30, mole ratio); **b** liposomes obtained after sequential binding of Anx5ZZ (60 Anx5ZZ/liposome, 6% of surface coverage), and then IgGs (120 IgGs/liposome); **c** liposomes obtained after binding of preformed Anx5ZZ–IgG complexes (60 Anx5ZZ–120IgG per liposome)

In conclusion, the order in which liposomes are functionalized with IgGs via the Anx5ZZ adaptor protein has a strong influence on the liposome dispersity, which is a critical parameter for drug delivery applications.

### 3.4 Binding Efficiency of Anx5ZZ–IgG Complexes to Liposomes, by Fluorescence Quenching

As our objective was to exploit the high affinity of Anx5 for PS-exposing membranes, we wondered whether Anx5ZZ–IgG complexes presented also a high affinity for PS-exposing membranes. This question was addressed by comparing the binding behavior of Anx5ZZ and Anx5ZZ–IgG to liposomes, by a fluorescence quenching assay.

We selected conditions corresponding to 60 Anx5ZZ molecules per liposome, because we showed previously that, in the case of 100 nm diameter liposomes, a ligand density of 60 to 120 proteins per liposome ensures maximal targeting efficiency towards model membrane systems [27]. This Anx5ZZ quantity corresponds to 6% of surface coverage, a density for which binding of Anx5ZZ is complete, as shown above. For fluorescence quenching experiments, we used Anx5ZZ labelled with one fluorescein per molecule, referred to as Anx5ZZ-Fluo, and liposomes labelled with a rhodamine-lipid (DOPC/DOPS/DOPE-Rh, 69/30/1, mole ratio), referred to as LUV-Rh. Figure 5 presents the fluorescence emission spectra of a mixture of Anx5ZZ-Fluo and LUV-Rh upon excitation at 490 nm. In the absence of $Ca^{2+}$, Anx5ZZ-Fluo are not bound to LUV-Rh and the emission spectra consisted of an intense peak at about 530 nm corresponding to fluorescein emission and a weaker peak at about 590 nm corresponding to rhodamine emission (Fig. 5a, dotted line). This points out that some emission from rhodamine dye takes place upon excitation at 490 nm, even though this is not the optimal excitation wavelength of rhodamine. After addition of 2 mM $Ca^{2+}$, which promotes binding of Anx5ZZ-Fluo to LUV-Rh, the intensity of the Anx5ZZ-Fluo peak decreased significantly (Fig. 5a, solid line). This indicates that upon binding of Anx5ZZ-Fluo to LUV-Rh, the two fluorescent dyes become close enough for energy transfer to occur, which results in a quenching of fluorescein emission.

Figure 5b shows that the results obtained with preformed Anx5ZZ-Fluo–IgG complexes (Anx5ZZ-Fluo/IgG, 1/2, mole ratio) are almost identical to those obtained with Anx5ZZ-Fluo, indicating that almost the same quantities of Anx5ZZ-Fluo bound to liposomes in both conditions.

This experiment indicates that Anx5ZZ–IgG complexes present a high affinity for PS-containing liposomes.

### 3.5 Targeting of Thrombus Associated with Atherosclerotic Plaques with Anx5ZZ–XIIF9 Functionalized Magnetoliposomes

To evaluate the capacity of IgG-functionalized liposomes to target specific cell receptors, we selected the system made of ruptured atherosclerotic plaques. The antibody used for liposome functionalization, called XIIF9 mAb, is directed against the $\beta$3 subunit of $\alpha$IIb$\beta$3 integrin, which is expressed by activated platelets present in thrombi observed in ruptured atherosclerotic plaques [26]. In order to detect liposomes in TEM, we used magnetoliposomes loaded with colloidal iron oxide particles [24], which give a characteristic signal in TEM images (Fig. 6f).

Magnetoliposomes were functionalized with preformed Anx5ZZ–XIIF9 complexes, namely 60 Anx5ZZ and 120 XIIF9 mAbs per liposome. Figure 6a presents a section of an atherosclerotic plaque (2) with an associated thrombus (3). TEM images of the thrombus area show the presence

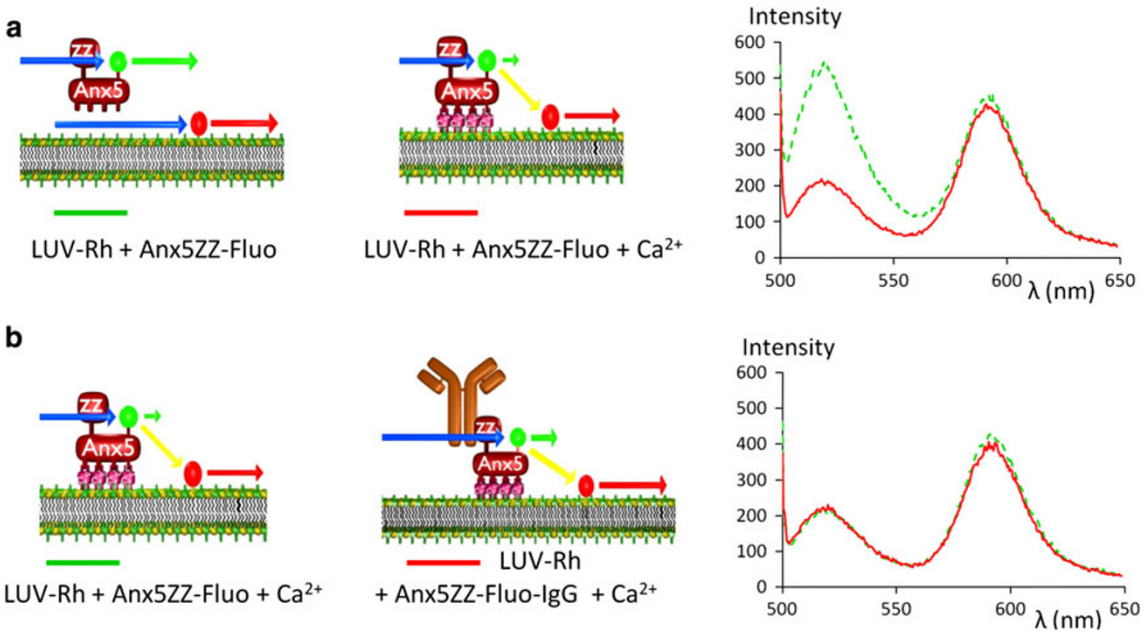


**Fig. 5** Efficacy of binding of Anx5ZZ–IgG complexes to liposomes, by fluorescence quenching. Fluorescence emission spectra of Anx5ZZ-Fluo (60 Anx5ZZ-Fluo/liposome) and LUV DOPC:DOPS:-DOPE-Rh (69:30:1, mole ratio) (LUV-Rh) at $\lambda$ excitation = 490 nm. **a** Comparison of spectra obtained before (*dashed lines*) and after (*continuous lines*) $Ca^{2+}$-dependant binding of Anx5ZZ-Fluo to LUV-Rh (60 Anx5ZZ/liposome); **b** comparison of spectra obtained after binding of Anx5ZZ-Fluo (*dashed lines*) and Anx5ZZ-Fluo-IgG (*continuous lines*) to LUV-Rh (60 Anx5ZZ–120 IgG/liposome)

of activated platelets, characterized by their numerous granulations (Fig. 6b). Objects exhibiting a characteristic granular structure are observed next to activated platelets (Fig. 6d, e red arrows). Control images of pure magnetoliposomes (Fig. 6f) establish that the objects observed in Fig. 6d, e are magnetoliposomes. The presence of magnetoliposomes in thrombus areas was not observed when anti-$\alpha$IIb$\beta$3 XIIF9 mAbs were replaced by isotype antibodies (Supplementary material). These results demonstrate that liposomes functionalized with anti-$\alpha$IIb$\beta$3 IgGs via the Anx5ZZ adaptor were able to recognize and bind firmly their target, at least in ex vivo situation.

## 4 Discussion

This study describes a platform of IgG-presenting liposomes based on Anx5ZZ, a bi-functional protein that acts as an adaptor between the liposome surface and the Fc domain of antibodies. This original approach is based on two affinity interactions and constitutes an alternative to commonly used approaches in which IgGs are covalently coupled to liposomes.

Annexin-A5 was chosen as membrane-anchoring moiety due to its high affinity towards anionic membranes in the presence of mM $Ca^{2+}$, while the ZZ tag was chosen for its high affinity and specificity for the Fc domain of IgGs. Both reactions, namely Anx5 binding to liposomes and ZZ binding to IgGs, are characterized by $K_D$ values in the pM or nM range [28, 29]. The strong binding of both Anx5 and ZZ moieties towards their recognition elements allows the formation of complexes by simple mixing of µg quantities, corresponding to tens of picomoles, in near stoichiometric amounts, followed by a short—less than 1 h—incubation period.

In practice, the monodispersity and size of drug delivery systems are critical parameters that determine their clearance behavior in vivo, hence aggregation must be avoided. We found that when liposomes were first functionalized by Anx5ZZ and then by IgGs, aggregation was observed by DLS. This is probably due to the fact that IgGs have a dimeric nature and may interact with ZZ moieties bound to different vesicles, thus creating bridges between liposomes. On the other hand, when Anx5ZZ–IgG complexes were formed first in solution and then adsorbed onto liposomes, IgG-functionalized liposomes remained monodisperse.

In order to characterize the binding efficiency of Anx5ZZ to IgG and Anx5ZZ–IgG complexes to liposomes, we used PAGE and fluorescence quenching assays, respectively. Each Anx5ZZ was shown to bind two IgGs, in agreement with the fact that Anx5ZZ contains a tandem Z repeat. The affinity coupling of IgGs to ZZ domains presents several major advantages; not only it is highly efficient and complete, but it involves un-modified IgGs, avoids possible alteration by chemical modification, and maintains an optimal orientation of IgGs towards their

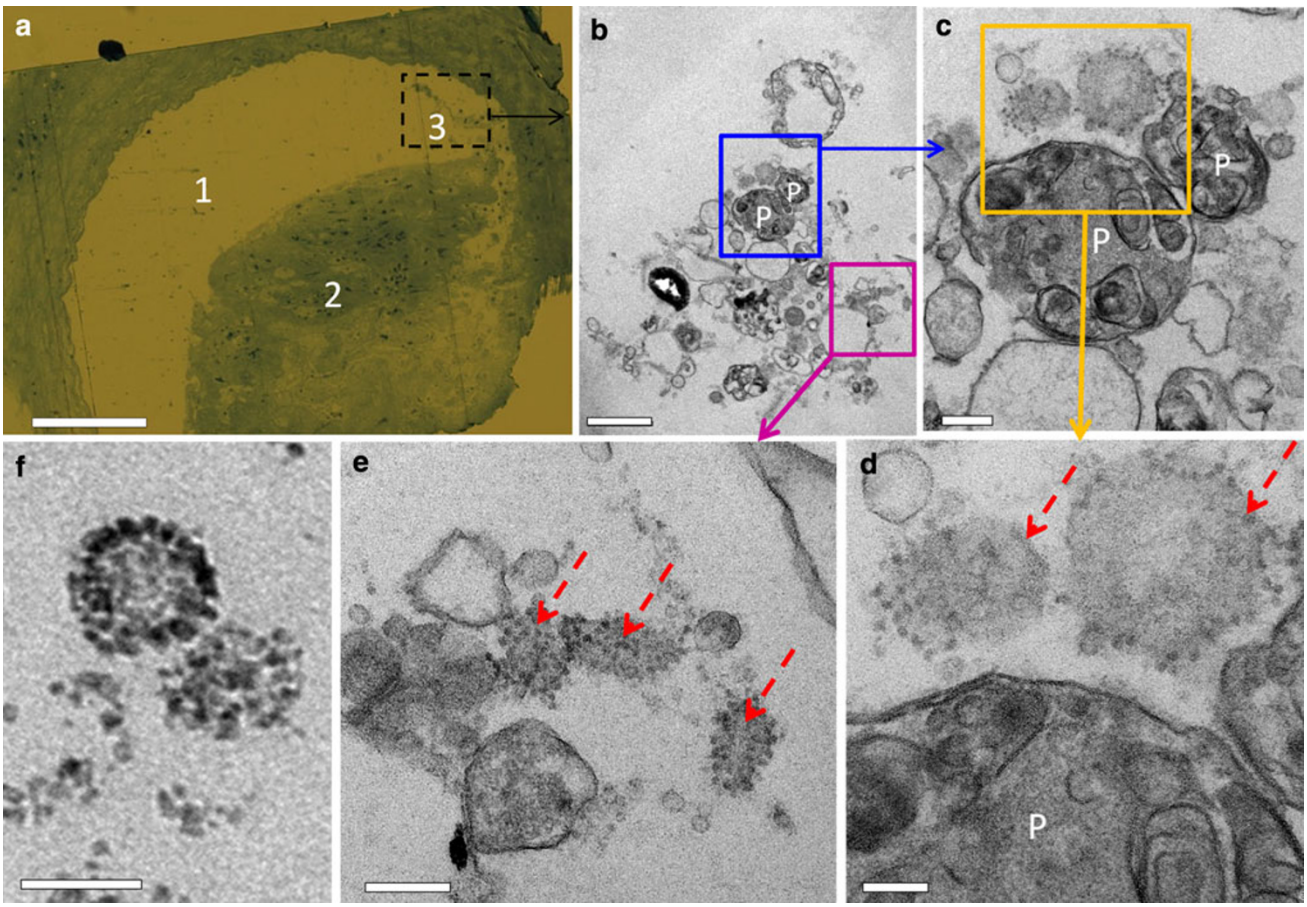


**Fig. 6** Targeting of atherosclerotic plaques with XIIF9-functionalized magnetoliposomes. **a** Optical microscopy image of a thick section of a rabbit femoral artery showing the vessel lumen (*1*), an atherosclerotic plaque (*2*) and a thrombus area (*3*, *dotted line* surrounding); **b**–**e** EM images of the thrombus area shown in (**a**), at increasing magnifications, showing platelets (*P*) constitutive of the thrombus and magnetoliposomes (*dotted arrows*); **f** TEM image of control magnetoliposomes embedded in resin. Characteristic electron-dense clusters are observed, closely associated in spheres. *Scale bars* **a** 100 μm; **b** 1 μm; **c**, **e** 200 nm; **d** 100 nm; **f** 50 nm

target site [20, 21]. In comparison, the covalent coupling of IgGs to lipid hydrazides via carbohydrate residues located in Fc domains constitutes another approach allowing the control of the IgG orientation, yet with low yield [15]. Another advantage of the non-covalent binding of Anx5ZZ to IgGs is that the reaction is complete in near stoichiometric conditions and thus does not require purification procedures, in contrast to most methods of protein derivatization which require an excess of crosslinkers in order to achieve a satisfactory yield, and consequently a purification step for eliminating un-reacted molecules.

Concerning the second step of the functionalization, the $Ca^{2+}$-dependent binding of Anx5ZZ–IgG to PS-containing membranes, Anx5ZZ was found to bind completely to anionic liposomes before total membrane coverage, in an almost irreversible reaction, as previously shown for Anx5 [19]. Anx5ZZ–IgG was also found to behave similarly to Anx5ZZ, binding quantitatively to anionic liposomes, at least at low surface coverage. The Anx5ZZ fusion protein constitutes therefore a simple, fast and versatile adaptor system for linking IgGs to liposomes, in a plug-and-use fashion. In comparison, several studies have reported variable coupling yields (from 100 to 10%) for the formation of immunoliposomes by covalent conjugation [15, 30–32], together with the need of excess lipid reactive groups to reach reasonable coupling rates.

Finally, we showed that IgG-presenting liposomes functionalized via the Anx5ZZ adaptor were able to reach their target, using the atherosclerotic plaque as a model system. In this example, magnetoliposomes functionalized with anti-αIIbβ3 mAbs were found to bind specifically to activated platelets constitutive of thrombi associated with atherosclerotic plaques. Due to the fact that thrombi are a hallmark of unstable plaques, these immuno-magnetoliposomes may be of potential interest as diagnostic tools for detection of atherosclerotic plaques at high risk of cardiovascular complications. The experiments presented here demonstrate the efficiency of the Anx5ZZ liposomal platform for ex vivo targeting. Several important issues have to be solved before similar platforms are used successfully in

in vivo applications, including the stability of the IgG–Anx5ZZ complexes in the presence of native antibodies or the capacity of the vectors to escape natural body filters.

## 5 Summary and Conclusions

This work describes an original strategy for coupling IgG antibodies to liposomes in a non covalent and versatile manner, by means of the Anx5ZZ adaptor fusion protein. The synthesis of IgG–Anx5ZZ-liposomes was shown to be simple, fast and efficient. The high binding efficiencies of Anx5 for negatively charged liposomes and of the ZZ domain for the Fc domain of IgGs ensure a quantitative attachment of IgGs to liposomes, with control of the IgG density, and prevent IgG waste and extensive purification steps. The addressing ability of IgG–Anx5ZZ-liposomes was demonstrated with the ex vivo targeting of thrombi sites from atherosclerotic plaques in a rabbit animal model. This strategy of producing immunoliposomes via the Anx5ZZ adaptor may present potential applications in molecular imaging and drug delivery.

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