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Secretory phospholipase A2 activity in blood serum: The challenge to sense

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ABSTRACT

Excess levels of secretory phospholipase A2 (sPLA2) is known to contribute to several inflammatory diseases including vascular inflammation correlating with coronary events in coronary artery disease. Thus a method to monitor sPLA2 activity in blood serum is urgently needed. Such method is still a challenge since existing fluorescent probes do not allow to monitor sPLA2 activity directly in blood serum. Here we analyze and overcome barriers in sPLA2 sensing methodology and report a fluorescent probe and a kinetic model of its hydrolysis by sPLA2. New probe is designed with a fluorophore and a quencher not interfering binding to the enzyme. At the same time phospholipid matrix bearing the probe promotes efficient initial quenching of the fluorophore. Kinetic model of probe hydrolysis takes into account signal change due to the side processes. The probe and the kinetic model applied together prove the concept that the activity of sPLA can be measured directly in blood serum.

1. Introduction

Secretory phospholipases A2 (sPLA2s) are a family of extracellular Ca^{2+}-dependent phospholipases, which catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position producing non-esterified fatty acids and lysophospholipids [1]. Multiple sPLA2 functions include and, probably, account for their role in pathophysiology of atherosclerosis. There is also a wealth of evidence suggesting sPLA2 is a biomarker of cardiovascular risk [1–5]. This has motivated the development of a set of kits for measurement of sPLA2 activity. In these kits the substrate, either fluorescently or radioactively labeled phospholipid, is hydrolyzed by the enzyme and the extent of decomposition is then monitored. Chromatography based methods are the most reliable in tracing the substrate hydrolysis since they measure unmodified substrate and hydrolysis products independently [6]. However, chromatography does not allow for monitoring substrate hydrolysis directly in serum because of the need for probe purification workup resulting in proportional time and information losses. Several kits were designed to be used with fluorescent microplate readers. Most often the kits are based on a lipid probe which bears a fluorescent label on one of the hydrocarbon tails and a quencher, on the other, for example, Red/Green BODIPY® PC from EnzChek kit (available from Life Technologies), in which quenching is based on fluorescence resonance energy transfer (FRET). The probe is incorporated into a phospholipid vesicular matrix often containing phosphatidylglycerol to promote enzyme binding to vesicular surface. While the probe is intact, the fluorescent dye and the quencher are located in a single molecule close to each other and the fluorescence is quenched. PLA2 cleaves either the dye or the quencher from the substrate, which results in fluorescence build-up.

To achieve reasonable quenching of a dye within the probe (and thus sensitivity of the assay), the distance between the dye and the quencher should be small. Careful analysis of commercial probes shows that quenching is efficient enough when the dye and the quencher are located not farther than at the fifth carbon atom of a hydrocarbon tail. Such probes demonstrate good performance in serum-free samples and are used to study isolated PLA2s from different sources [7]. The lowest PLA2 activity the assays report detected is 0.05 U/mL, which is significantly higher than the activity of sPLA2 in serum (below 0.01 U/mL). One possible reason for the low sensitivity of the assays is that the hydrophobic tails might

Abbreviations: BODIPY, boron-dipyrromethene; FRET, fluorescence resonance energy transfer; PC, phosphatidylcholine; PG, phosphatidylglycerol; sPLA2, secretory phospholipase A2.

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be too short to ensure strong enough binding of the probe in the enzyme active site on one hand and the dye and/or quencher at the extremity of the tails may interfere binding, on the other.

A probe with longer hydrocarbon chains binds the enzyme better, but the efficiency of dye quenching within the probe decreases. To overcome the problem, quenching should have stronger basis than FRET or dye-quencher collisions. In bis-BODIPY® FL C11-PC (available from Life Technologies), quenching is achieved through formation of dimers by BODIPY moieties. The energy of dimer formation is not high, but it increases the probability that two dye moieties within a single probe molecule in a bilayer stick together and thus are quenched. When the probe is hydrolyzed, the dyes become too far from each other in space to form a dimer. This smart methodology has a drawback that not only sPLA2 can interfere with the dimer formation. Among the 3000+ different proteins in serum, many have hydrophobic surfaces. Probe adsorption on these surfaces can affect the probability of dimer formation. Indeed, measured fluorescence response is complicated and does not follow the probe hydrolysis in serum [6].

Alternatively, hydrophobic surface of serum proteins can be used to regulate fluorescence of the probe. For example, an assay utilizing a pyrene monolabeled PC as a probe and albumin, rather than any phospholipid-based system, as a matrix has been reported [8,9]. In intact state the probe solely forms aggregates. Due to the high concentration of the dye inside the aggregates, fluorescence is quenched. It is assumed that aggregates do not interact with albumin (added as a reagent or with the serum). Upon hydrolysis with PLA2, pyrene labeled fatty acid is detached from the probe. The labeled fatty acid adsorbs on the hydrophobic surface of albumin, where it is no longer quenched by other pyrene residues. The approach is very productive, and its sensitivity is great. However, one should keep in mind that here PLA2 influences the signal indirectly. If something destroys the aggregates, fluorescence will increase without hydrolysis of the probe. In complicated environments, such as blood serum, this should be the case: the fluorescence signal, at least in part, should be determined by non-hydrolytic processes.

Thus, for a sensitive PLA2 assay, the challenges are (1) to design a probe structure which does not interfere with the enzyme binding site and (2) to develop a measurement methodology which would account for the effects of non-hydrolytic processes on the fluorescence signal. Below, we report a probe and a methodology which meet these challenges.

2. Materials and methods

2.1. Blood serum

The study was approved by the Ethics Committee of the Russian Cardiology Research and Production Complex. All patients provided written informed consent. Venous blood was taken from patients with coronary artery disease. Blood samples were centrifuged at 3000 rpm at 4 °C for 20 min. Serum was stored at −70 °C until use.

2.2. Fluorescent probe in vesicles

Probe synthesis is described in Supplementary materials, Section 3. Aliquots of lipids in organic solvent were mixed, evaporated and dried under high vacuum (40 min, 0.04 mmHg). Lipid films were hydrated in 0.1 M Tris–HCl, pH 8.8, 5 mM $CaCl_2$ and 0.1 M NaCl buffer. The suspension was sonicated for 20–30 min in an UM-1 ultrasound bath (Unitra-Unima). A set of formulations was tested as matrices for the probe (see Supplementary materials, Section 4, for details). Optimal composition contained phosphatidylcholine (ePC) and phosphatidylglycerol (ePG) from egg yolk and palmitoyl lysophosphatidylcholine (lyso-PC) (Sigma Aldrich); optimal molar ratio was found to be ePC/lyso-PC/ePG = 45/45/10 and probe/matrix molar ratio, 1/100.

2.3. Fluorescence measurement

Measurements were carried out in 96-well plates on a Fusion Alpha-FP HT multimode fluorescent microplate reader (Perkin Elmer) set at 485 nm excitation and 535 nm emission wavelengths. Final volume of reaction mixture in each well was 200 μL; kinetics of enzymatic reaction was followed by fluorescence detection every 5 min during 2 h.

Immediately before measurements, 20 μL serum (with unknown PLA2 activity x) and 10 μL bee venom PLA2 of known activity was added to 170 μL substrate solution. Thus, 200 μL final mixture contained the probe at final concentration of 0.91 μM, 10 vol% serum, and a PLA2 spike (eight concentration points, from $x + 0.02$ to $x + 0.8$ U/mL); three replicas of each point were prepared and analyzed.

2.4. Data analysis

Data were fitted with curves obtained by a mathematical routine written in python programming language. The routine fits simultaneously all experimental curves (x, $x + 0.02$, … $x + 0.8$ U) using the kinetic model which takes into account three simultaneous processes: escape of the probe from phospholipid vesicles and PLA2-driven hydrolysis of vesicle-associated and vesicle-free probe (see Section 3.2 for details). The routine yields PLA2 activity in serum x and rate constants for each of the three processes. The program code is placed in Supplementary materials (Section 5; open source) and is provided under GNU GPL (General Public License) which permits free usage, distribution and modification of the code for commercial and non-commercial purposes as long as the derived code is also open.

3. Results and discussion

3.1. Probe design

When challenged with fatty acids naturally occurring in human phospholipids, secretory PLA2 failed to show specificity for any particular fatty acid in the *sn*-2 position [10]. However, this may not hold true for fluorescently labeled or other modified fatty acids (e.g. halogenated phospholipids decrease sPLA2 activity [11].

Particularly, the active site of sPLA2 IIA is curved, so a substrate should either be flexible enough to adopt appropriate conformation or fit into the bend of the active site inherently. Indol-based sPLA2 inhibitors have indol and phenyl rings located in different planes to meet the curvature of the active site (see [12] and crystal structures, e.g. 1DB4 in RCSB Protein Data Bank; see also rendered image in Supplementary materials, Section 1). Another inhibitor has an ω-phenyl heptanoyl residue [13]. Carboxylic oxygen of this residue binds Ca^{2+} in the active site of the protein. Thus, the residue resembles the *sn*-2 acyl chain. It is bended near the C6 atom of the chain (see [13] and 1KQU in RCSB Protein Data Bank).

If *sn*-2 acyl chain contains a fluorescent dye or a quencher (which is also a bulky and rigid moiety) in the region of the first 10 carbon atoms of the chain, binding is disturbed due to the low ability of the substrate to adopt the required curvature. This is possibly the reason of low sensitivity of probes with quencher and/or fluorescent dye capping hydrocarbon tails. They simply do not bind effectively to the protein. To make the binding of fluorescent substrate to the protein as good as binding of natural

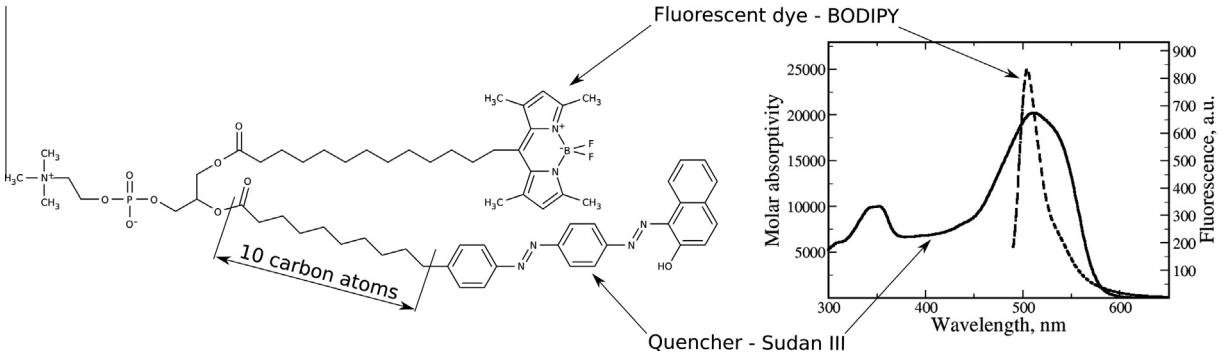

Fig. 1. Structure of the probe and dye emission–quencher absorption spectra overlap. Sudan III absorption (solid line, left axis) and BODIPY emission (dashed line, right axis).

phospholipids, the fluorescent dye should be located farther than 10 carbon atoms away from the beginning of *sn*-2 chain. In part this is supported by the data on cobra venom PLA2, which accommodates first 10 or so carbons of the *sn*-2 acyl chain of phospholipids in its catalytic site, where they are exposed to a very hydrophobic environment [14].

According to crystallographic structures of sPLA2 binding various inhibitors, *sn*-1 chain is not as buried into the protein as *sn*-2 chain is, but still is inside the binding site. Thus bulky dyes attached to *sn*-1 chain should also effect binding. In contrast to *sn*-2 chain, there is no data on how many carbon atoms of *sn*-1 chain are located inside the binding site. But it cannot be more than there is for *sn*-2 chain.

We designed a probe with sudan III quencher at the end of a 10 carbon atom-long *sn*-2 chain and a fluorescent BODIPY reporter group attached to the end of *sn*-1 chain, which is even longer than 10 carbon atoms (Fig. 1) to provide minimal if any restraints to binding. BODIPY, an acronym for boron-dipyrromethene, is a family of fluorescent dyes. The one used in this study—tetramethyl BODIPY—has maximum of absorption at 495 nm ($\varepsilon \sim 90{,}000\ M^{-1}\ cm^{-1}$) and a quantum yield of 0.95; its emission maximum is at 505 nm. Apart from the excellent brightness and photostability characteristics, BODIPY is a dye we are very well familiar with in terms of its behavior in lipid aggregates [15,16] (see Supplementary materials, Section 2).

Sudan III is a diazo dye traditionally used for staining of triglycerides in frozen sections. It has absorption maximum slightly above 500 nm. We have calculated its molar extinction coefficient to be $20{,}000\ M^{-1}\ cm^{-1}$ at 510 nm. Sudan III is a so called black quencher since it has no fluorescence and a good FRET quencher of BODIPY emission. Due to the strong overlap of absorption and emission spectra of sudan III and BODIPY, respectively (Fig. 1), the Förster distance for BODIPY–sudan III pair is as big as 51 Å. At this distance, a half of BODIPY emission should be absorbed by sudan III. On the other hand, the distance between geometrical centres of the fluorophore and quencher in the molecule in extended conformation (with chains being fully tilted) is about 40 Å. This is close to the Förster distance. Thus even with fluorophore and quencher being spaced with chains in extended conformation, the quenching efficacy should be high. Indeed, in chloroform/methanol solution the probe quantum yield was measured to be 0.49.

Quantum yield of the probe dramatically decreased if the probe binds to lipid bilayer. Playing with composition of lipid vesicles we found that the best quenching was achieved in lipid vesicles with low or moderate curvature, like 100 nm liposomes (see Supplementary materials, Section 4). In micelles (e.g. composed of short-tail dioctanoyl phosphatidylcholine or from pure lyso-PC) the decrease in quantum yield is almost negligible. Incorporation of lyso-PC (up to 50%) results in liposomal suspension of optimal size, even without the extrusion step, while keeping quantum yield low. In vesicles composed of ePC/lyso-PC, 1/1, with 10% of phosphatidylglycerol (to promote binding of PLA2 to vesicles) the quantum yield was measured to be 0.02. We named this phenomenon the *enhanced quenching in vesicles*. In solution the dye and the quencher can have any relative position in 3D space. In lipid layer relative position of the dye and the quencher is restricted to a 2D surface. This dramatically increases the probability of dye-quencher collisions. Lipid bilayer not only restricts relative position of dye and quencher, but orients them in space (see Supplementary Section 2). In the case of sudan III–BODIPY pair, this likely promotes *p–p* interactions between the two moieties, which leads to additional quenching, or enhanced quenching in vesicles.

3.2. Kinetic model of substrate hydrolysis

Interactions of numerous serum components with vesicles containing fluorescent probe are complex. In the model, we start from the fact that the process of interest—hydrolysis of a fluorescent lipid by PLA2—is accompanied by the destruction of vesicles in the presence of serum proteins, and both processes result in fluorescence build-up. Contribution of the latter process has been underestimated previously.

Before enzyme or serum are added to the vesicular system, there is only one population of a probe, that is, unhydrolyzed probe associated with vesicles, or fraction *A* (Fig. 2). Fluorescence of the probe in fraction *A* is strongly quenched according to enhanced quenching in vesicular carrier (quantum yield of the probe in fraction *A* is as low as 2%).

When PLA2 is added to the system (either purified or with serum), hydrolysis of the probe begins. Fluorescent lysolipid

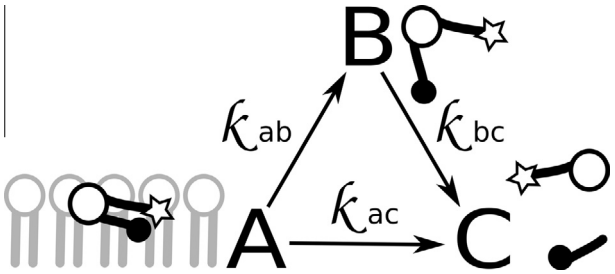

Fig. 2. Interaction of a fluorescent probe-containing vesicles with serum may be described as a complex process of transitions between three states of the system: state (A) fluorescent probe is in vesicles and fluorescence is strongly quenched; state (B) fluorescent probe is outside of the vesicles, possibly bound to serum proteins; fluorescence is partially dequenched; and state (C) quencher-free lysolipid is in solution; fluorescence is not quenched. Star represents reporter fluorophore; filled circle, a quencher.

emerges in the system. It can be associated with initial vesicles or with micelles of freshly formed lysolipids, or even remain free in solution. In any case, fluorescence is completely dequenched. Since quantum yield of the dye (BODIPY) does not depend on the polarity of the environment (in water and micelles the fluorescent lysolipid has a quantum yield of the pure dye, i.e. 95%), all forms of fluorescent lysolipid make up a single fraction, *C*. We describe the hydrolysis process as a formal first-order chemical reaction. Thus, fraction *A* transforms to fraction *C* with a rate constant k_{ac}:

$$A \xrightarrow{k_{ac}} C$$

Along with this, serum proteins destroy vesicles which carry the probe. The probe is released unhydrolyzed to form fraction *B*, which is also described by pseudo first order kinetics:

$$A \xrightarrow{k_{ac}} B$$

In fraction *B* the probe is either associated with serum proteins, like albumin, which have hydrophobic surface, or with micelles of freshly formed lysolipids, or free in solution. There is no enhanced quenching since there is no orienting effect of the lipid bilayer, so the distance between the dye and the quencher is random and the quantum yield of the probe is supposed to be the same as in the case of unhydrolyzed probe in solution, i.e. 49%.

The probe in fraction *B* can be further hydrolyzed by PLA2 to yield fraction *C* with a rate constant of its own:

$$B \xrightarrow{k_{ac}} C.$$

Assuming first order kinetics for all of the reactions above, corresponding differential equations are:

$$\frac{dA}{dt} = -k_{ab} \cdot A - k_{ac} \cdot A$$

$$\frac{dB}{dt} = k_{ab} \cdot A - k_{bc} \cdot B,$$

$$\frac{dC}{dt} = k_{ac} \cdot A + k_{bc} \cdot B$$

where *A*, *B* and *C* are concentrations of a probe in corresponding fractions. These equations can be solved analytically to yield concentrations of all fractions at time *t*:

$$A_t = A_0 \cdot e^{((-k_{ab}-K_{ac})\cdot dt)}$$

$$B_t = A_0 \cdot k_{ab} \cdot e^{-k_{ab}\cdot dt} \cdot \left(1 - e\frac{(k_{bc} - k_{ab} - k_{ac}) \cdot dt}{(k_{ab} + k_{ac} - k_{bc})}\right)$$

$$C_t = A_0 - A_t - B_t$$

During interaction of vesicles carrying fluorescent probe with serum, all three processes $A \to B$, $B \to C$ and $A \to C$ run simultaneously. Thus, in a sample there are three populations of the probe. The observed fluorescence intensity is proportional to a sum of signals provided by each population.

$$F \sim A_t \cdot q_A + B_t \cdot q_B + C_t \cdot q_c,$$

where q_A, q_B and q_C are corresponding quantum yields.

3.3. Experimental data and the model

Representative experimental data are shown in Fig. 3I. The curve shape is common for many reported assays utilizing fluorescent probes. Our data allow for calibration within the PLA2 activity range of 0.02–0.1 U/mL using the slopes of the initial regions of the curves. This surpassed sensitivity of the Red/Green BODIPY® PC from EnzChek kit with the lowest activity detected in serum to be 0.05 U/mL. However, due to the multiple effects of serum proteins on the vesicular system and the probe, such calibration curve requires at least five samples spiked with different amounts of PLA2 for each serum batch, which is not feasible, especially in clinical setting.

PLA2 activity in serum *x* calculated using the proposed model is in the range of 0–0.030 U/mL depending on the serum batch, which is also close to values reported in literature.

Independent reconstruction of the kinetics of three processes described in the model allowed for elucidation of the reasons for the failure of the fluorescent probe-based assays proposed thus far.

Before serum is added, the fluorescence intensity (with background subtracted) is proportional to C_A. Immediately after addition of the serum with PLA2 to the vesicles two processes ($A \to B$ and $A \to C$) start. After a while, when population *B* appears in the system, process $B \to C$ starts.

As follows from Fig. 3II and III, vesicles are almost completely destroyed during the first ten minutes. After that, no probe remains in fraction *A* of the system and the only process running is $B \to C$ (hydrolysis of the vesicle-free probe).

If compared to vesicle disruption ($A \to B$) and probe hydrolysis in vesicles ($A \to C$), the $B \to C$ process is rather slow, which is manifested through the small slope of the curves after 40 min of incubation (Fig. 3I). It also does not depend strongly on the concentration of the probe in fraction *B*. This is illustrated with almost parallel lines (Fig. 3I) in the 40–150 min interval for all PLA2 concentrations.

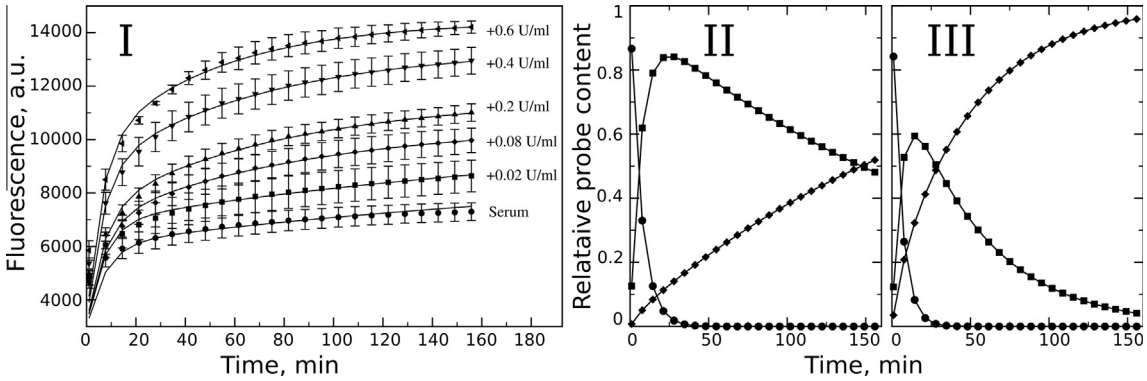

Fig. 3. (I) Fluorescence build-up after addition of blood serum supplemented with PLA2. Circles, serum; squares, serum +0.02 U/mL PLA2; diamonds, serum +0.08 U/mL PLA2; triangles up, serum +0.2 U/mL PLA2; triangles down, serum +0.4 U/mL PLA2; triangles left, serum +0.6 U/mL PLA2. Solid lines are the results of kinetic model fit to experimental data. (II and III) Relative content of the probe fractions *A* (circles), *B* (squares) and *C* (diamonds) in samples with 0.02 U/mL (II) and 0.6 U/mL PLA2 (III). At each time point, sum of all fractions is unity. Error bars represent standard deviations in three replica.

During a short time interval immediately after serum and PLA2 addition, the two processes, $A \rightarrow C$ and $A \rightarrow B$, run together. When PLA2 activity is low the $A \rightarrow B$ process dominates. For example, in a sample of serum supplemented with 0.02 U/mL PLA2, more than 84% of fraction *A* is converted to fraction *B* (Fig. 3II). Increasing PLA2 activity in serum makes $A \rightarrow C$ process run faster but still slower than vesicles destruction. With 0.6 U/mL PLA2 spike almost 60% of fraction *A* is still converted to fraction *B* (Fig. 3III). The activity of native sPLA2 in serum is ~0.01 U/mL, which is much lower than the activities at which the $A \rightarrow C$ process might affect considerably the fluorescence readings. This explains why calculated values *x* are too imprecise and outlines the next challenge in developing sPLA sensing assays – decreasing the speed of vesicles disruption should be brought into focus.

Long tail probe design, enhanced quenching effect and three-stage kinetic model allowed for investigation of interactions between PLA2-supplemented serum with vesicles carrying the fluorescently labeled phospholipid probes. It turned out that destruction of vesicles in non PLA2-mediated processes is very fast, competes with probe hydrolysis by PLA2 and interferes with sPLA2 activity determination. Probably, the same phenomenon has been observed by other researchers previously (e.g., [6]). The three-stage kinetic model can be used to take into account the side processes in determination of PLA2 activity. Moreover, the model can describe the behavior of other similar probes; for example, bis-BODIPY used by Lasch, since it has dyes located further than 10 carbon atoms from the glycerol backbone and they can form dimers in bilayer.

Further development of PLA2 assay will benefit from the enhanced quenching effect. Also, to increase vesicle stability in serum, pegylated lipids might be incorporated in the bilayer. The approach is already used to prolong life-time of drug-loaded liposomes in circulation [17]. However, care must be taken of the accessibility of the probe on vesicle surface to sPLA2.

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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.bbrc.2014.10.069.

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