Enhancement of Phospholipase A_2 Catalyzed Degradation of Polymer Grafted PEG-Liposomes: Effects of Lipopolymer-Concentration and Chain-Length

K. Jørgensen,[1,2,4] C. Vermehren,[2] and O. G. Mouritsen[1,3]

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INTRODUCTION

Incorporation of poly(ethylene-glycol)-phospholipids (PEG-PE) into liposomes significantly increases the intravascular circulation time without causing considerable leakage of incapsulated material to the blood stream. The use of PEG-liposomes as microcarrier drug-delivery vehicles furthermore involves major advances in therapeutic application due to an accumulation and release of encapsulated drugs from the PEG-liposomes in pathological tissue (1). Although PEG-liposomes have come into use as particulate drug-delivery systems with improved therapeutic profiles, the detailed mechanisms involved in the clearance and extravascular degradation are poorly understood (2–5). The stabilization and protective effect induced by the lipopolymers in the blood stream is generally considered to be the result of a repulsive steric barrier that prevents opsonization and degradation of the PEG-liposomes by blood proteins and phacocytic cells (1,4). However, very little is known about the mechanisms involved in the destabilization and release of incapsulated material after extravasation through gaps in the blood vessel walls and passive targeting of the PEG-liposomes to pathological tissue (1,2). Both the macroscopic phase behavior of liposomes as well as the lipid-membrane microstructure are of importance for the in vitro behavior and the in vivo fate and interaction of liposomes with biological components (6). In the present work we have investigated the activity of PLA_2 towards DPPC liposomes incorporated with submicellar concentrations of PE-PEG_{750}, PE-PEG_{2000}, and PE-PEG_{5000} lipopolymers. Above a certain lipopolymer threshold-concentration, that is determined by the length of the polymer chain, a lamellar to micellar transition of the lipid system will take place (7). PLA_2 belongs to a group of lipid-membrane active and liposome-degrading enzymes which are present in extra- and intracellular compartments of human tissue where they take part in a number of different physiological and pathophysiological processes (8,9).

[1] Department of Chemistry, Technical University of Denmark, DK-2800 Lyngby, Denmark.

[2] Department of Pharmaceutics, The Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark.

[3] OGM is an associate fellow of the Canadian Institute for Advanced Research.

[4] To whom correspondence should be addressed at Department of Chemistry, Technical University of Denmark. (e-mail: jorgense@kemi.dtu.dk)

ABBREVIATIONS: DPPC, dipalmitoylphosphatidylcholine; PLA_2; 14kD low molecular weight phospholipase A_2 from *Agkistrodon piscivoros piscivoros*; HPLC, high performance liquid chromatography, PE-PEG_{750}, polyethyleneoxide-distearoylphosphatidylethanolamine with 17 monomers in the polymer chain; PE-PEG_{2000}, polyethyleneoxide-dipalmitoylphosphatidylethnolamine with 45 monomers in the polymer chain; PE-PEG_{5000}, polyethyleneoxide-dipalmitoylphosphatidylethanolamine with 114 monomers in the polymer chain.

MATERIALS AND METHODS

Materials and Liposome Preparation

Unilamellar liposomes of narrow size distribution were made from various concentrations of PE-PEG_{750}, PE-PEG_{2000}, PE-PEG_{5000}, and DPPC (Avanti Polar Lipids, Birmingham, AL) by extrusion of the liposome suspension through 100nm polycarbonate filters (5). Purified snake-venom PLA_2 (*Agkistrodon piscivoros piscivoros*) has been isolated according to the procedure of Maraganore *et al.* (10). The structural similarity between mammalian and snake-venom PLA_2 indicates common molecular mechanisms of the phospholipid catalyzed hydrolysis at the lipid-membrane interface (8).

Phospholipase A_2 Lag-Time Measurements

Assay conditions for the PLA_2 lag-time measurements were 0.15 mM unilamellar liposomes (total lipid concentration), 150 nM PLA_2, 150 mM KCL, 10 mM HEPES (pH 7.5), 1 mM NaN_3, 30 μM $CaCl_2$, and 10 μM EDTA. The catalytic reaction was initiated by adding 8.3 μL of a 45 μM PLA_2 stock solution to 2.5 mL of the thermostated liposome suspension equilibrated for at least 20 min at 40.1°C prior to addition of PLA_2. The time elapsed before the onset in rapid enzymatic activity and lipid hydrolysis takes place (~lag-time), is determined by a sudden increase in the intrinsic fluorescence from PLA_2 at 340 nm after excitation at 285 nm followed by a decrease in the 90° static light scattering from the lipid suspension (11). A strong temporal correlation exists between the intrinsic PLA_2 tryptophan fluorescence, the 90° static light scattering, and the lipid hydrolysis as determined by pH-stat titration of fatty acids produced (12). The total amount of hydrolyzed DPPC lipids 20 min after onset of rapid PLA_2 hydrolysis was determined by HPLC using a 5 μm diol column, a mobile phase composed of chloroform/methanol/water (730:230:25, v/v), and an evaporative light scattering detector.

RESULTS AND DISCUSSION

Typical PLA_2 hydrolysis time-course profiles obtained at 40.1°C are shown in Fig. 1 for DPPC unilamellar liposomes incorporated with 0 mol%, 1 mol% PE-PEG_{5000}, 3 mol% PE-PEG_{2000}, and 6 mol% PE-PEG_{750} lipopolymers. The lag-time, τ, reflects a sudden increase in the rate of lipid hydrolysis followed by a change in the morphology of the lipid system due to the production of non-bilayer-forming fatty acids and

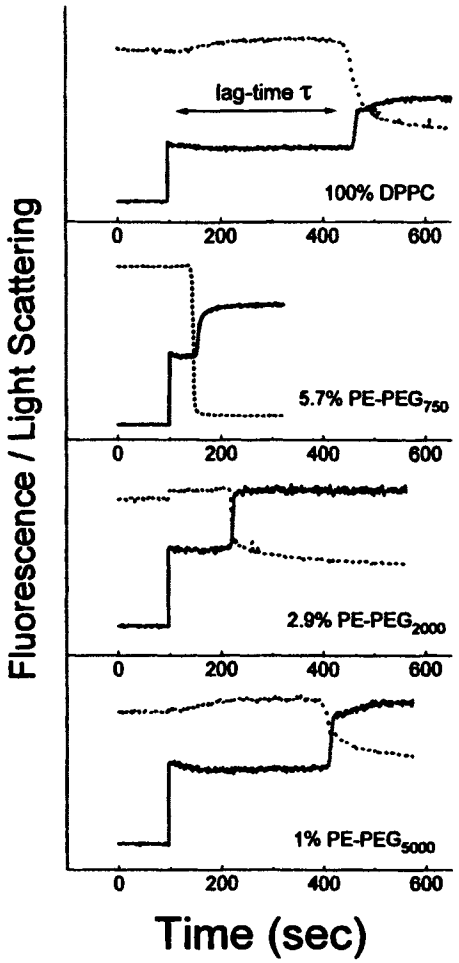

Fig. 1. Reaction time-coarses obtained at 40.1°C for PLA_2 catalysed hydrolysis of 100 nm unilamellar DPPC liposomes incorporated with 0 mol%, 1 mol% PE-PEG_{5000}, 2.9 mol% PE-PEG_{2000}, and 5.7 mol% PE-PEG_{750} lipopolymers. The PLA_2 hydrolysis reaction is monitored by intrinsic fluorescence (solid line) from the enzyme and 90° static light scattering (dotted line) from the lipid suspension. After adding PLA_2 to the liposomes at t = 100 s, a characteristic lag-time, τ, follows before a sudden increase in the catalytic activity takes place (11).

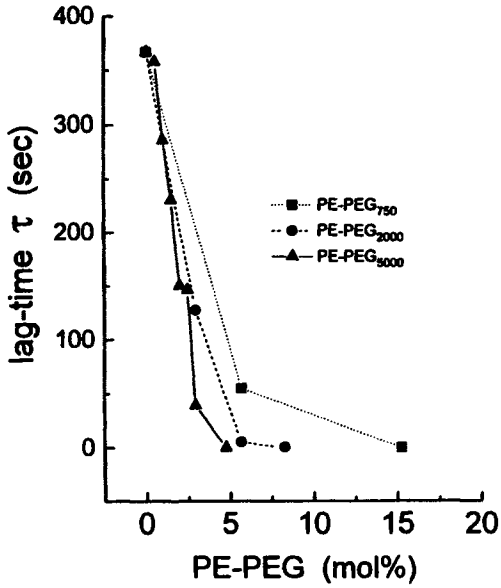

Fig. 2. PLA_2 lag-time, τ, obtained at 40.1°C as a function of lipopolymer concentration for unilamellar DPPC liposomes incorporated with PE-PEG_{750}, PE-PEG_{2000}, and PE-PEG_{5000}.

lysolipids (11). Figure 2 shows the PLA_2 lag-time results as a function of submicellar amounts of the PE-PEG_{750}, PE-PEG_{2000}, and PE-PEG_{5000} lipopolymers incorporated into the unilamellar DPPC liposomes. Each of the three lag-time curves shown in Fig. 2 displays a concentration-dependent decrease of τ reflecting an enhancement of the PLA_2-catalyzed lipid hydrolysis of the polymer-grafted PEG-liposomes. At similar amounts of the three different lipopolymers incorporated into the DPPC liposomes, the most pronounced influence on the lag-time is observed for the longest PE-PEG_{5000} lipopolymer. All the liposome suspensions were characterized by differential scanning calorimetry and the obtained heat capacity curves showed a pronounced effect on the melting enthalpy and a weak freezing point depression in accordance with previous results (7). Moreover, a reduction of the transition temperature moves the liposomes further away from the temperature region of maximal PLA_2 activity (11).

When the PE-PEG lipopolymers are incorporated into the liposomes in submicellar concentrations the polymers will exist in either a mushroom or brush conformation depending on the surface density. For low densities the polymer will adopt a mushroom conformation and cover a region next to the surface that is given by the Flory radius, $R_F = aN^{3/5}$, where a is the monomer size (3.5 Å) and N is the number of monomers (N = 17, 45, and 114) in the polymer chains for the PE-PEG_{750}, PE-PEG_{2000}, and PE-PEG_{5000} lipopolymers (14,15). The effective surface areas occupied by the the three lipopolymers in the mushroom conformation then become 288, 927, and 2828 $Å^2$, respectively. Assuming that the area of a single phospholipid molecule is 65 $Å^2$, the lipid-membrane surface becomes completely covered by, e.g., PE-PEG_{750} lipopolymers in the mushroom conformation at 23 mol% before the mushroom to brush transition takes place (14,15). If the PLA_2 lag-time is analyzed as a function of the polymer coverage of the lipid-membrane surface it becomes clear that the influence on the PLA_2 lag-time is stronger for the shortest PE-PEG_{750} lipopolymer as seen in Fig. 3.

Previous results have demonstrated that the catalytic cleavage of phospholipids and the overall activity of PLA_2 depend strongly on the microstructure of the lipid membrane. In particular, it has been shown that PLA_2 lipid hydrolysis is significantly enhanced in the phospholipid main-transition region due to the formation of a heterogeneous lateral microstructure composed of coexisting dynamic lipid-domains (11). It is likely that the enhanced PLA_2 activity towards PEG-liposomes reflects an intimate relationship between composition and microstructure of the lipid-membrane substrate, e.g., a change in the lateral membrane organization when the PEG-lipids are incorporated into the liposomes. Furthermore, the enhanced PLA_2 activity might involve a change in the interfacial hydration level of the PEG-liposomes (3) as well as and an increase in the binding and contact time of PLA_2 with the membrane surface when the anionic PEG-PE lipopolymers are present in the lipid membrane.

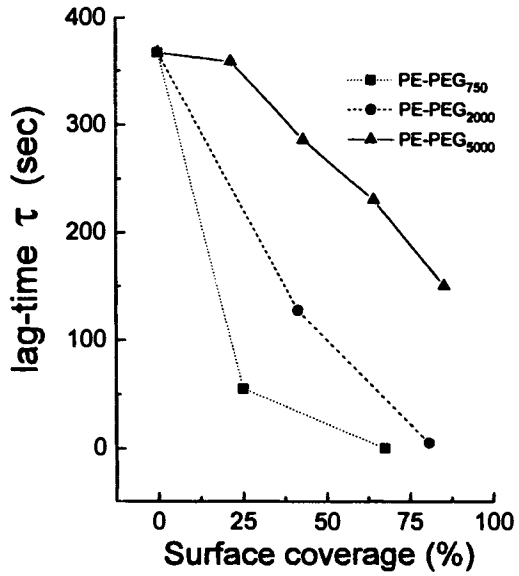

Fig. 3. PLA_2 lag-time, τ, obtained at 40.1°C as a function of the lipid-membrane surface coverage of polymers in the mushroom conformation for unilamellar DPPC liposomes incorporated with $PE\text{-}PEG_{750}$, $PE\text{-}PEG_{2000}$, and $PE\text{-}PEG_{5000}$.

CONCLUSIONS

It has been argued that the incorporation of PEG-lipids into liposomes establishes a repulsive barrier that prevents close approach and interaction of lipid-membrane destabilizing biological components with the surface of the polymer-grafted liposomes (4,16). However, the results presented above clearly reveal that a small water-soluble enzyme, such as PLA_2, can reach the surface of the polymer-grafted PEG-liposomes and promote the lipid-membrane degradation process. In fact, our results demonstrate increasing amounts of the PE-PEG lipopolymers incorporated into the liposomes lead to an increase in the PLA_2-catalyzed lipid hydrolysis resulting in a degradation of the lipid-membrane structure and a concomitant destabilization of the liposomes (17). In our PLA_2 lag-time measurements we found that the total amount of hydrolyzed DPPC lipids 20 min after the onset of rapid PLA_2 hydrolysis was between 50 and 60% as determined by HPLC.

Long circulating PEG-liposomes are able to leave the blood stream and accumulate at pathological sites where they may release their contents by nonspecific leakage or due to a specific enzymatic degradation of the lipid-membrane structure by certain phospholipases as strongly suggested by our in vitro results. Important drug-delivery aspects are related to an improved understanding of the influence of lipid composition on functional biomaterial properties of liposomes that are of relevance for a rational modification and optimization of the PEG-liposomes as drug-carrier systems. The PLA_2 activity results reported above suggest the possibility of using a certain PE-PEG lipopolymer-concentration and polymer-chain-length in order to obtain an optimized degradation and controlled drug release at certain pathological sites that are characterized by an increased concentration of lipid-membrane degrading enzymes such as PLA_2.

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