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Determination of the activity of maleimide-functionalized phospholipids during preparation of liposomes

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ABSTRACT

Numerous examples exist in the literature for the use of maleimide-thiol-reactions in the area of functionalized nanoparticles. Although the hydrolysis tendency of maleimides is well-known, qualitative and quantitative information on the stability and reactivity of maleimide groups during preparation and in final formulations are missing. This is surprising, since hydrolysis of maleimides prevents nanoparticle functionalization and results in an increase of negative surface charge due to the hydrolysis product maleic acid. In this study we investigated the stability of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[maleimide-2000] (DSPE-PEG$_{2000}$-Mal) during the preparation of liposomes via two common preparation methods, which can be distinguished by the insertion of DSPE-PEG$_{2000}$-Mal during or after the liposome formation process (pre-insertion and post-insertion process). The liposomes prepared by the pre-insertion method had 63% active maleimide groups remaining on their surface. The activity decreased dramatically during the purification process down to 32%. The preparation by post-insertion showed minimal effects with regard to maleimide activity. 76% of maleimide groups were active and therefore available for coupling reaction. By identifying active maleimide groups on the surface of the final formulations, the presented work revealed the dramatic impact of preparation methods on the activity of maleimide groups.

1. Introduction

The maleimide-thiol reaction gained great popularity in the field of surface modification of drug delivery systems (DDS) (Kim et al., 2013; Tao et al., 2012) and bioconjugation of drugs to antibodies (Christie et al., 2015). Maleimides react with thiols resulting in the formation of stable thioether bonds. The specificity to thiols, fast aqueous reaction kinetics and mild reaction conditions explain their broad pharmaceutical application in DDS functionalization (Hermanson, 2013; Nair et al., 2014). A great pitfall however is the pH-dependent hydrolysis of maleimides, which is often neglected. With increasing pH (pH > 8), maleimide groups are hydrolyzed to maleic acid, a ring-open hydrolysis product that lacks an electrophile carbon and, therefore, unable to react with nucleophile thiols (Brinkley, 1992; Hermanson, 2013; Khan, 1984; Mattson et al., 1993). This hydrolysis carries two inherent risks for functionalized DDS, as the DDS does not obtain the desired functionalization but furthermore, resulting in an increase of free carboxyl groups on the outer surface. This fact becomes especially critical in active targeting strategies, where the functionalization should enable a controlled and specific uptake into cells. The absence of ligands and increase of negative charge on the outer surface can result in unspecific uptake pattern of nanoparticles (Fröhlich, 2012; Kelf, 2010). Although the risk of the hydrolysis of final functionalized DDS is intensively discussed in the literature, the impact of the preparation on maleimide activity is completely overlooked (Patterson et al., 2014; Tumey et al., 2014).

Facing the risk of maleimide hydrolysis during preparation, the use of maleimide functionality for liposome functionalization is an interesting example. The complex composition of liposomal formulations as well as the multi-step character of the preparation are critical parameters with potential impact on the activity of maleimides. In that regard, we had a closer look at two common functionalization methods for liposomes modified with maleimide-containing PEGylated phospholipid 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[maleimide-2000] (DSPE-PEG$_{2000}$-Mal). We investigated the maleimide acitivty of DSPE-PEG$_{2000}$-Mal during preparation of liposomes by solvent-injection

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and their functionalization via pre-insertion or post-insertion. The distinct difference feature between both methods is the insertion of DSPE-PEG_{2000}-Mal, which is either during (pre-insertion) or after liposome formation (post-insertion) (Nag and Awasthi, 2013). Both methods have potential risks with respect to maleimide activity. Therefore, tracking maleimide activity during the complex preparation processes was the main purpose of our studies.

2. Material and methods

2.1. Material

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino-2000] (DSPE-PEG_{2000}) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (CHO), Ethylenediaminetetraacetic acid (EDTA) and Dulbecco's phosphate buffered saline (PBS) (D1408) were obtained from Sigma-Aldrich (St. Louis, USA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[maleimide-2000] (DSPE-PEG_{2000}-Mal) was purchased from Avanti Polar Lipids (Alabaster, USA). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from AMRESCO LLC (Solon, USA). Rabies virus glycopeptide and EPRNEEK peptide, both modified with terminal sulfhydryl-groups and supposed to enable blood-brain barrier targeting, were used as model ligands (Kumar et al., 2007; Liu et al., 2014). The peptides were modified with an additional cysteine on the C-terminus and were synthesized by Novo Nordisk A/S (Bagsvaerd, Denmark). Float-a-lyzer G2 (50 kDa) was purchased from SPECTRUM® LABORATORIES Inc. (Rancho Dominguez, USA). Methanol, isopropanol (HPLC grade), ethanol, dichloromethane, ammonium acetate, L-cysteine, disodium hydrogen phosphate, sodium dihydrogen phosphate and 2,2′Dinitro-5,5′-dithio-dibenzoic acid (DTNB), also referred to as Ellman's reagent, were obtained from Merck KGaA (Darmstadt, Germany). Purified water was produced by a Millipore-Milli-Q integral water purification system (Merck KGaA, Darmstadt, Germany).

2.2. Methods

2.2.1. Preparation of functionalized liposomes by pre-insertion method

The preparation of ligand-functionalized liposomal formulations was a multistep process (Fig. 1, Table 1). Liposomes were prepared using a solvent injection method (Batzri and Korn, 1973). DSPE-PEG_{2000}-Mal, DMPC and CHO were dissolved in ethanol with a total lipid concentration of 77 mM (step 1). The ethanol solution was injected at a flow rate of 12 ml/min into PBS (pH 7.0–7.5) with a flow of 80 ml/min and resulting in a total lipid concentration of 10 mM (step 2). Dilution with ethanol led to a controlled precipitation of the lipids at the injection site, which were subsequently rearranged to form liposomes (step 3) (Batzri and Korn, 1973; Dua et al., 2012). According to the common protocol for the preparation of plain liposomes, the removal of solvent was carried out directly after preparation with a 50.000 MWCO float-a-lyzer over 5 h and three buffer changes (step 4). The coupling reaction was performed with a molar ratio of 2:1 peptide to DSPE-PEG_{2000}-Mal for 1 h at room temperature under nitrogen atmosphere at pH of 7.0 (step 5). The model peptide RVG was reduced prior to the coupling reaction with TCEP (10-fold molar excess).

2.2.2. Preparation of functionalized liposomes by post-insertion method

The preparation by post-insertion method was described by several groups (Moreira et al., 2002; Uster et al., 1996). Liposomes composed of DOPC and CHO were prepared according to the protocol described in Section 2.2.1 by solvent injection method and concentrated to a final total lipid concentration of 20 mM by centrifugal filtration (Fig. 2, Table 2). DSPE-PEG_{2000}-Mal and DSPE-PEG_{2000} were dissolved in methylene chloride in a molar ratio of 1:4 with total lipid concentration of 2 mM (step 1). A lipid-film was generated under nitrogen stream by the removal of solvent (step 2). The micelles were formed by hydration of the lipid-film with PBS to a concentration of 2 mM at 40 °C for 5 min in a water bath. The model peptide EPRNEEK was dissolved in PBS and reduced with TCEP prior to coupling (10-fold molar excess). The peptide solution was added to the micellar solution and incubated for 1 h at room temperature at pH 7.00 with a molar ratio of 2:1 peptide to

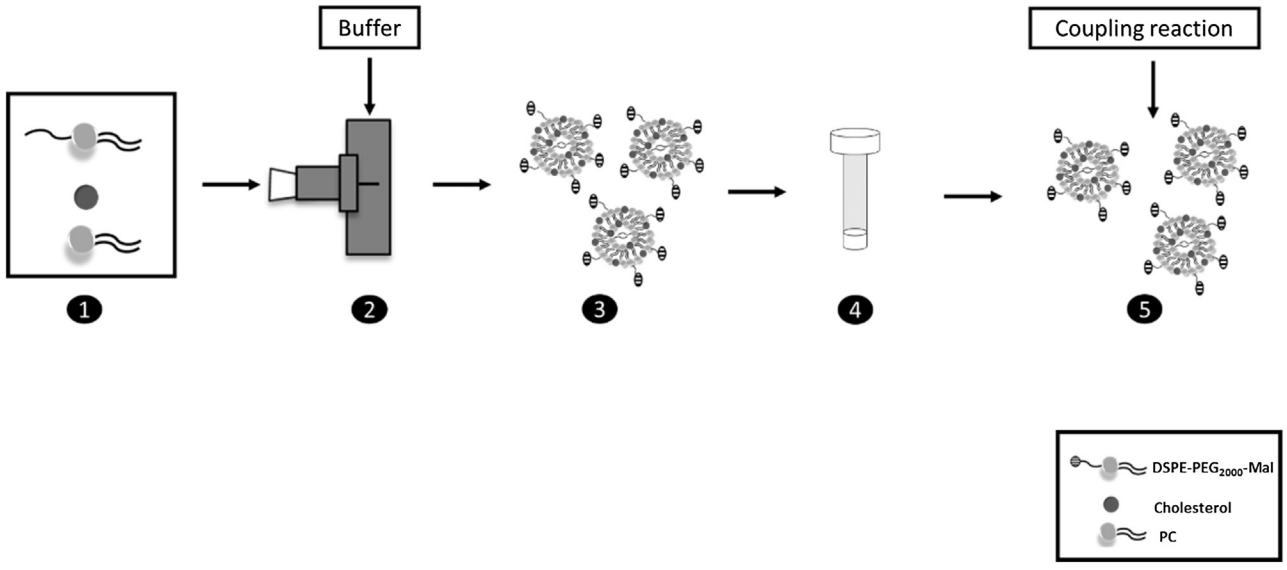

Fig. 1. Graphic illustration of the pre-insertion method. Liposomes were prepared by solvent injection method. DSPE-PEG_{2000}-Mal, DMPC and CHO were dissolved in ethanol with a total lipid concentration of 77 mM (step 1). The ethanol solution was injected at a flow rate of 12 ml/min into a stream of PBS (pH 7.0–7.5) with a flow of 80 ml/min (step 2), resulting in a total lipid concentration of 10 mM. The dilution of the ethanol led to a controlled precipitation of the lipids at the injection site, which were subsequently rearranged to form liposomes (step 3). Removal of solvent was carried out directly after preparation with a 50.000 MWCO float-a-lyzer over 5 h and three buffer changes (step 4). The coupling of ligand was performed after a purification step (step 5).