Journal of Colloid and Interface Science 390 (2013) 267–274

Contents lists available at SciVerse ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

Surface energy of phospholipid bilayers and the correlation to their hydration

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ARTICLE INFO

Article history:
Received 22 June 2012
Accepted 12 September 2012
Available online 21 September 2012

Keywords:
SLB
PEG
Surface energy
Contact angle measurement
Hydration

ABSTRACT

Supported lipid bilayers (SLBs) were prepared on glass and silicon slides grafted with polyethylene glycol (PEG) and covalently bound cholesteryl anchors to fix the lipid bilayer on the surface. Phospholipid bilayers and bilayers modified by addition of covalently bound PEG were investigated. Using contact angle measurements, the surface energy components of bilayer surfaces were analyzed using van Oss' and Owens–Wendt's methods. A quantitative correlation between the polar proton acceptor component of the surface energies and the respective hydration densities was proven for SLBs of pure lipids. We could show that the presence of PEG in the SLB produces a significant change of the proton acceptor component. Regarding the correlation between the surface energies and the hydration densities of SLBs with PEG, we were able to show a dependency on the PEG conformation.

1. Introduction

Drug delivery systems like liposomes, polymer nanocapsules, solid lipid nanoparticles or hydrogels are increasingly employed to improve drug targeting and to reduce adverse side effects [1–3]. The surface protection of these drug delivery systems against opsonizing blood proteins to avoid phagocytosis and rapid blood clearance is a topic of intensive investigation [4–8]. Poly(ethylene glycol) (PEG) is one of the most widely used protecting polymers, where PEG coating (or PEGylation) of liposomes strongly decreases adsorption of human blood proteins and the uptake by liver and spleen. However, recent clinical studies [9,10] provide evidence that the induction of anti-PEG antibodies adversely affects therapy and is the cause of accelerated blood clearance upon repeated injection [11]. Therefore, future research will focus on the replacement of PEG by protecting polymers, which avoid both unspecific (opsonization and phagocytosis) and specific immune reactions (antibodies). To enable an efficient search for alternatives to PEG, it is necessary to characterize the advantages of PEG covered surfaces with respect to uncovered lipid surfaces. In preceding publications, it was assumed that PEG changes the hydrophilicity of lipid surfaces to avoid the activation of the complement system. However, no data were published so far to evaluate this hypothesis quantitatively. Therefore, it is the aim of this study to characterize the hydrophilicity (i.e. the surface energies) of different lipid surfaces and their modification by covering them with covalently bound PEG in different concentrations. One of the most accurate methods to quantify surface energies is to study contact angles on precisely defined planar surfaces. The surfaces of drug delivery systems like liposomes and other phospholipid and PEG coated nanoparticles are therefore modelled by air stable supported lipid bilayers (SLBs) on silicon and glass slides. In order to perform contact angle measurements, we have utilized air stable SLBs [12–16]. Liposomes, deposited from a suspension, fuse and self-organize on glass or silicon surface with grafted PEG and tethered cholesteryl anchors to form a bilayer. The resulting polymer-cushioned SLB is air stable due to cholesteryl anchors [17]. The air stable SLBs consist of either pure phospholipids or phospholipids with an additional quantity of PEG covalently bound to the SLB.

We have characterized the surface energy components of SLBs through contact angle measurement using polar and nonpolar liquids. There are different methods to calculate the surface energy. In this investigation, we consider the van Oss [18] model, which is currently most frequently used. In Supplementary material, we additionally quote the Owens–Wendt model [19] for comparison. Both models require contact angle measurements of different liquids on a dry lipid bilayer surface to enable the calculation of polar and nonpolar surface energy density components of the underlying SLBs. In contrast to Owens–Wendt, the van Oss model divides the polar term into proton donor and proton acceptor densities, which leads to additional information about the surface polarity. This more detailed calculation method is preferable for a more specific

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0021-9797/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jcis.2012.09.027

characterization of the lipid layers. However, both the donor and the acceptor component of different liquids can only be determined relative to one primary liquid. In order to obtain absolute values of both components, the balance of donor and acceptor components for one primary liquid, e.g. water, has to be assumed hypothetically. A set of contradicting hypotheses exists in literature based on different assumptions regarding the donor/acceptor balance of water. Therefore, in Supplementary material, we provide a comparison between the data sets for the testing liquids reported by van Oss [18] who claims an equal partition of donor and acceptor components for pure water, and Della Volpe and Siboni [20] who assume an unequal partition. Similar measurements of the surface energies of pure phospholipid SLBs without PEG have been recently reported by Jurak and Chibowski [21]. However, they do not provide experimental evidence for the air stability of their SLBs. To the best of our knowledge, no study characterizing air stable SLBs with covalently bound PEG has been published yet.

The aim of our study is to investigate the properties of PEG covers and their protective function for the underlying phospholipids (and the liposomes they constitute) against plasma proteins, which would initiate the clearance from the human body and an immune response. Therefore, we compare the surface energies of SLBs of different phospholipids with and without a PEG layer. We correlate the proton donor and acceptor components with the hydration densities of these surfaces reported in the literature to elucidate the mechanisms underlying the protecting effects of PEG reported from in vivo studies of drug delivery systems.

2. Materials and methods

2.1. SUV preparation

The surface properties of one saturated phospholipid, DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), and two unsaturated phospholipids, DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) and POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), were investigated. Additionally, DSPE-mPEG2000 (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]) and DSPE (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine) were mixed in different concentrations with POPC lipids. All lipids were obtained as a gift from Lipoid (Ludwigshafen, Germany). Lipids were dissolved in ethanol (99% purity, AppliChem GmbH, Darmstadt, Germany), which was then removed by rotary evaporation at 175 mbar and ~45 °C for 2–4 h. The obtained dried lipid film was resuspended in Tris buffer (pH 7.4; 50 mM Tris and 100 mM NaCl) (tris(hydroxymethyl)-aminomethane, 99.8% purity, Fluka, Buchs, Switzerland) to form multilamellar vesicles with a lipid concentration of 20 mM, containing 40 μM sodium azide (NaN_3, Fluka) for microbiological stability. This vesicle suspension was extruded with a hand extruder (Avestin, Ottawa, Canada) through two 50 nm polycarbonate membranes (Whatman, Maidstone, UK) at 40–50 °C. The size of liposomes was measured by dynamic light scattering (Zetasizer ZS90, Malvern, Herrenberg, Germany). The diameters were 80–90 nm with a polydispersity index smaller than 0.1. The four binary mixtures: (a) 99 mol% POPC + 1 mol% DSPE-mPEG2000, (b) 95 mol% POPC + 5 mol% DSPE-mPEG2000, (c) 99 mol% POPC + 1 mol% DSPE and 95 mol% POPC + 5 mol% DSPE, will be referred to subsequently as: (a) POPC + 1% PEG, (b) POPC + 5% PEG, (c) POPC + 1% DSPE and d) POPC + 5% DSPE.

2.2. Formation of SLBs

Air stable SLBs were prepared on commercial silicon surfaces (1″ × 1″) and glass cover slides (22 × 22 mm), both grafted identically with PEG and tethered cholesteryl anchors (Microsurfaces Inc., Austin, USA). To check comparability, contact angle measurements were performed on both supports (silicon and glass) and proved to be identical. Contact angles were measured at different locations on the same slide and on different slides. For the coating process, liposomes, calcium chloride ($CaCl_2$, Carl Roth, Karlsruhe, Germany) and Tris buffer were mixed to a final concentration of 1 mM lipids, 2 mM $CaCl_2$ in a 3 ml suspension. Grafted silicon or glass slides were tempered at a temperature higher than 45 °C (which is above the highest transition temperature of all lipids used) while incubating for 1 h with the suspension, which was freshly mixed for each incubation step. After incubation, excess vesicles were removed from the surface by flushing with Tris buffer (6×) and water (3×) to remove salt crystals. For each flush, 3 ml of the mentioned liquid was filled into the dish and shaken very gently and horizontally for 1 min before aspiration of the cleaning fluid without touching the slide. The SLB surface was placed face upwards at the bottom of the dish and horizontally to minimize shear forces on the SLB. This method avoids inhomogeneities, which could arise from contact lines of liposome suspensions. The coated surfaces were dried for ~1.5 h under an argon atmosphere. For all steps bidistilled water with the quality for injectable drugs was used. Liquids were always degassed. Samples were kept in an argon atmosphere at 37 °C. The relative humidity inside the measurement cell was measured with a digital hygrometer (HT-315, Lutron Electronic Enterprise Co., Taipei, Taiwan) and was kept constant at 20% by varying the argon flow rate. We verified the existence, integrity and homogeneity of the SLBs by characterizing the bilayer fluidity on the solid support using fluorescence recovery after photobleaching technique (FRAP) and the surface roughness using atomic force microscopy (AFM), described in detail in Section 2.3.

2.3. Surface characterization

2.3.1. Fluorescence recovery after photobleaching (FRAP)

Two mole percent NBD-PE (*N*-(7-nitrobenz-2-oxa, 1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phospho-ethanolamine, triethylamonium salt, Invitrogen, Karlsruhe, Germany) was used to fluorescently label the lipid headgroup (excitation/emission maxima 463/536 nm). FRAP experiments were carried out using an Andor Revolution XD spinning disk laser scanning microscopy system (Bfi OPTiLAS, München, Germany). It is based on an inverted microscope (Olympus IX81S1F-ZDC, Tokyo, Japan) with an oil immersion objective (APON 60XOTIRF, numerical aperture *NA* = 1.49, Olympus, Tokyo, Japan), temperature control (37 °C, Tokai Hit, Shizuoka-ken, Japan), a CSU-X1 scan head (Yokogawa, Tokyo, Japan) and a DU897 EMCCD camera (Andor, Belfast, UK). Bleaching was done using an exposure time of 0.1 s and the radius of the bleached spot was 20 μm. To normalize the FRAP curve and for evaluation of the diffusion coefficients, the model published by Kapitza was used [22]. Therefore, the normalized fluorescence intensity f_d was calculated by

$$f_d = \frac{F_{\text{bleached},0}}{F_{\text{unbleached},0}} - \frac{F_{\text{bleached}}(t)}{F_{unbleached}(t)}. \quad (1)$$

$F_{\text{bleached},0}$ and $F_{\text{unbleached},0}$ denote the mean value of the fluorescent intensity directly after and before the bleaching pulse for the bleached and unbleached reference area, respectively. The experimental results of f_d can be fitted by a decaying exponential function

$$f_d(t) = A^*\left(1 - e^{-\frac{\omega^2}{4Dt}}\right) + B, \quad (2)$$

where A and B are the fractions of mobile and immobile components. The parameter ω refers to the radius of the bleached spot.