



Ferritin-supported lipid bilayers for triggering the endothelial cell response



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ABSTRACT

Hybrid nanoassemblies of ferritin and silica-supported lipid bilayers (ferritin-SLBs) have been prepared and tested for the adhesion, spreading and proliferation of retinal microvascular endothelial cells (ECs). Lipid membranes with varying surface charge were obtained by mixing cationic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-ethylphosphocholine (POEPC) with zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) at increasing POPC/POEPC ratios. The supported bilayer formation and their subsequent interaction processes with ferritin were studied at the pH of 7.4 at different protein concentrations, by using the quartz crystal microbalance with dissipation monitoring and by atomic force microscopy. Both kinetics and viscoelastic parameters of the protein-lipid membrane interface were scrutinized, as well as surface coverage. Phase-contrast optical microscopy analyses of the ferritin-SLBs substrates after their interaction with endothelial cells evidenced the highest cell adhesion (2–4 h of incubation time) and proliferation (from 24 h to 5 days) for the membranes of POPC/POEPC (75:25 ratio). Moreover, ferritin increased both cell adhesion and proliferation in comparison to control glass (respectively 1.5- and 1.75-fold) as well as proliferation in comparison to bare POPC/POEPC (95:5 ratio) (2 fold). Results are very promising in the goal of modulating the endothelial cell response through the interplay of viscoelastic/charge properties of the solid-supported membranes and the SLB-conditioned ferritin activity.

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1. Introduction

Solid-supported lipid bilayers (SLBs) might act as multi-functional and versatile nanoplatform for cell adhesion and proliferation studies. SLBs generally exhibit a low protein affinity, especially for some lipid types, and can readily be used to simultaneously present multiple ligands [1,2]. Moreover, the lipid membrane exhibits fluidity properties that are assumed to play an active role for the effective exposure of the ligand moieties at the target site [3]. Exhibition of ligands within SLBs at temperatures above the lipid melting transition temperature (T_m) allows for ligand rearrangement to complement the cell receptors pattern, and assists cell-mediated clustering and redistribution of receptors and ligands [4]. The combination of the lateral mobility and the low

protein affinity are expected to be relevant to the frequently low cell affinity of SLBs.

In a previous work we demonstrated that SLBs might serve as well-defined substrates for immobilization of ferritin, with a dominant role played by electrostatic interactions [5]. In particular, other than the pure effect of charge matching between the protein and the charged lipids in the membrane, a very interesting case of membrane rearrangement was demonstrated for ferritin-SLBs with the lipid membrane having a mixed composition of zwitterionic POPC and cationic POEPC, in a 3:1 ratio.

Ferritin is a protein best known for its role in intracellular iron storage and detoxification. Ferritin stores the unreactive Fe^{3+} form inside its shell and serves as an antioxidant protein, preventing excess iron from taking part in the Fenton reaction that would cause ROS production [6].

The ferritin protein is made by a shell of 24 protein subunits (apoferritin), about 10 nm of diameter, and a core Fe^{3+} ions with a peptide MW of 440 kDa. The isoelectric point (pI) of horse spleen

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ferritin has been reported to be 4.1–5.1 [7]. Other than in multiple locations within the cell, ferritin is ubiquitous in tissues and serum, and the presence of ferritin-binding proteins on the cell surface (ferritin receptors) is known for many cell lines [8].

Extracellular ferritin has been demonstrated to be involved in several metabolic processes such as cell proliferation and differentiation, angiogenesis, neoplastic transformation, immunosuppression, and iron delivery [9]. The supply of iron to all tissues of the body is necessary for cellular metabolism as the iron is present in many enzymes containing heme groups. In particular, in the retina the enzyme guanylate cyclase requires iron for the synthesis of cGMP, which acts as a second messenger in the phototransduction pathway [10]. The iron reaches the retina mostly through serum transferrin, which binds to its receptor on the surface of vascular endothelial cells [11,12]. It has been demonstrated that ferritin plays also an important role in iron transport to the retina [13].

Since the blood-retinal barrier (BRB) prevents access of a large number of molecules that circulate in the blood [14], the entry of ferritin in the retina occurs via specific receptors such as Scara5 [15], transferrin receptor 1 (TfR1) [16] and TIM-2 [17]. These receptors are also involved in pathological processes such as cancer, placing the regulation of ferritin in the broader context of cell damage and impaired growth [18]. High levels of ferritin were found in some tumour tissues such as in colon cancer [19], breast cancer [20] and in neuronal tumour cells [21]. These findings bring ferritin in close relationship with important signals for growth and cell cycle progression.

In this context, the primary retinal microvascular endothelial cell culture represents a strategic model to scrutinise ferritin-‘conditioned’ lipid membranes as suitable substrates for endothelial cell adhesion, spreading and proliferation processes. Indeed, on the one hand, there is a significant need of developing new strategies to enhance endothelization, with faster wound-healing around the biomaterial. On the other hand, there is a challenging requirement for triggered cell response, with spatial and temporal resolution, for higher implant stability both in the short- and in long-term.

In the present work we studied the interaction between negatively charged ferritin (at the pH of 7.4), in different concentrations, and cationic SLBs at varying surface charge. A ferritin supported by a mixed POPC/POEPC lipid bilayer is an attractive model system since the POEPC fraction should control the ferritin concentration/density [5] and the POPC fraction is – based on earlier reports – likely to be rather inert towards protein adsorption [5,22] and cell attachment [23].

The paper scrutinises the assembly process of ferritin-modified silica supported lipid bilayers (ferritin-SLB), through the physicochemical characterization of adsorption kinetics, viscoelastic and topographical properties, for applications at the interface with retinal microvascular endothelial cells. Results point to the very promising potential for application of ferritin-SLBs systems to tune the cellular response at the artificial bio-nanointerface.

The purpose of this study is to demonstrate the synergy between the lipids charge effect and the viscoelastic properties in the interaction of supported bilayers with ferritin and, subsequently, with endothelial cells. In fact, while the merely electrostatic charge effect can be trivial (e.g., zwitterionic SLBs are known to be “inert” to a certain extent to adsorption of proteins and attachment of cells [1,2], the SLB + ferritin assembly, where the protein immobilization is reached through the weak interaction forces involved in the physisorption process [5], makes available a panel of possible substrates for the modulation of cell response, both adhesion and proliferation. This aspect is very relevant in the design and fabrication of advanced materials for biomedical applications where, for example, the relative rates of endothelial cell

Table 1

List of the cationic SUVs with the relative composition in POEPC and POPC lipids.

Name	POEPC (mg/mL)	POPC (mg/mL)
EPC5	0.05	4.75
EPC20	1.00	4.00
EPC25	1.25	3.75
EPC30	1.67	3.33
EPC50	2.50	2.50
EPC100	5.00	0

adhesion/proliferation can be very crucial in the wound healing and scar repair process.

2. Material and methods

2.1. Preparation of small unilamellar vesicles (SUV) dispersions

Small unilamellar vesicles (SUVs) were prepared from chloroform solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0–18:1) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-ethylphosphocholine (16:0–18:1) purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were purchased from Sigma-Aldrich (USA). Water was deionized (resistivity $>18 \text{ M}\Omega \times \text{cm}^{-1}$) and purified using a MilliQ unit (MilliQ plus, Millipore, France). Phosphate Buffer Saline (PBS) solution was prepared from tablets (0.01 M phosphate buffer containing 0.003 M KCl and 0.14 M NaCl, pH 7.4). The buffers were filtered and degassed. Zwitterionic vesicles were prepared from pure POPC. Gradient positively charged vesicles were prepared by mixing POPC with cationic POEPC lipids as described in Table 1.

As fluorescent probe, rhodamine-DHPE (1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, Invitrogen, Carlsbad, CA, USA) was added (1 wt.%) to 5 mg/mL solutions of lipid in a round glass flask. The solvent was evaporated under a flow of nitrogen, while rotating the round-bottomed flask, to form a film on the wall of the flask. The lipid film was emulsified in buffer at room temperature, vortexed, and extruded 11 times through a 100 nm polycarbonate membrane, followed by 11 times through a 30 nm membrane (Avanti Polar Lipids Inc., AL, US).

2.2. Silica-supported lipid bilayer formation and their interaction with ferritin

SLBs were prepared by adsorption of the SUVs on polar hydrophilic silicon oxide surfaces, followed by vesicle rupture-fusion processes and the formation of a homogeneous silica supported lipid membrane. The used substrates were both QCM-D sensor crystals (5 MHz) reactively sputter-coated with 50 nm silicon oxide (QSense, Biolin Scientific AB, Sweden) and plasma treated-thin films of polyhydroxymethylsiloxane deposited by spin coating on the gold coated QCM-D sensor crystals [24] or glass bottom 96-well plates (uncoated, γ -irradiated, MatTek Corporation, Ashland, MA, USA). Immediately before the experiments the surfaces were cleaned by 20 min of UV ozone treatment, rinsing with ultrapure water ($3 \times$, 1 mL) and drying under Ar stream. Horse spleen ferritin solution was purchased from Sigma and used as received by dilution in PBS at the final concentrations of 0.05, 0.1, 0.15 and 0.2 mg/mL. For the physicochemical characterization and the cellular assays, the vesicles were diluted to a final concentration of 0.1 mg/mL and allowed to adsorb onto the silica surfaces. After rinsing with PBS buffer, the ferritin solution was added and, after 30 min of incubation time, samples were rinsed with PBS buffer. Each experiment was replicated three times.

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