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Polymersomes with engineered ion selective permeability as stimuli-responsive nanocompartments with preserved architecture

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ABSTRACT

Following a biomimetic approach, we present here polymer vesicles (polymersomes) with ion selective permeability, achieved by inserting gramicidin (gA) biopores in their membrane. Encapsulation of pH-, Na^+- and K^+- sensitive dyes inside the polymersome cavity was used to assess the proper insertion and functionality of gA inside the synthetic membrane. A combination of light scattering, transmission electron microscopy, and fluorescence correlation spectroscopy was used to show that neither the size, nor the morphology of the polymersomes was affected by successful insertion of gA in the polymer membrane. Interestingly, proper insertion and functionality of gA were demonstrated for membranes with thicknesses in the range 9.2–12.1 nm, which are significantly greater than membrane lipid counterparts. Both polymersomes with sizes around 100 nm and giant unilamellar vesicles (GUVs) with inserted gA exhibited efficient time response to pH- and ions and therefore are ideal candidates for designing nanoreactors or biosensors for a variety of applications in which changes in the environment, such as variations of ionic concentration or pH, are required.

1. Introduction

Compartmentalization is crucial for cell stability, whilst it mediates the transport of substances, signaling, or energy production via a complex variety of membrane proteins [1,2]. For example, an eukaryotic cell has half of its total volume confined by intracellular membranes in separate compartments known as organelles, which have unique functions [3]. In an analogous way, compartmentalization through physical confinement of compounds in nanoscale enclosed structures can transport sensitive molecules in a conventional drug delivery approach [4,5], or function as nanoreactors [6], biosensors [7], or artificial organelles [8]. For example nanocompartments that contain active compounds in their inner structure can increase the safety and efficacy of polymerization reactions [9] and catalysis [10,11] offer protected space for cascade reactions [12], serve as source “on demand” for reactive oxygen species [13], or provide dual functionality (oxygen storage and detoxification of peroxinitrites) [6]. Polymeric vesicles (polymersomes) are an example of compartments with high potential for a variety of applications, because they are more robust and versatile than their lipid counterparts [14]. In addition, it is possible to modulate their properties (size, morphology) by changing the hydrophilic to hydrophobic ratio, functionalizing their external surfaces for targeting approaches, or inducing stimuli-responsiveness in their membranes.

Appreciable efforts have been made to design 3D polymer assemblies to respond to specific stimuli, such as pH [15], temperature [16], light [17], enzyme [12,18] or a combination of thereof [19,20]. Stimulus responsive polymer carriers enhance the efficacy of transported active compounds by providing a homogenous spatial distribution, and increasing their localization in desired regions through a triggered release [21]. Stimuli-responsive 3D assemblies can be obtained by various approaches, including: (i) selection of responsive homopolymers, (ii) selection of copolymers with stimulus-responsive blocks (for example polydimethylaminoethyl methacrylate PDMAEMA [22]), and (iii) use of responsive groups on the main chain, on side chains of polymers, or as linkers between different blocks [23–25]. A particularly important category of stimuli-responsive 3D polymer assemblies are those that are pH responsive, because a pH change is an essential signaling factor accompanying pathological conditions [26]. Such 3D assemblies have been obtained by selecting a pH responsive

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polymer, such as PDMAEMA [22] or 4-vinylbenzyl)-pentane-1,5-diamine dihydrochloride (VBPDA) [27], and introducing amino acids (histidine) [28] or carboxyl groups in the polymer main or side chains [20]. The majority of pH sensitive 3D polymer assemblies are designed to dissociate or degrade into polymer chains or small molecules, in order to release their payloads [29–31]. There are only very few examples of pH responsive 3D polymer assemblies maintaining their structural integrity [32,33]. These have been obtained by using a cross-linked homopolymer PDMAEMA but they lack controlled permeability, and have limited stability [33].

Preserving the structural integrity of compartments is essential if polymer vesicles are to serve as nanoreactors, biosensors or artificial organelles. Although polymersomes have lower membrane permeability than their lipid counterparts [34–36], there are various possible approaches for increasing it, such as: (i) selection of polymers that intrinsically form porous membranes (for example poly(styrene)-*block*-polyisocyanoalanine(2-thiophene-3-yl-ethyl) amide [7] or boronic acid-based block copolymers [37]), (ii) chemical modification of the membrane with a hydroxyalkylphenone [38] and (iii) insertion of channel proteins [35,39–41]. However, to the best of our knowledge, none of these examples have reported polymersomes with selective membrane permeability, the large diameter of their pores allowing a passive exchange of small mass molecules with a cut-off resulting from the pore size.

Here we describe the design of polymersomes with membranes able to permit proton and ion exchange without changing their morphology (Fig. 1). Selective permeability of polymersome membranes was achieved by inserting biopores, in a similar manner to biological cell membranes, where various proteins serve for ion transport, or stabilization of the internal environment of the cell. We selected as biopore model, gramicidin (gA), which allows a controlled passage of protons and monovalent cations [42]. This ion channel was chosen, because of its simplicity (only 15 amino acids in its compositions), and versatility [43]. gA has been extensively studied in lipid membranes [44–47], surfactants mimics of unsaturated lipids [48,49], and liposomes [49–53], in which a successful insertion is favored by a reduced thickness mismatch between gA size and lipid membranes. However, there is only one report on the insertion of gA in symmetric block copolymer membrane arrays, and this gave no indication of the thickness of the membrane, the number of inserted gA pores or their localization inside the membrane [54].

We have used a library of poly(2-methyloxazoline)-*block*-poly(dimethylsiloxane)-*block*-poly(2-methyloxazoline) ($PMOXA_x$-$PDMS_y$-$PMOXA_x$) triblock copolymers resulting in polymersomes with membrane thickness ranging from 9.2 to 16.2 nm, in order to understand whether the membrane thickness affects the biopore insertion. Depending on the hydrophilic-to-hydrophobic ratio, this type of amphiphilic polymer self-assembles in dilute solutions, and generates vesicles with sizes in the nanometer range, with high mechanical stability and flexibility [55]. Their membranes are known to be permeable only to reactive oxygen species and oxygen [56], and they are highly impermeable to ions, and low molecular mass molecules [34–36]. We used a combination of light scattering, transmission electron microscopy, and fluorescence correlation spectroscopy to characterize the polymersomes without and with inserted gA to establish if the biopore insertion affects their architecture and size. Exchange across the membranes of protons, Na^+ and K^+ was studied using fluorescence spectroscopy by monitoring changes in fluorescence intensity of an encapsulated sensitive dye as a result of pH change or the presence of ions in the environment of the polymersomes. In addition, gA was inserted in single giant unilamellar vesicles (GUVs), and the change of fluorescence intensity of the encapsulated dye was visualised in real time. The design of polymersomes with membranes permeable to protons and ions, such as Na^+ or K^+, represents a new approach for producing compartments with specific responses, whilst preserving their morphology, and therefore the encapsulated active compounds. They represent ideal candidates for developing applications in which specificity and responsiveness are key factors, as for example in biosensors, or specialised artificial organelles.

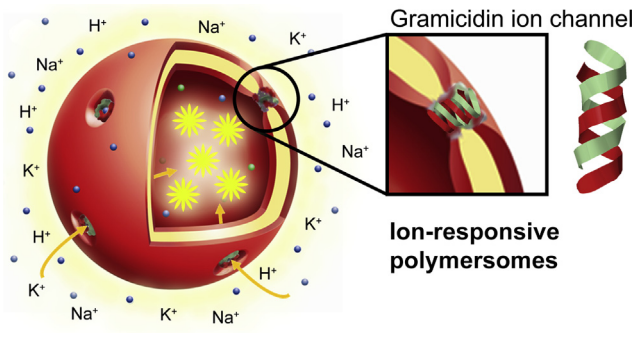

Fig. 1. Design of polymersomes engineered for ion selective permeability based on gramicidin (gA) biopore insertion.

2. Materials and methods

Gramicidin from Bacillus aneurinolyctus (Bacillus brevis), linear polypeptide antibiotic complex A mixture of gramicidins A, B, C, and D, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (pyranine), 5(6)-carboxyfluorescein, monosodium phosphate (NaH_2PO_4), potassium phosphate dibasic (K_2HPO_4), potassium phosphate monobasic (KH_2PO_4), sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl), ethanol (EtOH), and dimethyl sulfoxide (DMSO), C_2H_6OS, were obtained from Sigma Aldrich and were used as received. 6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl) aminohexanoic acid, succinimidyl ester (BODIPY) 630/650 fluorophore was purchased from Molecular Probes. Asante Natrium Green-2 (ANG-2) and Asante Potassium Green-2 (APG-2) were purchased from TEFLabs Inc.

2.1. Preparation of polymersomes and insertion of gA

Polymersomes were prepared at room temperature from different triblock copolymers $PMOXA_x$-$PDMS_y$-$PMOXA_x$, by film hydration method [57]. To produce a thin film, 5 mg of the block copolymer was dissolved in 1 mL of ethanol and subsequently evaporated under reduced pressure (150 mbar) to render a thin film. This film was further rehydrated with: a) 1 mL of pyranine solution 0.5 mM in 10 mM NaH_2PO_4, 132 mM NaCl buffer (pH 8.2); b) 1 mL of 250 μg/mL APG-2 solution in sodium buffer (10 mM NaH_2PO_4, 132 mM NaCl, pH 7.0), or c) 1 mL of 250 μg/mL ANG-2 solution in potassium buffer (10 mM KH_2PO_4, 132 mM KCl, pH 7.0). The polymer suspensions were stirred overnight at RT and afterwards extruded through a 200 nm pore-size polycarbonate (PC) membrane (Merck, Germany), using an Avanti mini-extruder (Avanti Polar Lipids, USA). Any free dye was separated from polymersomes containing entrapped dye by passage through HiTrap desalting columns (Sephadex G-25 Superfine, GE Healthcare, UK) or 20 cm^3 in-house prepacked column (Sepharose 2B, Sigma Aldrich). gA biopores were inserted in polymer membrane by direct addition of 10 μL 0.08 mg/mL to 0.45 mg/mL gA in DMSO:EtOH, 1:1 ratio (solvent) to the pyranine (ANG-2, or APG-2) loaded polymersomes solutions.

The concentration of pyranine encapsulated in polymersomes was determined by UV-vis spectroscopy, $\lambda = 454$ nm. Encapsulation efficiency of the process (EEP) [13] was calculated as a percentage of the ratio between the concentration of pyranine in polymersomes after encapsulation and purification (c_{ep}) and the initial concentration of pyranine used for the film rehydration (c_i):

$$EEP[\%] = \frac{c_{ep}}{c_i} \quad (1)$$

The concentration of encapsulated pyranine was determined using an extinction coefficient of 2.55×10^{-3} M^{-1} cm^{-1}, obtained experimentally at 454 nm wavelength.

2.2. Encapsulation efficiency

The encapsulation efficiency (EE[%]) was experimentally determined by fluorescence correlation spectroscopy (FCS) and calculated using Equation (2):

$$EE[\%] = \frac{\#Pyr_{exp}}{\#Pyr_{calc}} \quad (2)$$

where #Pyr was calculated using Equation (3):