Polymeric Hybrid Microstructures for Smart Targeting and Drug Delivery

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ABSTRACT

A new approach of a hybrid artificial cell (AC) is presented, consisting of an impermeable non-biological scaffold in combination with a bilayer lipid membrane (BLM) as a means to incorporate functioning bio-molecules required to give the AC its functionality, e.g. specific targeting and active drug release. We report on the fabrication and loading of the scaffold and the functionalization of the AC. Single nano-holes were introduced into the sub-micrometer polystyrene scaffolds in a controlled way using a focused ion beam. A hydrophilic solution is loaded in these hydrophobic scaffolds via the nanoholes. In addition, artificial BLM of roughly 6 nm thickness, as measured with atomic force microscopy, are deposited on a surface in a controlled way by manipulating unilamellar vesicles with optical tweezers and collapsing them on a surface. The results presented here is a first step towards the development of a biomimetic AC using hybrid technology.

Keywords: artificial cell, polystyrene microcapsules, nanofabrication, biomimetic

1 INTRODUCTION

Artificial cells (AC) refer to (sub) micro-vehicles that can perform a specific bio-function in the body, without being rejected from the immune system [1]. They are intended to supplement or replace defective or deficient cells or cell functions. Another application of the AC is smart targeting and drug delivery systems. They are aimed for use in medicine (e.g. in gene therapy [2]), studying fundamental biological processes (e.g. biomimetic [3]), diagnostics [4], etc. Depending on the applications, there is a wide variety of AC's. Here we can mention liposome-based AC, polymer based (biodegradable and nondegradable), micells, inorganic AC, etc as small micro-containers for specific targeting and as drug delivery systems [5, 6, 7].

It is the aim of our study to develop an AC for the purpose of specific targeting and controlled active drug release. Our approach of the AC is based on a hybrid scaffold: an impermeable polymeric scaffold that will have a window to communicate with the in- and outer medium of AC and a bilayer membrane, which temporarily blocks that window and serves as a natural supporter for the biomolecules required to functionalise the AC. In addition, this is a unidirectional drug-releasing system and can offer local targeting of e.g. a disease cell and local drug release. The stability of the polymeric structure facilitates the use of different techniques that might be required to manipulate, functionalise, and characterize our AC. Implementation of biomolecular motors such as ATPase is the first goal of our work. In this paper we report on the fabrication and loading of the scaffold and the functionalization of the AC.

2 METHODS AND IMPLEMENTATION

2.1 Elements of Design

In Figure 1, a preliminary design of the AC is schematically presented. The design is based on an impermeable hollow polymer scaffold that will carry a certain load e.g. a drug or biomolecules.

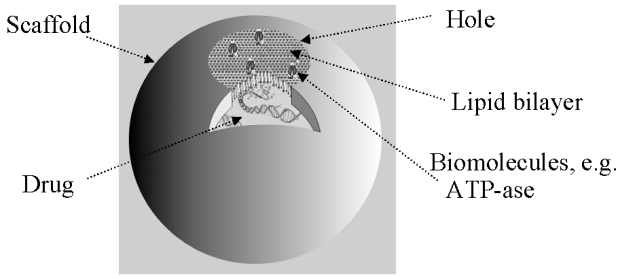


Figure 1: Preliminary design of an AC.

The advantage of a hollow structure is that more drug can be encapsulated inside the AC compartment comparing to drug immobilization on the surface or in the scaffold. Furthermore, the scaffold separates the inner content of the AC from the environment. In the scaffold, a hole is implemented that will connect the inner medium of the AC with the outer environment. The hole is used to load the scaffold and is then sealed by a lipid membrane. The small dimensions of the hole encounter a high stability of the artificial membrane which controls the release of the AC contents and supports the biomolecules required for the functionality of the AC, like sensing ability, motility, etc. Sensing and diagnosing functionality of the AC in a bio-environment could be based on bio-molecular interaction

(e.g. antibody-antigen, ligand-receptor, enzymatic interaction, etc), and hence it is required to incorporate specific bio-molecules in the membrane. Directed movements of the AC could also be realized by incorporation of bio-molecules in the membrane that produce bio-energy (e.g. ATPase combined with photosynthetic complexes) in combination with motor proteins like e.g. flagellar motors, which convert the bio-energy into mechanical movement. Nowadays, there are several known methods for oriented incorporation of biomolecules in artificial membranes, e.g. based on hydrophobic phase [3], pH of the solution, etc. In order to inhibit the binding of plasma proteins and thus increasing the plasma circulation time, a PEG coating of the AC is necessary to have a long AC functioning time. Studies have shown that the PEG surface modification highly decreases non-specific binding of proteins, and hence prevents the recognition of the AC by the immune system [8].

2.2 Materials and Sample Preparation

Materials. Hollow polystyrene (HPS) beads having an inner diameter of 900 nm and wall thickness of 100 nm were purchased from Polysciences Inc. Rhodamine123 and DiO were obtained from Molecular Probes. Lipid (1,2-Diphytanoyl-sn-Glycero-3-Phosphocholine), cholesterol, and 1-Stearoyl-2-Oleoyl-sn-Glycero-3-Phosphate were purchased from Avanti Polar-Lipids Inc., prepacked Sephadex G-25 columns were obtained from Pharmacia Biotech AB.

Preparation of samples for focused ion beam (FIB) experiments. HPS beads, which are suspended in water and also contain water inside (as purchased from the company), are diluted to a particle concentration of 10^7 beads/ml. A transparent supporter for the beads is prepared using a coverslip of 150 μm thickness, on the top of which a very thin titanium (20 nm) layer is deposited. The suspension of HPS beads is sonicated and a droplet from this solution is placed on the prepared transparent supporter and left there under room temperature (28 ^{O}C) for 24 hours in order to dry.

Preparation of giant unilamellar vesicles. A thin and uniform layer of dried lipid film is formed by mixing in a round flask lipids (1.25 mg), cholesterol, and phosphate acid (respective ratio of 1: 0.26 : 0.02 mw) with chloroform and putting them in a rotorvapor under vacuum conditions. 400mM aqueous glucose solution (4mL) is added into the flask in order to swell the film. Freezing-thawing method is applied to the formed vesicles by placing the flask solution 7 times once in liquid nitrogen and then in water of room temperature in order to form unilamellar vesicles. Than the vesicle solution is loaded into the Sephadex column and allowed to flow through it. Vesicles are labeled fluorescently afterwards by adding 3μl DiO solution (concentration of 10μg/ml in MeOH) per ml vesicle solution.

Glass surface modification. Glass surface (coverslips) at the bottom of a dedicated cuvette was modified by incubating it over night with 0.1% aqueous poly-L-lysine solution. Before use, the glass supporters were washed with distilled water.

2.3 Techniques and Experimental Procedures

The FIB is used to make the holes in the HPS beads. For that purpose, the prepared sample with dried HPS beads on the conductive surface of a transparent holder (see previous section) is placed in the working chamber of the FIB equipment. A low current of gallium ions (1 pA) is generated in order to have a low etching rate of the polystyrene material and the accelerated ion beam is focused on the bead surface, etching the polystyrene (PS) material mechanically. Furthermore, the FIB was used to make images of the beads before and after the etching process.

Fluorescence microscopy is used to monitor the filling of the fabricated scaffolds. Fluorescent measurements are performed using a Confocal Laser Scanning Microscope (CLSM) (Zeiss LSM 510). Rhodamine 123 (15 μg/ml) is used as water tracer in these measurements.

A dedicated optical tweezers set-up combined with EPI fluorescence is used to deposit an artificial BLM on a certain position and to image the process. Optical tweezers (OT) technique is used to manipulate the glucose-loaded vesicles and drive them to rupture on a desired position on the modified coverslip, forming in this way controlled BLM patches. A high laser output power of 1W (wavelength of 1064 nm) was used to trap the vesicles. While rupturing the vesicles at the bottom of the cuvette, the DiO labelled vesicles were fluorescently monitored first using EPI fluorescence and then oriented to a certain desired position using the OT. A low laser power is used for the fluorescent imaging in order to avoid bleaching of the dye.

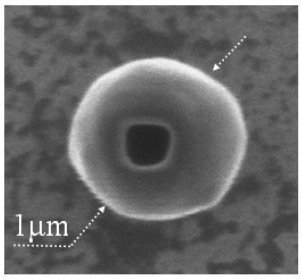
A home made Atomic Force Microscope (AFM) system, as described previously [9], is used to characterize the thickness of artificial lipid membrane patches. First the membrane patches were formed by collapsing the vesicles on a glass surface and then kept under water condition. The AFM measurements were performed using a soft taping mode.

3 RESULTS AND DISCUSSION

3.1 Fabrication of the Scaffold

Square holes (Figure 2A) of desired dimensions, varying from 100nm to 400 nm, were drilled on several PS scaffolds in time duration of 7 to 42 sec per hole, respectively. The HPS scaffolds were stable and no shift in the structure position occurred due to the ionic charging of PS surface while using the FIB. In order to check the HPS scaffold inside, we etched one half of the scaffold and

(A)



(B)

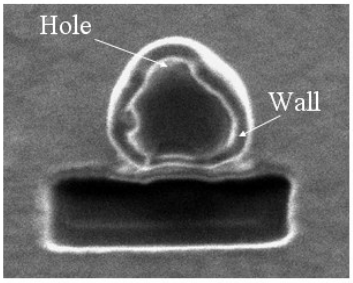


Figure 2: Scanning electron micrograph of a HPS scaffold with a hole of 250 nm x 260 nm in it (A); the HPS scaffold with a hole, which is half etched and rotated under an angle (B).

imaged afterwards the scaffold from an angle (Figure 2B). Observations showed that the scaffold remains hollow after the drying and etching process and the hole exists only on the top of the scaffold, as aimed for. In fact, no structural damage to the scaffold, apart from the holes could be observed. It appeared also possible to fabricate cells with two holes without damaging the integrity of the structure.

3.2 Loading of the Scaffold

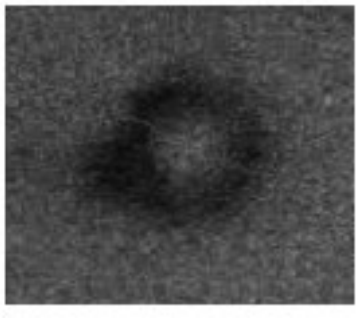
In Figure 3A, the fluorescent image of a scaffold with a hole, which is loaded with aqueous Rhodamine 123 solution containing ethanol, is presented. This image refers to a confocal slice through the centre of the scaffold. It is chosen from a stack of images made at different depths (step size of 250 nm). Loading occurs within a few seconds after immersing the dried HPS scaffolds in the dye solution. The addition of ethanol (2%) was required to lower the surface tension. No filling was observed when using water only. A fluorescent intensity profile across the fluorescent image of Figure 3A (i.e. through the scaffold) as a function of the position is presented in Figure 3B. As expected, the fluorescent intensity shows a Gaussian peak intensity, which indicates that the inner volume of the scaffold is filled with dye. The minimum intensity occurs at the position of the shell. It should be noted that the dimensions of the scaffold are close to the optical resolution of the microscope, which prevents to observe sharp transitions between non-fluorescent wall and fluorescent solution. Furthermore, the level of the intensity inside the scaffold (Figure 3B) is comparable with the level intensity of the background, as expected.

As a control, scaffolds that did not contain a hole were observed as well. No fluorescence could be observed in these scaffolds indicating that the loading of the scaffolds is accomplished by transport through the holes only. Additional measurements are performed to further test the permeability of the wall of the scaffold. HPS beads, which contain water inside, were incubated in a cuvette with an ethanol containing (2%) water-dye solution for more than a week. Fluorescent measurements were performed in several scaffolds for different time intervals and no change of the fluorescent intensity was observed inside the scaffolds. Hence, no observable diffusion occurs through the wall of the PS scaffold, indicating the impermeability of the scaffold.

3.3 Artificial Lipid Membrane Formation and Characterization

Figure 4 presents an image of a pattern of 4 equidistant membrane patches formed by controlled rupture of 4 vesicles. The rupture occurs very fast and the formed patches appeared to be uniform.

(A)



(B)

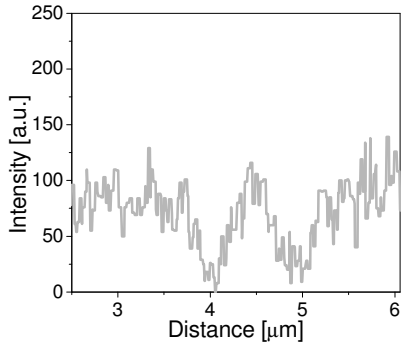


Figure 3: (A) CLSM image in the middle of a HPS scaffold filled with aqueous dye solution; (B) Intensity profile across the image (line) in Figure 3A.

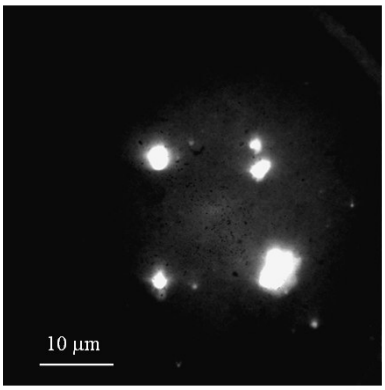


Figure 4: Fluorescent image of a pattern of collapsed vesicles positioned by using OT.

A more detailed and closer view of these membrane patches is obtained by atomic force microscopy. In Figure 5, an AFM image of a membrane patch is shown. The membrane appears to be homogeneous. According to the measurements performed on several patches, it results an average membrane thickness of roughly 6 nm ± 1nm, which is comparable with the thickness of a real cell membrane.

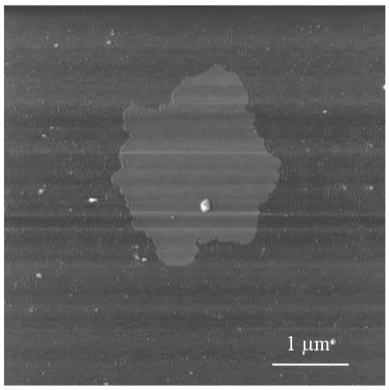


Figure 5: AFM image of a collapsed unilamellar vesicle on a flat surface.

4 CONCLUSIONS

In this paper we present a new design of an artificial cell for the purpose of local and controlled drug delivering system. The scaffold of our AC is based on hollow polystyrene beads of inner diameter of 0.9 μm and wall thickness of 100 nm. However, this approach could also be used using other polymer material, with more specific properties like biocompatibility and biodegradability. Small square holes have been drilled on several scaffolds by using FIB and these holes could be controlled in number and size (100-400 nm).

Loading of the scaffold with aqueous dye solution containing ethanol is visualized using CLSM. In principle we are able to load any sort of hydrophilic artificial drug in our hydrophobic scaffold. Furthermore, measurements showed that the used HPS beads are impermeable for small molecules like Rhodamine 123.

Artificial lipid membrane is deposited on a surface in a controlled way. AFM measurements show a thickness of these membranes approximately 6 nm that is comparable with the thickness of the real cell membranes.

Future work will be directed to the functionalisation of the scaffold by depositing in the nanofabricated hole a bilayer membrane modified with different biomolecules, such as ATPase, etc.

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