ARTIFICIAL BACTERIAL FLAGELLA FUNCTIONALIZED WITH TEMPERATURE-SENSITIVE LIPOSOMES FOR BIOMEDICAL APPLICATIONS

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ABSTRACT

Inspired by flagellar propulsion of bacterial such as *E. coli*, artificial bacterial flagella (ABFs) are magnetic swimming microrobots with helical shapes. ABFs can perform precise three-dimensional (3D) navigation in liquids under low-strength rotating magnetic fields making them attractive tools for drug delivery applications. Further functionalization of these swimming microrobots is necessary to optimize their performance of biomedical tasks. We report here for the first time the successful functionalization of titanium-coated ABFs with temperature-sensitive dipalmitoylphosphatidylcholine (DPPC) liposomes. Adsorption of intact liposomes on titanium was assessed using quartz crystal microbalance with dissipation monitoring (QCM-D). The adsorption of fluorescently labeled liposomes on the surface of ABFs was confirmed with confocal laser scanning microscopy (CLSM) images. Functionalized ABFs (f-ABFs) can be loaded with both hydrophilic and hydrophobic drugs, and controlled drug release triggered by temperature. ABFs have a great potential to be used in targeted and controlled drug delivery and for *in vivo* sensing.

KEYWRODS

Artificial bacterial flagella (ABFs), liposomes, DPPC, biomedical applications, controlled release

INTRODUCTION

Magnetic micro- and nanorobots, wirelessly powered by magnetic fields, have potential to be used in biological and medical applications such as *in vitro* cell manipulation, targeted therapy and *in vivo* sensing [1-6]. Artificial bacterial flagella (ABFs) are magnetic helical microrobots, which use a cork-screw strategy to propel themselves and are of similar size as real bacteria such as *E. coli* [7]. ABFs are capable of precise 3D navigation in liquids under weak magnetic fields (1000 times lower than the fields used in MRI systems). Flagellar propulsion has been proposed as a promising approach for *in vivo* applications [8, 9].

Previous work has shown that ABFs can be used to manipulate cellular and sub-cellular objects in liquids by mechanical contact [10, 11] and non-contact (controlled fluidic drag forces when an ABF is rotating) methods without surface functionalization [12, 13]. For biomedical applications such as drug delivery and sensing, further functionalization with specific chemicals, such as drug molecules and sensitive chemicals, is required [14]. Furthermore, biological modification of the surface of nano/micro motors have been applied to DNA separation and drug delivery [2].

In biology and medicine, liposomes have been extensively studied and used in various applications such as drug delivery systems and cell membrane science [15]. A liposome is a lipid vesicle consisting of a self-assembled lipid bilayer, in which DNA, drugs and/or chemicals can be encapsulated. Liposomes range in size from 20 nanometers to several hundred micrometers. Furthermore, depending on the lipid composition of liposomes, their entrapped materials can be locally and remotely trigger-released by different stimuli, such as enzyme, pH, ultrasound, light and temperature [16]. Among them, dipalmitoylphosphatidylcholine (DPPC) has a phase transition temperature of 41℃ and DPPC liposomes can be temperature-triggered to release their cargo [17].

In this paper, we report on a process to functionalize ABFs with DPPC liposomes by coating the ABF surfaces with liposomes. Quartz crystal microbalance with dissipation monitoring (QCM-D) was used to investigate the adsorption of DPPC liposomes onto TiO_2 surface. Confocal laser scanning microscopy (CLSM) was used to detect fluorescently labeled or calcein loaded liposomes on the surface of ABFs.

EXPERIMENTAL

Fabrication process of ABFs

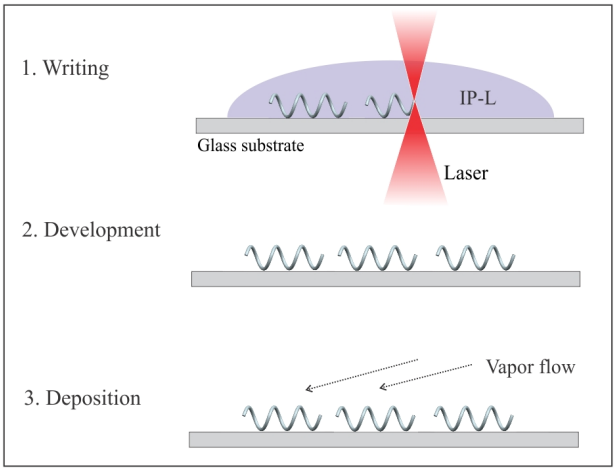

Figure 1: Fabrication flow of ABFs. Step 1: Writing helical arrays in IP-L photoresist; Step 2: Development in IPA; Step 3: Coating the Ni/Ti bilayer using electron beam deposition.

ABF arrays were fabricated using direct laser writing (DLW) and e-beam deposition methods. The process consisted of three steps: Step 1, writing helical structures in a photoresist IP-L (a commercial negative photoresist from Nanoscribe GmbH, Germany) using DLW, which is based on two-photon polymerization mechanism [18]; Step 2, developing the written sample in isopropyl alcohol (IPA) to remove un-polymerized resist; Step 3, coating the sample with Ni/Ti layers (25 nm Ni and 15 nm Ti) using electron beam deposition. The Ti layer is naturally oxidized to TiO_2 when it is exposed to oxygen. The detailed fabrication process can be found in other literature [10]. Figure 2 shows the scanning electron microscopy (SEM) image of horizontal ABF arrays, where the length of a single ABF is 16 μm and the diameter is 5 μm.

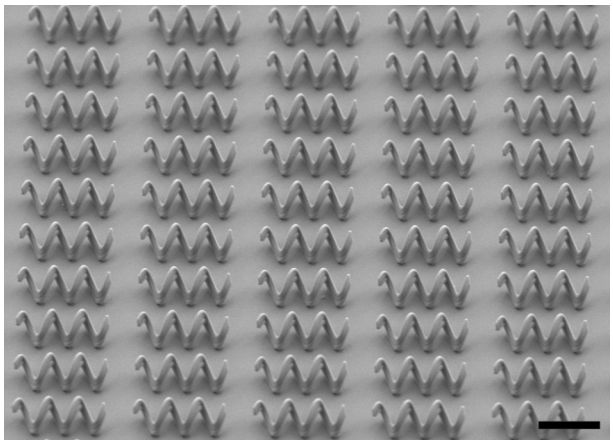

Figure 2: SEM image of an ABF array. The image was taken by a SE2 detector. The scale bar is 10 μm.

Preparation of liposome-coated ABFs

Figure 3 shows the three-part preparation flow of liposome-coated ABFs. The first part is the preparation of unilamellar DPPC liposomes, the second part is the preparation of the ABF suspension, and the last part is the mixture of two suspensions and washing to generate functionalized ABFs (f-ABFs).

The unilamellar DPPC liposomes were prepared by extrusion [19, 20]. DPPC lipids (Avanti Polar Lipids, Inc.) in chloroform were completely dried in a glass vial under a gentle N_2 flow for 30 minutes, and rehydrated with HEPES buffer. In this step, fluorescent molecules can be dissolved in HEPES to be incorporated within the liposomes. The glass vial was subsequently vortexed to create multilamellar vesicles. The multilamellar vesicle suspension was transferred into a glass syringe, and assembled to form the extruder (Figure 4a). The lipid solution was extruded 31 times through two packed polystyrene membranes (Figure 4b) to form uniform-sized (200 nm) unilamellar vesicles. Extra care was taken to keep the whole extruder including the lipid solution above the transition temperature (41 ℃) during the extrusion by pre-warming them at 65 ℃ in an oven.

After metal coating of the ABF arrays, the substrate with arrays was placed in UV/ozone cleaner for 30 minutes to remove organic contaminations on the surface. The arrays were then batch-released by sonication in HEPES buffer to get an ABF suspension [9]. The solution was transferred into a centrifuge tube.

The last step was to mix the liposome and ABF suspensions together and incubate for at least two hours with gentle rotating to get a saturated adsorption of liposomes on the ABF surfaces. The incubation time was determined by QCM-D data, and the final concentration of DPPC lipids in buffer was 2.5 mg/ml.

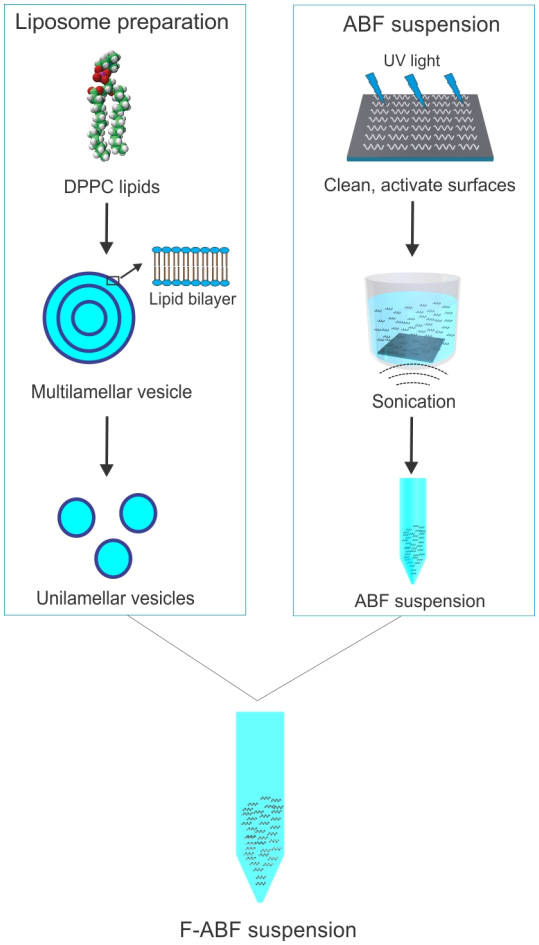

Figure 3: Preparation flow for coating ABFs with unilamellar DPPC liposomes.

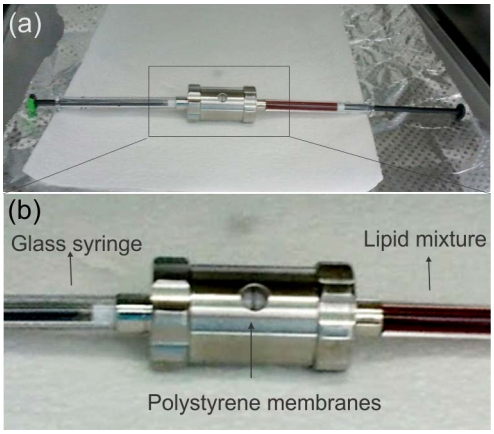

Figure 4: (a) Assembled extruder used to obtain the desired size of liposomes in an oven. (b) The magnified image of extruder. Two polystyrene membranes with 200 nm pore size were packed in the middle of the extruder. And the lipid mixture was loaded in the right syringe.

QCM-D measurement

Lipids can form a range of different structures on a solid surface, including monolayers, intact vesicles or bilayers. In this study the goal was to coat ABFs with intact vesicles which allowed entrapment of water-soluble drugs. QCM-D is a commonly used tool to measure adsorption of lipids and their structure on surfaces [21]. When lipids adsorb on the QCM-D crystal, the resonant frequency of the crystal decreases and the dissipation increases. By monitoring the change of frequency and dissipation, the structure, mass and viscoelastic properties of attaching lipids can be determined.

Since the top surface of ABFs is TiO_2, the TiO_2-coated crystal was used to simulate the adsorption of DPPC liposomes on ABFs. The crystal was cleaned in UV/ozone cleaner for 30 min followed by washing with Milli-Q water. After the crystal was assembled in the chamber, HEPES buffer was injected into the cell and left until a stable baseline was observed. The liposome solution (0.5 mg/ml) was then injected, and the changes of frequency and dissipation were recorded to monitor the adsorption and stability of lipids on the surface. After the adsorption saturated, buffer was quickly injected three times to check the stability of the adsorbed liposomes. The QCM-D experiment was repeated three times (n=3).

CLSM imaging

In order to confirm the coating of liposomes on ABFs, fluorescent probes, calcein loaded liposomes or rhodamine B labeled liposomes were used. Calcein (50 mM in HEPES) was entrapped inside of the liposomes, and rhodamine B labeled lipids were incorporated in the liposome lipid bilayer by adding 2% (w/w) to the DPPC initial lipid solution. The mixture of two suspensions was centrifuged (4000 g, 3 min) and washed at least 5 times to remove the background signal from the resident dyes in solution. The calcein signal was detected using a 488 nm excitation laser and a 505-550 nm band-pass filter. For rhodamine B, the laser wavelength was 561 nm, and the filter was BP 575-615 IR.

RESULTS AND DISCUSSION

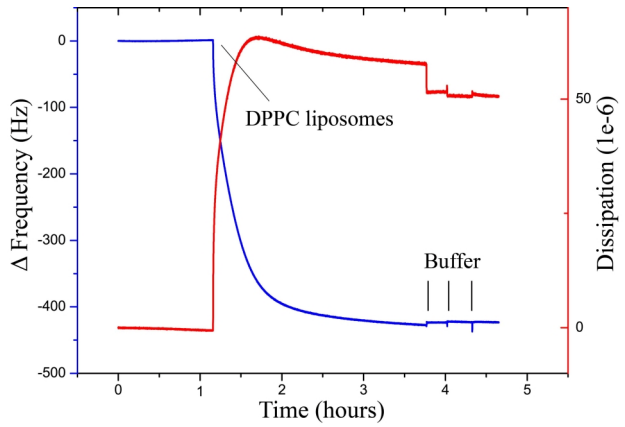

Figure 5: The QCM-D curve of DPPC liposomes deposited on a TiO_2 crystal. The blue and red curves are signals of frequency and dissipation of the crystal, respectively.

Figure 5 shows the QCM-D results of DPPC lipids on TiO_2-coated crystals. The data presented were measured at the third overtone. The frequency decreased 425 Hz while the dissipation increased up to 50 in the first 30 minutes and reached a plateau after 2h. This indicates the adsorption of intact DPPC liposomes and is consistent with previous literatures [21-23]. There were no significant changes in the frequency or dissipation after washing the crystal three times with buffer, which suggests that DPPC liposomes were stable on the TiO_2 surface. For coating DPPC liposomes on the surface of ABFs, we incubated liposomes with ABFs for 3 hours to ensure a saturated adsorption.

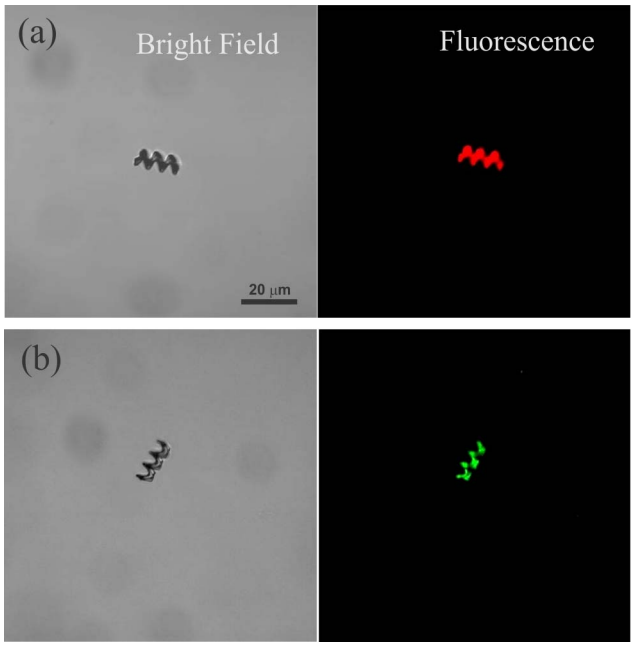

Figure 6: Fluorescent images of DPPC-coated ABFs. (a) Liposome-coated ABF labeled with rhodamine B. (b) Liposome-coated ABF labeled with calcein.

QCM-D results showed a stable adsorption of DPPC liposomes on the flat surface of the crystal. In order to confirm the adsorption of liposomes on 3D-shaped ABFs, rhodamine B labeled lipids (red) were used as a model for a lipid-soluble drug. The rhodamine B-tagged lipids are embedded within the liposome lipid bilayer. Calcein, a green dye, was entrapped within liposomes, mimicking a water-soluble drug. Figure 6 shows CLSM images of f-ABFs. We used uncoated ABFs as controls to calibrate the intensity of the laser. We thereby ensured that any signals were a result of the fluorescent dye, and not associated with the autofluoresence of the ABFs themselves. Strong signals from both rhodamine B (Figure 6a) and calcein (Figure 6b) show that liposomes were bound to the ABF surface, which confirms the QCM-D data. It shows that both hydrophobic and hydrophilic drugs can be incorporated in liposomes. The release of the trapped drugs can be temperature-triggered [24]. This study presents the feasibility of using f-ABFs as a controlled drug delivery vehicle. Furthermore, the fluorescent signal provides a way to track f-ABFs when they swim inside of human body.

CONCLUSION

In conclusion, ABFs were successfully functionalized with DPPC liposomes. The functionalization was proved by QCM-D and CLSM results. These f-ABF systems can be wirelessly controlled by low-strength rotating magnetic fields, which are harmless to cells and tissues. They show the ability to load both hydrophilic and hydrophobic drugs, and the ability to release the cargo. F-ABFs are stable for at least two weeks in physiological aqueous conditions. Functionalized ABFs are promising robotic tools for biomedical applications. Our next step is to use f-ABFs for single cell targeted drug delivery and sensing.

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