Replenishable dendrimer-nanoparticle hybrid membranes for sustained release of therapeutics

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Supplementary Information

Materials and Methods

1. Materials and instruments

The fourth generation (G4) poly (amido amine) (PAMAM) starburst dendrimers, trimethoxysilyl propylene diamine, CS_2, and doxorubicin hydrochloride (Dox) were purchased from Aldrich. Sylgard 184 silicon elastomer kit, anodic aluminum oxide (AAO) membranes (Anodisc 25, 0.02 μm pore size & 60 μm film thickness), and a peristaltic pump were purchased from Fisher Scientific**.** The preparation of water soluble AuNPs (~ 7 nm core diameter) and the expression of green fluorescent protein (GFP) were performed according to known procedures. For *in vitro* cell studies, human cervical cancer (HeLa) cells were purchased from American Type Culture Collection. Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich), fetal bovine serum (FBS, Thermo Scientific), antibiotics (Cellgro) were prepared according to standard protocols**.** Phosphate buffer solution (PBS) was purchased from Thermo Scientific. Photoluminescence was recorded on a Photon Technology International fluorescence spectrometer with Felix 32 and the thickness of nanocomposites was investigated using a profilometer (Dektak 150). Circular dichroism (CD) spectra of the protein were recorded on a Jasco J-715 spectropolarimeter equipped with a thermostatic temperature controller. The transmission electron microscopy (TEM) image was acquired on a JEOL 1000CX operating at 80 KeV. Optical and fluorescence microscopy imaging were performed using an Olympus IX51 microscope and cell viability was determined on a SpectroMax M5 microplate reader (Molecular Devices).

2. Preparation of water soluble gold NPs (AuNPs) with ~7 nm diameter

Water soluble AuNPs were prepared according to a known procedure using a N,N,N-trimethyl (11 mercaptoundecyl) ammonium (TMA) ligands as a capping agent. First, AuNPs (~1.5 nm) were prepared using two-phase (water-toluene) reduction of $AuCl_4^-$ (0.6 mmol) by sodium borohydride (6 mmol) in the presence of a dodecanethiol (0.6 mmol) and TOAB (1.2 mmol) at room temperature, allowing vigorous stirring overnight. The organic phase of the resulting solution was then separated, evaporated completely in a rotary evaporator at 40 ^oC, and dried in vacuum at 30 ^oC for a day. The crude solid obtained was heat-treated at 170 ^oC at the heating rate of 2 ^oC/min, and held 30 min at this temperature. The heat-treated product was dissolved in 30 mL toluene and mixed with 300 mL methanol to remove excess dodecanethiol and TOAB. The dark brown precipitates were filtered off, washed with methanol, and redispersed in toluene. The place exchange reaction was carried out by adding 60 mg TMA ligands in 20 mL dry dichloromethane into a toluene solution of 20 mg dodcanethiol-capped AuNPs, allowing vigorous stirring for two days. The precipitates in the reaction mixture were centrifuged and further washed with ether and dichloromethane twice to remove free thiols. The resulting precipitation was dried in vacuum and was dissolved in MilliQ water to produce the water soluble AuNPs (a diameter ~ 7 nm) with a wine red color. The core size and concentration of AuNPs were achieved using TEM and UV-Vis analysis.

3. Expression of green fluorescence protein (GFP)

Starter cultures from a glycerol stock of GFP [enhanced GFP (eGFP) cloned into the pET21d vector (Novagen) where 6-His tag was located at N-terminal] in BL21(DE3) was grown overnight in 50 mL of 2 × YT media with 50 μL of 1000× ampicillin (16 g tryptone, 10 g yeast

extract, 5 g NaCl in 1 L water). The cultures were shaken overnight at 250 rpm at 37 ^{o}C. The following day, 10 mL of the starter cultures were added to a Fernbach flask containing 1 L of 2 × YT and 1 mL 1000× ampicillin and shaken until OD_{600} = 0.7 was reached. The culture was then induced by adding isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM final concentration) and shaken at 28 ^{o}C. After 3 h, the cells were harvested by centrifugation (5000 rpm for 15 min at 4 ^{o}C). The pellet was then resuspended in lysis buffer (2 mM imidazole, 50 mM NaH_2PO_4, 0.3 M NaCl). The cells were lyzed using a microfluidizer. Once lyzed, the solution was centrifuged at 15,000 rpm for 45 min at 4 ^{o}C. The supernatant was further purified using HisPur Cobalt columns from Pierce (cat. # 89969). The imidazole was removed by dialyzing in 5 mM sodium phosphate buffer (pH = 7.4).

4. Circular dichroism (CD) studies of GFP collected from hybrid films

The CD spectra of proteins were recorded on a Jasco J-715 spectropolarimeter equipped with a thermostatic temperature controller and interfaced to a computer. Measurements were performed in a quartz cell with 10 mm path length. Spectra were recorded at 20 °C, with an average of 3 scans between 195 and 250 nm, with a 2.0 nm bandwidth, a scanning rate of 50 nm/min, a wavelength step of 0.2 nm, and a time constant of 4 s. Protein samples were prepared in 5 mM sodium phosphate buffer solution (pH= 7.4).

5. Fabrication of Dox/GFP loaded membranes

The Den-NP films were coated on both sides of an amine functionalized AAO substrate using the method described in our previously published paper for deposition on non-porous surfaces.[1] In typical reactions, the amine-functionalized AAO was prepared by immersing an

oxidized AAO membrane into a 10 mM solution of trimethoxysilyl propylene diamine in ethanol for 12 h under nitrogen gas. This procedure was followed by rinsing with dichloromethane and ethanol and drying with nitrogen gas. The amine functionalized AAO disk was placed at the bottom of small vial containing a 4 mL mixture consisting of 2 mL of a 250 nM AuNP (7 nm) aqueous solution at pH 9.5, 1 mL of a 1 mM CS_2 methanol solution, and 1 mL of a 50 μM G4 PAMAM dendrimer methanol solution. The composite material precipitated as they were formed onto the AAO substrate at the bottom of the vial. Following completion of the reaction, the remaining solution was removed. The hybrid membrane was dried completely, rinsed with ethanol, and then dried with nitrogen gas. The AAO disk was turned over and the coating process was repeated on the opposite side. The hybrid membrane was then immersed into a solution of 0.05 wt% Dox or 40 μM GFP for loading. After 8 h, films were removed from the loading solution, rinsed with water, and dried with nitrogen gas.

6. Fabrication of PDMS fluidic channels with loaded hybrid membranes

A polydimethylsiloxane (PDMS) replica was fabricated by pouring a 10:1 (v/v) mixture of Sylgard 184 elastomer and a curing reagent over a glass channel mold. The mixture was cured in the oven for 12 h at 60 ^{o}C and then peeled off from the mold. Dox- or GFP-loaded membranes were then fitted to the middle of the PDMS replica and then covered with a flat PDMS surface to form the sealed PDMS fluidic channel. One side of the fluidic channel (that possessed the loaded membrane) was attached to a peristaltic pump that provided a continuous flow of PBS (pH =7.4), while the other side was fitted with collection containers for fluorescence measurements of the released drugs (Figure 2a). The flow rate of PBS was held at 4 mL/h and fractions were collected after passing through the channel. The release profiles of a Dox/GFP-

loaded hybrid film were generated by analyzing the collected PBS fractions using a fluorescence spectrometer.

7. Fabrication of a refillable reservoir in a fluidic channel

Unloaded hybrid membrane films were inserted into an injectable apparatus using the fluidic channel system mentioned above. Additionally, an injection line was harnessed to a reservoir behind the surface of the reusable membrane. The reservoir was fitted with a drainage line to prevent pressure buildup behind the membrane. A Dox solution (0.1 wt% in PBS) was injected through the injection line, and excess solution was allowed to flow out. Once the reservoir solution has been adequately flushed and replaced with fresh solution, both the injection and drain lines were closed. The reservoir was refreshed with Dox solution numerous times, allowing the membrane to release Dox in a sustained manner for 24 h. The release was monitored by a fluorescence spectrometer mentioned above.

8. In vitro cell analysis using a fluidic system

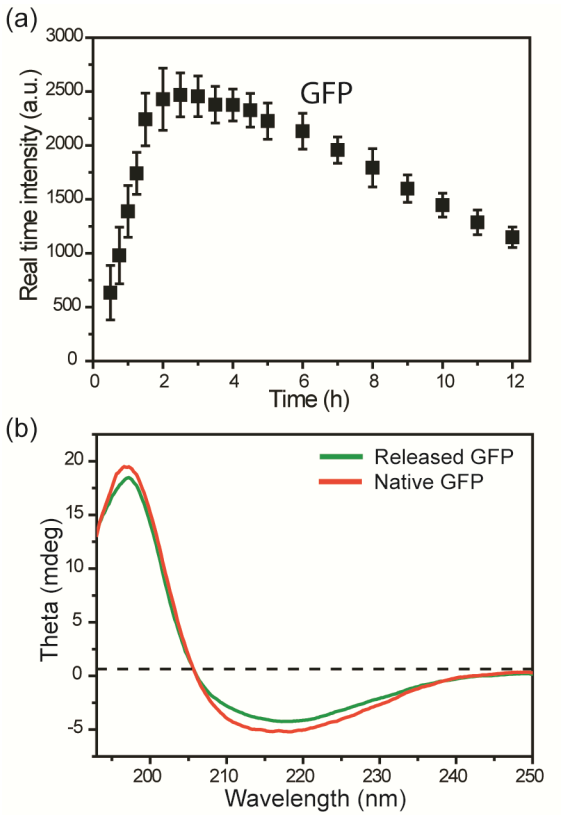
HeLa cells were cultured in DMEM that was supplemented with 10% FBS and 1% antibiotics under a humidified atmosphere of 5% CO_2 at 37 ^{o}C. Cells were seeded at $2x10^5$ cells/well in a 6 well plate for 24 h before introducing them to the fluidic system. Cell culture medium was circulated for 20 h and the apparatus (except for the peristaltic pump) was placed in a CO_2 incubator to maintain proper pH and temperature (Figure 4a). Cells were removed from the apparatus after a 4 h incubation in the presence of the circulating medium, washed with PBS, and observed using a fluorescent microscope to assess Dox loading. Cell viability was assessed by an alamarBlue® (AbD Serotec, UK). After 20 h of incubation, the cells were washed three times with PBS and then incubated with fresh culture medium supplemented with 10 vol%

alamarBlue® solution. Following incubation, 0.1 mL of medium from each well was transferred into a 96 well plate. Fluorescence was measured (excitation 560 nm, emission 590 nm) using a microplate reader (SpectraMax M2, Molecular Devices).

Sustained release and CD of GFP

Having demonstrated successful sustained release of Dox from the Den-NP membrane, we further explored the delivery of biologics, using GFP as a model for therapeutic protein release.[2] A significant challenge in such delivery applications is the proper maintenance of biomolecular structure and activity through the delivery process. Both AuNP and dendrimer scaffolds have been well known for their non-toxicity in biomedical applications,[3, 4] making the Den-NP membrane promising for protein delivery. In practice, the GFP-loaded membrane was tested using the same fluidic procedure mentioned above **(Supplementary Fig. 1a)**.

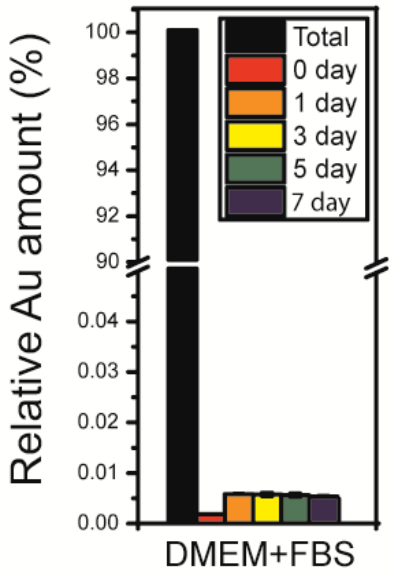
To assess protein stability during the release process, the collected GFP solutions were studied by circular dichroism (CD) analysis. Both native and released GFPs generated similar CD spectra **(Supplementary Fig 1b)**, demonstrating that GFP released from these hybrid films remained in its native and active form during loading and following release.



Supplement Fig. 1. (a) Sustained release profile of GFP ($\lambda_{ex}/\lambda_{em}$: 490/509 nm) collected after having passed through the PDMS channel containing the GFP loaded membrane and (b) CD profiles of released and native GFP.

Stability of the hybrid Den-NP membranes under the physiological condition

To determine long-term stability, the hybrid membrane was stored for 1 week in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). During the week, samples (0.2 mL) were taken every other day (0, 1, 3, 5, and 7 day) to analyze gold (Au) amount. The total amount of Au on the membrane was also measured using inductively coupled plasma mass spectrometry (ICP-MS, NexION 300X, PerkinElmer). Extremely small amounts of Au were detected in 10% FBS-DMEM (Supplement Fig. 2). The relative amounts of Au leached in the medium were below 0.01% after the one-week incubation. These data demonstrate that over 99.99% of AuNPs was still attached to the membrane. Based on the results, the hybrid membrane is stable with no significant leaching of AuNPs within one week.



Supplement Fig. 2. Stability of the hybrid Den-NP membrane in 10% FBS-DMEM. For 1 week, amounts of Au leached in 10% FBS-DMEM were quantitatively analyzed using ICP-MS.

References

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