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Functionalization of Imprinted Nanopores in Nanometer-Thin Organic Materials**

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Keywords

Membranes; Nanopores; Liposomes; Nanocapsules; Self-Assembly

Recently, we described the method for creating nanometer-thin organic materials with programmed size nanopores.[1] Controlling the pore geometry and mass transfer was identified as key to advances in DNA sequencing,[2] microreactors,[3] molecular electronics,[4] and drug-delivery devices.[5] Nanocapsules with selective permeability gained considerable attention in biomedical applications.[6] Controlling the chemical environment of nanopores is critical for realizing the full potential of nanoscale porous materials.[7] Herein, we describe an efficient method for creating uniform nanopores with programmed chemical environment and demonstrate successful quantitative conversion of functional groups.

Using lipid bilayers as temporary self-assembled scaffolds, we directed the assembly of sub-nanometer thin crosslinked organic polymer with embedded pore-forming templates (Figure 1). Previously, we used this method for creating nanocapsules with programmed size pores. [1] Modular construction of the template offers great versatility in varying the pore shape and size as well as the nature and number of functional groups.

Although liposomes[8] made of dimyristoylphosphatidylcholine (DMPC) were used in this work to demonstrate feasibility, we expect the method to be applicable to many other bilayers [9]

The pore-forming template (**1**) was synthesized in one step from commercially available materials. Coupling of 1,2,3,4-tetra-O-acetyl-D- β -glucopyranose with 4-vinylbenzoic acid was performed by a standard protocol and produced the desired product in 85% yield (Scheme 1).

At lipid/template molar ratio of 34 or higher, all template molecules (**1**) are incorporated in the bilayer (Table 1). Following the loading of t-butylstyrene and divinylbenzene (1:1) and **1** into DMPC liposomes and UV-initiated polymerization, methanol was added to precipitate the nanocapsules and remove the lipids; nanocapsules were washed with methanol, resuspended in benzene, and freeze-dried. The FTIR spectrum of the capsules (Figure 2) revealed a characteristic peak at 1759 cm^{-1} corresponding to the C=O stretching of the ester groups. All template molecules (**1**) incorporated in the bilayer were found in the

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nanocapsules after the removal of lipids and multiple methanol washings (Table 1). FTIR spectra of nanocapsules revealed a shift of the band corresponding to the carbonyl group of **1** to higher wavenumbers (1759 cm^{-1} for **1** in nanocapsules vs. 1748 cm^{-1} for free **1**, see Supporting Information), which is common for functional groups incorporated in bulk polymer.[10] These data agree with embedding **1** into the nanothin film as shown on Figure 1.

Alkaline hydrolysis of templates produced pores with free carboxylic groups (Figure 2). In control experiments, nanocapsules made without pore-forming templates and capsules made with glucose pentaacetate (GPA, a structural analog of **1** but without a polymerizable moiety) showed no signals corresponding to the carbonyl group after alkaline hydrolysis (Figure 2). Quantitative FTIR measurements (see Supporting Information) using aromatic C–H signal as an internal standard showed complete conversion of esters into carboxylic acids. We conclude that all templates **1** incorporated in the bilayer co-polymerized with the *t*-butylstyrene/divinylbenzene polymer matrix.

We converted the carboxylic acid groups into the acid chloride by treatment with excess thionyl chloride and then formed two amides by the reaction of acid chloride with benzyl amine or 4-(aminomethyl)benzonitrile (Figure 3). We selected the latter to quantify the amount of amide using C≡N stretching vibration band at 2226 cm^{-1} . The FTIR spectrum of the acid chloride shows the shift of the C=O band to 1774 cm^{-1} compared with 1763 cm^{-1} for the free acid (Figure 3) and absence of a band at 1215 cm^{-1} corresponding to the C(O)–O vibrations (see Supporting Information). The FTIR spectrum of the amide shows the C=O stretching vibrations at much lower frequency (1650 cm^{-1}) than that of the free acid. Using the intensity of cyano group absorption band, we found that the carboxylic acid was completely converted to the amide.

If 34:1 ratio of DMPC to **1** is used, considering that area of each DMPC molecule is 62 Å^2 , [11] the resulting nanoporous material has an estimated pore density of 9.5×10^{16} pores per m^2 with an average distance between centers of pores of 3.2 nm and 3×10^3 pores per 100 nm capsule.

Electron microscopy images demonstrated preservation of shape and integrity of nanocapsules (Figure 4). The size and shape of liposomes loaded with **1** and monomers (Figure 4a) are similar to those of liposomes containing polymerized capsules (Figure 4b). In agreement with previous reports,[1,8g] the detergent-assisted lipid removal did not affect the size distribution of nanocapsules (Figure 4d). Remarkably, SEM image shows clusters of nanocapsules with the same size after the lipid removal, hydrolysis, precipitation in methanol, multiple washings, resuspension in benzene, and freeze-drying (Figure 4c). These results suggest that the nanocapsules with nanometer-thin walls are stable under regular handling conditions such as solvent exchange.

We used the previously described colored size probe retention assay to demonstrate the successful formation of nanopores with narrow size distribution.[1] We encapsulated a mixture of molecules with different colors and sizes in a liposome, carried out the polymerization, and separated the capsules from released probes on a size exclusion column. We used 0.6 nm yellow probe (methyl orange), 1.1 nm red probe (Procion Red), and 1.6 nm blue probe (1:1 β -cyclodextrin-Reactive Blue conjugate) to gauge the pore size.[1] All probes were retained in the capsules prior to template removal. After opening the pores, we observed complete release of 0.6 nm probes and retention of 1.1 nm and 1.6 nm probes (Figure 5) suggesting 0.8 ± 0.2 nm pore size. Considering that size probe release from 100 nm capsules would occur faster than the chromatographic separation, quantitative retention of

1.1 nm and 1.6 nm probes allows us to conclude that very few capsules, if any, contain pinholes or pores larger than 1.1 nm.

The pore size is preserved even after freeze-drying the nanocapsules and resuspending them in water. When porous capsules containing 1.1 nm probes were dried, solubilized in 2% Triton X-100 solution and passed through a size-exclusion column, no release of the encapsulated size probes was observed. Combined with SEM images (Figure 4) this provides strong evidence that the materials preserve both their structure and function upon solvent exchange and drying.

In summary, we demonstrated an efficient method for controlling the chemical environment of molecular-size pores in nanometer-thin organic materials. This method combines the use of temporary self-assembled scaffolds with molecular imprinting, which was widely used to fabricate functional materials.[12] We created uniformly sized pores with a single carboxylic functional group and quantitatively converted the carboxylic group into an acid chloride and subsequently into an amide. This opens opportunities for further functionalization for controlling the mass transfer across the pore, e.g. with stimuli-responsive moieties or creating arrays of functional groups that may potentially act as molecular recognition sites.

Experimental Section

1: 1,2,3,4-tetra-O-acetyl-D- β -glucopyranose (522 mg, 1.5 mmol), DMAP (37 mg, 0.3 mmol) and DCC (310 mg, 1.5 mmol) were added to a stirred solution of 4-vinylbenzoic acid (222 mg, 1.5 mmol) in anhydrous CH_2Cl_2 (5 ml)/DMF (4 ml) mixture at 0 °C. The reaction mixture was stirred for 5 min at 0 °C and then 48 h at ambient temperature. The reaction was monitored by TLC (hexane/ethyl acetate, 1:1). The precipitated urea was filtered off and the filtrate was evaporated in vacuum. The residue was taken up in CH_2Cl_2 and the solution was washed with 3% aqueous NaHCO_3 and dried over molecular sieves. The solvent was evaporated to yield the product as clear oil, which crystallized within 15 minutes. Yield 85%; mp 121-122 °C; ^1H NMR (270 MHz, CDCl_3): δ 7.6-7.4 (m, 2H) 8.1-7.9 (m, 2H), 6.9-6.6 (m, 2H), 6.00-5.80 (m, 2H), 5.80-5.70 (m, 2H), 5.35-5.10 (m, 2H), 4.60-4.45 (m, 2H), 4.40-4.20 (m, 2H), 4.15-3.80 (m, H), 1.96, 2.01, 2.05, 2.10 (4s, 12H); ^{13}C NMR (270 MHz, CDCl_3): δ 20.6, 20.8, 20.9, 21.1 (CH_3), 62.2 (d, $J_{\text{C,C}} = 44.2$ Hz, 1C, C-6), 68.2, 69.3, 72.8, 73.0 (C-2,3,4,5), 91.8 (d, $J_{\text{C,C}} = 44.2$ Hz, 1C, C-1), 116.7, 136.1 (C=C), 128.3, 130.2, 133.7, 142.3, (Ph), 166.8 169.0, 169.5, 169.6, 170.2 (C=O). Elemental analysis calculated for $\text{C}_{23}\text{H}_{26}\text{O}_{11}$: C, 57.74; H, 5.48. Found: C, 57.64; H, 5.61.

Nanocapsules with functionalized nanopores

t-Butylstyrene (17.64 μL , 9.63×10^{-5} mol), p-divinylbenzene (13.70 μL , 9.62×10^{-5} mol), and 2,2-dimethoxy-2-phenyl-acetophenone (a UV initiator; 3 mg, 0.117×10^{-5} mol) were added to a solution of DMPC (5.9×10^{-5} mol, 20 mg/ml in chloroform) and **1** (0.87×10^{-5} mol, 2.08 mg/ml in chloroform). The monomers were purified before addition on a column of neutral alumina. Chloroform was evaporated using a stream of purified argon to form a lipid/monomer film on the wall of a culture tube. The lipid film was further dried under vacuum for 30 minutes to remove traces of chloroform. GC and UV-vis analysis confirmed that the concentration of monomers after drying remained the same. The dried film was hydrated with DI water giving a dispersion of multilamellar vesicles, which was then extruded at 32 °C through a polycarbonate Nucleopore track-etch membrane (Whatman) with 0.1 μm pore size using a Lipex stainless steel extruder (Northern Lipids). Prior to the polymerization, oxygen was removed by passing purified nitrogen or argon through the solution. The sample was irradiated ($\lambda=254$ nm) in a photochemical reactor equipped with a stirrer (10 lamps, 32W each; 10 cm distance between the lamps and the sample) for 60 min.

UV and GC analyses confirmed that >90% of monomers were polymerized. Triton X-100 (0.5 ml, 2%) and NaOH (0.5 ml, 1M) were added, and the mixture was stirred for 1 h at ambient temperature. Methanol (10 ml) was added, and the precipitate was washed 3-5 times with methanol, resuspended in benzene and freeze-dried. The nanocapsules with carboxylic groups (10 mg) were suspended in toluene (3 ml), mixed with thionyl chloride (5 ml) and refluxed for 12 hours. The mixture was evaporated to dryness and washed with toluene to yield nanocapsules with acid chloride groups. These capsules were suspended in toluene (3 ml), then triethylamine and either 4-(aminomethyl)benzonitrile (0.2 g) or benzylamine (3 ml) were added, and the reaction mixture was refluxed overnight to produce nanocapsules with amide groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Synthesis of polymerizable and degradable pore-forming template (**1**).

Figure 1.

Directed assembly of nanothin polymer membranes with uniform imprinted nanopores. A) The self-assembled phospholipid bilayer is loaded with hydrophobic monomers and pore-forming templates. The template is a single molecule consisting of three parts: a polymerizable moiety (gray) to covalently anchor the template to the polymer matrix, a degradable linker (red) to create a functionalized pore, and bulky hydrophobic unit (blue) to define the pore size. B) Polymerization produces a nanothin film with co-polymerized template molecules in the bilayer interior. C) Phospholipids are removed with the help of a detergent or solvent exchange. D) Pore-forming templates are removed by chemical degradation to yield nanothin films with nanopores containing a single functional group.

Figure 2.

FTIR spectra of the nanocapsules without pore-forming template (blue), nanocapsules with GPA after hydrolysis (purple), nanocapsules with **1** before (green) and after (red) hydrolysis.

Figure 3.

FTIR spectra of the nanocapsules with free carboxylic groups (blue), nanocapsules after reflux with thionyl chloride (red) and subsequent treatment with 4-(aminomethyl)-benzonitrile (green).

Figure 4. Electron micrographs of nanocapsules. a) TEM images of liposomes loaded with monomers, and b) polymer nanocapsules after polymerization. c) SEM image of polymer nanocapsules after hydrolysis and freeze-drying. d) TEM image of polymer nanocapsules after polymerization and treated with TX-100.

Figure 5.

Selective permeability demonstrates successful formation of 0.8 ± 0.2 nm pores.

Nanocapsules were prepared with encapsulated colored size probe mixtures and separated on a size-exclusion column to remove released probes. The nanocapsule fraction is shown. Sample composition is as follows: 1) 0.6 nm (yellow) probe before template removal; 2) same as 1 after template removal (complete release of 0.6 nm probe); 3) 0.6 nm and 1.1 nm (red) probe before template removal; 4) same as 3 after template removal (complete release of 0.6 nm probe and retention of 1.1 nm probe); 5) 0.6 nm, 1.1 nm, and 1.6 nm (blue) probes before template removal; 6) same as 5 after template removal (release of 0.6 nm probe and retention of 1.1 nm and 1.6 nm probes); 7) 0.6 nm and 1.6 nm probes before template removal; 8) same as 7 after template removal (release of 0.6 probe and retention of 1.6 nm probe).

Table 1Incorporation of **1** into the liposomal bilayer and nanocapsules.

Initial molar ratio lipid/template	Found in liposomes ^[a] % of initial amount	Found in capsules ^[b] % of initial amount
41	99±2	98±3
34	98±2	96±5
28	94±4	84±7
21	77±5	67±4
14	59±2	56±4
7	41±4	36±2

^[a] measured by HPLC^[b] measured by FTIR