NANOPOROUS SILICA COLLOIDAL FILMS WITH MOLECULAR TRANSPORT GATED BY APTAMERS RESPONSIVE TO SMALL MOLECULES

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Dedicated to Dr. Zdeněk Havlas on the occasion of his 60th birthday.

We report the preparation of colloidal nanoporous silica films whose function mimics that of protein channels in gating the transport of small molecules across a cell membrane. Specifically, we report a means of controlling the molecular flux through colloidal nanopores that employ aptamer oligonucleotides binding to a specific organic small molecule (cocaine). These biomacromolecules have been introduced onto the nanopore surface by attaching pre-made oligonucleotides to the activated nanopore surface. The aptamers change their conformation in response to the binding events, and thus alter the free volume of the colloidal nanopores available for molecular transport.

Keywords: Silica; Nanopore; Membranes; Aptamers; Cocaine; Surface chemistry; Colloids; Nanopores; Electrodes.

Nanoporous films and membranes are of great interest as materials with the ability to control molecular transport on the nanoscale by controlling both nanopore size and surface functional groups. Many types of nanoporous membranes have been prepared using polymers[1], carbon nanotubes[2–4], zeolites[5] and alumina[6,7]. Such nanoporous membranes have been applied for the separation of biomacromolecules[8] and chiral drug molecules[9] and are also used as chemical sensors[10] or for the controlled release of therapeutic agents[11,12]. These materials, however, have a number of limitations. The most significant include low molecular flux due to low porosity, and

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the inability to precisely control the pore size of – and thus flux through – the membrane. Previous studies in our group have focused on aptamer-modified single glass nanopores. While that approach has afforded us with a system in which the molecular flux through the nanopore is controlled by the cocaine binding event, the single nanopore is not suitable for separations. In the present work, we report the fabrication of a nanoporous silica membrane surface-modified with the cocaine-sensing aptamer. In this work, the membrane is supported by a microelectrode and the through-pore flux, modulated by the presence or absence of a specific small molecule (cocaine), is monitored using cyclic voltammetry.

As the substrate for our membranes we employ silica colloidal crystals, which are characterized by high molecular flux, easily and accurately controllable nanopore size, and well-developed surface chemistry[13]. These silica colloidal crystal membranes are formed via self-assembly of silica nanoparticles into a close-packed face-centered cubic (fcc) lattice[14]. They contain ordered arrays of three-dimensional interconnected nanopores whose size and thickness can be easily controlled[15].

In order to generate actively controllable pore sizes, we modify our nanopores with a responsive DNA aptamer. Since the development of the SELEX process in the early 1990's, aptamer oligonucleotides exhibiting selective and specific binding properties towards molecules or proteins have been developed for uses in medicine, analytical chemistry, and materials science[16,17]. These aptamers can be selected for affinity to small molecules, biopolymers, surfaces, or even whole cells[18], and can be of use in the preparation of nanoporous materials whose function mimics that of protein channels in gating the transport of a small molecule across a cell membrane. Specifically, these aptamers should provide the ability to control the molecular transport through a nanoporous colloidal film by utilizing conformational changes in a biopolymer in response to small molecule binding.

The aptamer used in our work is based on an oligonucleotide binder selective for the detection of cocaine which was previously engineered by Stajonovic et al.[18]. The secondary structure for the 32-base cocaine aptamer possesses a three-way junction, in the middle of which there is a cavity which binds the target molecule (Fig. 1). In the absence of a target, however, the aptamer is thought to remain partially unfolded, with only one of the three junctions folded. We expected that the conformational change of the oligonucleotide inside the nanopores in the presence of cocaine would cause a reversible increase in the rate of diffusion through the nanopore.

To demonstrate the feasibility of this approach, we introduced the cocaine responsive aptamers into the nanopores by attaching pre-made biomacromolecules to the activated nanopore surface[19]. Previous work in our group showed that such surface modifications do not perturb the colloidal crystal lattice[20]. We then measured the transport rates of a neutral redox-active probe molecule (ferrocene dimethanol) through the resulting nanoporous films using cyclic voltammetry as a function of cocaine concentration.

EXPERIMENTAL

Materials and Reagents

(3-Aminopropyl) triethoxysilane (99%) and tetraethoxysilane (TEOS, 99.999+%) were obtained from Aldrich and used as received. 1,1′-Ferrocenedimethanol, $Fc(CH_2OH)_2$ (98%, Aldrich), potassium chloride (99%, Mallinckrodt) were used as received. Acetonitrile (Mallinckrodt) was distilled before using. 18 MΩ cm water was obtained from a Barnsted "E-pure" water purification system. Mercaptohexanol was obtained from Aldrich and used as received. Diethyl ether was obtained from Mallinckrodt and used as received. Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, SMCC (>99%) and tris(2-carboxyethyl)-phosphine hydrochloride, TCEP·HCl, were from Pierce (Rockford, IL) and used as received. *N*,*N*-Dimethylformamide (DMF, 99.9%) was obtained from Fisher Scientific and was dried over molecular sieves. 4-Dimethylaminopyridine, DMAP (>99%, Aldrich), was used as received. Tris-EDTA buffer solution (Sigma) was used as received. Cocaine hydrochloride was received from Sigma–Aldrich and stored at 2–8 °C until use. 3-Aminopropyldimethylethoxysilane, $EtO(Me)_2Si(CH_2)_3NH_2$, (Gelest, Inc.) was used as received.

The DNA aptamer was synthesized (Biosource International, Camarillo, CA) for the preparation of the electrochemical cocaine biosensor. The aptamer sequence was based on the oligonuclotide sequence developed by Stojanovic et al.[18] The sequence, AGACAAGGAAAATCCTTCAATGAAGTGGGTCG, was modified with a six-carbon disulfide group at the 5′ terminus. The aptamer was purified by HPLC and PAGE, and its sequence was verified by mass spectrometry.

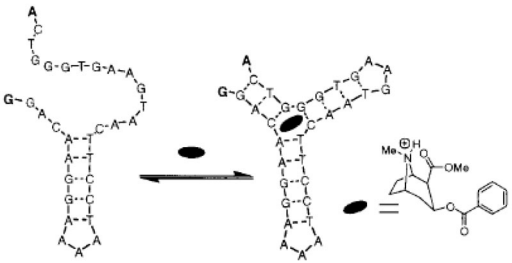


FIG. 1
Cocaine-sensing aptamer binding to cocaine

Preparation and Modification of Silica Spheres in Solution

Silica nanoparticles were prepared following the literature procedure[21]. A solution of tetraethoxysilane (TEOS) in absolute ethanol was rapidly poured into a stirred mixture of ammonia and water in absolute ethanol at room temperature. The final concentrations of the reagents were 0.2 M TEOS, 0.4 M ammonia and 17 M water for 290 nm silica nanoparticles, and 0.2 M TEOS, 0.2 M ammonia and 17 M water for 100 nm nanoparticles. The reaction mixture was stirred for 18 h. The silica spheres were isolated by repeated centrifugation and re-suspension in absolute ethanol. The diameter of the spheres was found to be 290 ± 2.5 nm using dynamic light scattering (DLS).

Amine fictionalization of silica spheres was achieved by treatment with a solution of 0.056 M (3-aminopropyl)triethoxysilane in dry acetonitrile at room temperature for 17 h followed by centrifugation and resuspension in acetonitrile. The presence of amino groups was confirmed by treating the silica spheres with dansyl chloride, followed by fluorescence measurements, as previously described[22].

Colloidal Film Electrodes

Pt microdisk electrodes (25 μm in diameter) shrouded in glass were prepared by first attaching a 1.0-mm-diameter Cu wire (Alfa Aesar) to a 25 μm-diameter Pt wire using Ag paint (DuPont). The Pt wire was then flame sealed in a glass capillary which was then bent into a U-shape and cut orthogonal to the length of the capillary with a diamond saw to expose the Pt disk. The resulting electrodes were polished with Microcut Paper disks (Buehler), from 240 to 1200 grit in succession, until the surface was free from visible defects.

The colloidal films were deposited onto the Pt and the glass shroud by placing the electrodes vertically into 1.5 wt.% colloidal solution of silica spheres in ethanol. The vials were placed under a crystallization dish and the solutions were allowed to evaporate for 3 days in a vibration-free environment.

Preparation of Maleimide-Activated Silica Spheres[23]

Amine-functionalized silica nanoparticles (1 mg) were first dried under vacuum for 24 h. SMCC (2.8 mg, 6.7 μmol) and DMAP (1.1 mg, 8.9 μmol) were added to the dried nanoparticles in DMF (5 ml) and the reaction mixture was stirred in the dark at room temperature for 24 h. The resulting maleimide-acitivated silica was collected by centrifugation and washed with DMF (10×), and 10 mM potassium phosphate buffer pH 7.0 (3×) and used immediately.

Activation of the Aptamer[24]

The activated aptamer was prepared by combining 0.2 mM aptamer stock solution in ×1 Tris buffer with 10 mM aqueous TCEP·HCl at a 1:1 (v/v) ratio and allowed to react at 4 °C for at least 2 h and used in the preparation of aptamer-modified silica nanoparticles without further purification.

Preparation of Aptamer-Modified Silica Nanoparticles[24]

The aptamer/TCEP solution (6.75 ml) was combined with 100 mM NaCl/10 mM potassium phosphate buffer pH 7.0 (18.9 ml) to make the aptamer solution. The maleimie-activated

silica nanoparticles (~0.5 mg) were then dispersed into the solution and the reaction was stirred in the dark for 25 min.

Passivation of Silica Surface[24]

Passivation of the area of the amine-modified silica surface still exposed after aptamer immobilization was achieved by stirring the nanoparticles in 2 mM mercaptohexanol solution in 1 M NaCl/10 mM potassium phosphate buffer pH 7.0 in the dark at room temperature for 3 h. Upon completion of passivation, the nanoparticles were washed (3×) with 1 M NaCl/10 mM potassium phosphate buffer pH 7.0 and collected by centrifugation.

Preparation of Maleimide-Activated Silica Colloidal Film[24]

First, amine modification of the colloidal films was achieved by immersing the colloidal film electrode in a 0.056 M solution of (3-aminopropyl)triethoxysilane in dry acetonitrile at room temperature for 17 h. The electrodes were then soaked in dry acetonitrile for 1 h.

Next, the surface of the colloidal film on the Pt electrodes was dried under vacuum for 24 h before immersing the electrodes vertically into a stirred solution of DMF containing DMAP (0.5 mg, 4.5 μmol) and SMCC (1.4 mg, 3.4 μmol). The solution was stirred in the dark at room temperature for 24 h. The resulting maleimide-acitivated silica colloidal film was washed with DMF in the dark for 1 h and then 100 mM NaCl/10 mM potassium phosphate buffer pH 7.0 in the dark for 1 h and used immediately.

Preparation of Aptamer-Modified Silica Colloidal Film[24]

Aptamer/TCEP solution (6.75 ml) was combined with 100 mM NaCl/10 mM potassium phosphate buffer pH 7.0 (18.9 ml) to give the aptamer solution. The maleimide-activated colloidal film electrode was then immersed vertically into the stirred solution in the dark at room temperature for 25 min.

Passivation of Silica Colloidal Film[24]

Passivation of the area of the amine-modified silica surface still exposed after aptamer immobilization was achieved by stirring the electrode in 2 mM mercaptohexanol solution in 1 M NaCl/10 mM potassium phosphate buffer pH 7.0 in the dark at room temperature for 3 h. Upon completion of the passivation, the electrode was washed with 1 M NaCl/10 mM potassium phosphate buffer pH 7.0.

Characterization

The molecular flux across the colloidal film was measured voltammetrically using a Par Model 175 Universal Programmer and Dagan Cornerstone Chem-Clamp potentiostat. The voltammetric responses of the bare, amine-modified, maleimide-modified, and aptamer-modified colloidal electrodes were measured in solutions of 10 mM monobasic/dibasic potassium phosphate buffer pH 7 with 1.6 mM $Fc(CH_2OH)_2$ and 0.2 M KCl as supporting electrolyte with 1 ml methanol/5 ml redox solution. The voltammetric response of aptamer-modified colloidal electrodes to cocaine was measured in a solution of 10 mM monobasic/dibasic potassium phosphate buffer pH 7 with 1.6 mM $Fc(CH_2OH)_2$ and 0.2 M KCl with 1.0 mg/ml cocaine hydrochloride solution in methanol with 1 ml/5 ml redox solution.

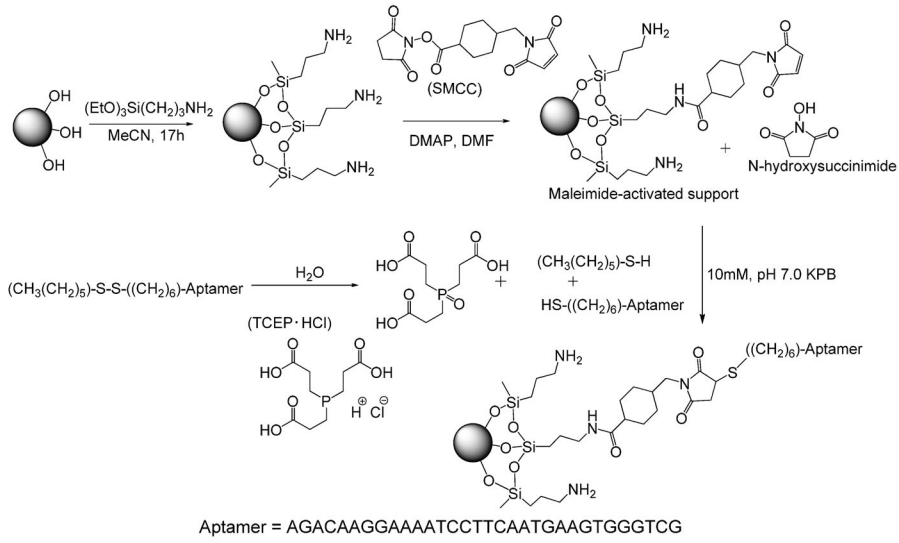
Aqueous solutions were prepared using 18 MΩ cm water and purged with nitrogen to remove dissolved oxygen. Dynamic light scattering (Brookhaven ZetaPALS, measurements conducted in water at 25 °C) were employed to perform size characterization of aptamer-modified silica spheres. UV-Vis spectra were measured in chloroform and recorded on an Agilent 8453 diode array spectrophotometer in order to determine the coverage of the aptamer on the surface of the colloidal nanoparticles.

RESULTS AND DISCUSSION

Aptamer Modification of Silica Spheres in Colloidal Solution

It has been shown that silica can be prepared with sulfhydryl-reactive functional groups for the immobilization of proteins for the use in high-performance affinity chromatography[23]. However, to our knowledge, there have been no reports on modification of silica nanoparticles with aptamer-based receptors. Thus, we developed the following procedure to attach the aptamers to the silica surface. Our approach was adapted from the previously reported work on the activation of silica with maleimide[23].

First, we modified the 290 nm silica nanoparticles with maleimide by treatment with SMCC in DMF in the presence of DMAP (Scheme 1). The resulting sulfhydryl-reactive group activates the surface and allows it to react



SCHEME 1
Preparation of aptamer-modified silica spheres

with the free thiol on the aptamer. In order to attach the aptamer to the maleimide-activated silica nanoparticles, the disulfide end group was cleaved with TCEP·HCl. The maleimie-activated silica nanoparticles were then subjected to the aptamer/TCEP solution.

In order to determine the surface coverage of the aptamer-modified silica particles, they were sonicated in chloroform for 30 min and the UV spectra were obtained. Based on the absorbance at 260 nm, the surface coverage was estimated using 2.07 g cm^{-3} density for silica spheres and extinction coefficient of the aptamer at 260 nm provided by the manufacturer. Surface coverage was calculated to be 0.5–2 molecules nm^{-2}. We assume that similar surface coverage will be achieved for silica colloidal films.

Maleimide Activation and Aptamer Modification of Colloidal Films

The colloidal films were assembled on the surface of Pt microelectrodes shrouded in glass using 1.5 wt.% solution 290 nm silica spheres. The surfaces were modified with amines, and maleimide activation was performed on the modified colloidal film electrodes in DMF solution. The electrodes were removed from solution and rinsed with DMF and potassium phosphate buffer solution. The aptamer was activated by the reducing agent TCEP·HCl in order to cleave the disulfide group on the aptamer to generate a free thiol for reaction with the maleimide-functionalize colloidal film. The electrodes were then modified with the activated aptamer solution and washed again with potassium phosphate buffer. After the aptamer addition, the surface of the nanoparticles still exposed was passivated with mercaptohexanol to create an aptamer/mercaptohexanol monolayer on the surface of the nanoparticles.

The limiting current of $Fc(CH_2OH)_2$ was measured for the electrodes after each modification, and compared to the initial limiting current, as shown in Fig. 2. The limiting current measured for the electrodes decreases after each modification, suggesting that an organic layer has been formed inside the nanopores. Calculated[25] nanopore sizes and the corresponding organic film thicknesses are given in Table I. The increase in the organic film thickness for APTES and maleimide modification is consistent with the formation of one or two layers on the surface of the nanopores. This appears to be true also for the aptamer surface modification. While the size of unfolded aptamers can only be estimated, multiple NMR[26] and X-ray structures[27] have been reported for aptamers bound to small molecules, with the size ranging from 3.5 to 6.8 nm. In the case of the larger nanopores it appears that clearly more than a monolayer of the aptamer is introduced,

based on the calculated thickness. This may result from the ability of the larger nanopores to accommodate more aptamer macromolecules.

Cocaine Response for Aptamer-Modified Colloidal Films

We investigated the molecular transport through the aptamer-modified colloidal films to the presence of cocaine using cyclic voltammetry. To exclude the possibility that the observed changes in the molecular transport would result from electrostatic effects[28,29] we examined the response of the colloidal film electrodes for a neutral redox probe, $Fc(CH_2OH)_2$. As can be seen in

TABLE I
Relative limiting current for colloidal film Pt electrodes as a function of the surface modification, the corresponding effective nanopore radius and organic film thickness inside the nanopore

Initial radius nm	Surface modification	$i_{lim}/i_{lim(0)}$	Resulting radius nm	Film thickness nm
22.5	APTES	0.93	21.0	1.2
	maleimide	0.91	20.2	1.6
	aptamer	0.32	14.3	12.1
7.8	APTES	0.69	6.0	1.8
	maleimide	0.68	5.9	1.9
	aptamer	0.28	3.4	4.4

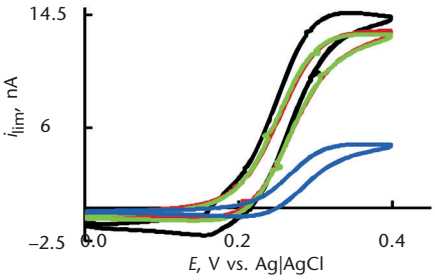


FIG. 2
Representative $Fc(CH_2OH)_2$ voltammetric responses for unmodified colloidal film Pt electrode (black), amine-modified (brown), maleimide-modified (green), and aptamer-modified silica colloidal film Pt electrode (blue)

Fig. 3, the limiting current for the aptamer-modified colloidal film electrodes is affected by the addition of cocaine to the solution. A 9.0 ± 3.5% change in the limiting current was observed as a result of the cocaine addition (measurements were performed in triplicate to ensure reproducibility), corresponding to 0.6 nm increase in effective nanopore radius (Table I). The cocaine-dependent change in the limiting current is reversible and the nanoporous aptamer-modified colloidal films could be regenerated by immersing the electrode into a potassium phosphate buffer. We attribute this behavior to the conformational change in the secondary structure where the aptamer changes from partially unfolded conformation with only one of the three junctions folded to a conformation with a three-way junction containing the cocaine molecule in the internal cavity (Fig. 1). With the formation of the three-way junction, the space that the aptamer occupies inside the nanopores is reduced, allowing for increased transport through the nanopores.

To verify that the responsive behavior described above results from the nanopore surface modification, the limiting current for glass-shrouded platinum microelectrodes carrying the unmodified colloidal film was examined. The resulting electrodes did not exhibit any significant response to the presence or absence of cocaine (data not shown). Previously, studies with this aptamer have been conducted in order to assess its ability to detect the cocaine molecule in complex, tainted samples[30]. These studies concluded that in the presence of biological fluids and other contaminants, the conformational change of the aptamer remains selective to cocaine.

In order to determine the effect of nanopore size on the aptamer-modified film response to cocaine, colloidal films were assembled using

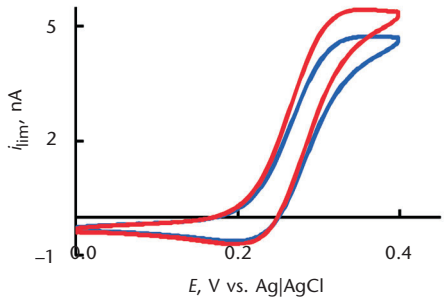


FIG. 3
Representative $Fc(CH_2OH)_2$ voltammetric responses for an aptamer-modified colloidal film Pt electrode in the absence (blue) and in the presence of cocaine (red)

100-nm silica spheres. The surfaces were then modified with amines, followed by modification with the maleimide linker and aptamer as well as passivated, as described above. The limiting current of $Fc(CH_2OH)_2$ was measured for the electrodes after each modification, and compared to the initial limiting current. The limiting current measured for the electrodes decreases after each modification, suggesting that an organic layer has been formed inside the nanopores.

We investigated the response of the aptamer-modified colloidal films with smaller nanopores to the presence of cocaine using cyclic voltammetry, as described above. We found that the change in limiting current resulting from cocaine binding is indeed affected by the size of the nanopore, as shown in Table II. For the nanopores that are ca. 2.9 times smaller, the limiting current increase is ca. 2.6 times higher compared to the larger nanopores. This observation can be rationalized by assuming that the aptamer change in size as the result of cocaine binding remains constant regardless of the nanopore size, while having a greater effect for the smaller nanopores. Indeed, the calculated change in the organic layer thickness inside the nanopores resulting from cocaine binding is essentially identical for both nanopore sizes studied (0.4 nm for the smaller and 0.6 nm for the larger pores). This thickness change is presumably proportional to both the conformational change the aptamer undergoes in the process of the small molecule binding and its packing density on the nanopore surface.

Response for aptamer-modified colloidal films with respect to nanoparticle size was also measured. Colloidal films comprised of 290 nm diameter nanoparticles exhibited near saturated gating at ca. 235 μM cocaine, which, while significantly higher than the dissociacion constant of 90 μM reported for this aptamer, is consistent with previous results published for nanopores modified with this aptamer[31]. Concentration response was also measured for 100 nm modified colloidal films with an observed near saturated gating of ca. 588 μM. This increased gating may be a result of the

TABLE II
Relative limiting current for colloidal film Pt electrodes in response to cocaine and the change in the organic film thickness inside the nanopore as a function of the nanopore size

Nanopore radius, nm	Ave.% increase i_{lim}	Film thickness change, nm
22.5	9.0 ± 3.5	–0.6
7.8	23.0 ± 8.0	–0.4

smaller nanopores formed from 100 nm nanoparticles, contributing to less efficient aptamer modification and thus, less efficient gating.

CONCLUSIONS

We have prepared aptamer-modified silica colloidal nanopores using maleimide activation of amine-modified silica surfaces. Aptamer modification of the colloidal films did not perturb the colloidal lattice. We then observed the response of the molecular flux through aptamer-modified colloidal films comprising 100 and 290 nm silica nanoparticles (7.8 and 22.5 nm radius nanopores, respectively) to cocaine using cyclic voltammetry. The molecular flux reversibly increases in the presence of cocaine indicating conformational changes (folding) of the aptamer as the result of the cocaine binding leading to ca. 0.5 nm increase in nanopore radius. This response is greater for colloidal films containing smaller nanopores.

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