Reversible Biochemical Switching of Ionic Transport through Aligned Carbon Nanotube Membranes

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Synthetic nanopore membranes can be used to mimic ion channels provided that molecular transport through membranes is precisely gated with selective and reversible chemical interactions. Aligned nanotubes of carbon or other inorganic materials can be assembled to construct higher-order supramolecular architectures using polymer films to force chemical flux through hollow cores. Open tips of carbon nanotubes (CNT) can be activated to have carboxylic groups, which can be easily derivatized with a molecule that binds to a bulky receptor that can open/close the pore entrance. In particular, the core entrances of an aligned CNT membrane were functionalized with a desthiobiotin derivative that binds reversibly to streptavidin, thereby enabling a reversible closing/opening of the core entrance. Ionic flux through the CNT membrane was monitored using optically absorbing charged marker molecules. The flux is reduced by a factor of 24 when the desthiobiotin on the CNT is coordinated with streptavidin; release of streptavidin increases the flux, demonstrating a reversible ion-channel flow. Analysis of solutions of released streptavidin shows approximately 16 bound streptavidin molecules per CNT tip.

Introduction

Nanoporous membranes have emerged as promising materials for ion channel mimetics, drug delivery systems and chemical separation units.[1−5] A major challenge associated with the use of synthetic membranes to mimic biological systems is instilling them with reversible gating properties. A case in point of such a reversibility is the nicotinic acetylcholine receptor, one of the most widely studied ligand-gated ion channels.[6] When acetylcholine binds to the α subunit of the acetylcholine receptor, the channel opens to allow the flow of sodium and potassium ions. Dissociation of acetylcholine from the receptor leads to channel closing. Hydrogels have been proposed as materials for use in gated membranes because of their reversible phase change, which is mostly controlled by external chemical stimuli, such as pH (swelling/deswelling of the hydrogel), temperature, or variation of electric charge.[7−10] Hydrogels, however, have some disadvantages.[11,12] For example, molecular diffusivity is low in hydrogels because of restricted chain mobility, volume changes in the hydrogel take longer time (lag time), and hydrogels typically lack mechanical stability.[13,14] In contrast, aligned carbon nanotube (CNT) membranes, the subject of the present study, are better suited as mimics of protein ion channels. These artificial channels could be designed to have desired molecular selectivity and transport properties.[15,16]

Recently, there is a high interest in biointerfaces that involve nanomaterials, such as quantum dots, fullerenes, and nanotubes/nanofibers. Controlling the interactions between biomolecules and these nanomaterials could enhance the range and functionality of the latter.[17−19] For example, proteins, nucleic acids, and other biologically active compounds can be covalently attached to chemically functionalized carbon nanotubes to form vehicles for drug delivery.[20,21] In that regard, CNTs functionalized with a peptide

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from the α subunit of the G_S protein could translocate across cell membranes.[22,23] An aligned array of CNTs impregnated in a polystyrene matrix has recently been reported to form a membrane structure with well-ordered, nanometer-scale pores.[21] Importantly, the open tips of the carbon nanotubes in this membrane terminate in carboxylates that can be easily derivatized with a molecule that binds to a bulky receptor, which subsequently can be used to open/close the pore entrance. In the present study, our aim is to switch the functionalized nanopore membranes between on handoff states reversibly by attaching and releasing the receptor in a controlled fashion (i.e., receptor-stimulated switching). In the present study, we have used a desthiobiotin derivative that binds reversibly to streptavidin to modify the entrance of the through pores of membrane-embedded CNTs, thereby enabling a reversible on/off system. Transport through the nanotube pores is switched off when streptavidin binds to the functionalized membrane and turned on when streptavidin is released.

Experimental Section

Materials. DSB-X Biotin hydrazide (a desthiobiotin derivative), streptavidin, and fluorescein-isothiocyanate-labeled streptavidin were purchased from Molecular Probes, (Eugene, OR). Methyl viologen dichloride (MV^{2+}) and tris(2,2′-bipyridyl)dichlororuthenium ($Ru(bpy)_3Cl_2$) were purchased from Aldrich, (Milwaukee, WI). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Pierce (Rockford, IL).

Membrane Fabrication. CNT membranes were fabricated using a previously described method.[21] Briefly, an aligned array of multiwalled CNTs having a mean pore diameter of 7.5 ± 2.5 nm was grown by chemical vapor deposition using a ferrocene/xylene feed gas. The volume between the CNTs was filled with polystyrene, and the composite film was removed from the quartz substrate with hydrofluoric acid. Excess surface polymer and Fe catalyst nanocrystals at the CNT tips were removed by H_2O plasma oxidation, resulting in a membrane structure with CNT cores traversing the polystyrene film and having carboxylate functionalization at the CNT tips. Figure 1 shows an SEM image of the aligned multiwalled CNT membrane. Our previous report[20] demonstrated that streptavidin could block the pore entrance of the CNTs that had been prefunctionalized with biotin. However, this process was not reversible and thus not well suited for sensor or ion-channel mimetic applications.

Conjugation of DSB-X Biotin Hydrazide. CNT membranes were functionalized with DSB-X biotin hydrazide (Figure 2, inset), which is a derivative of desthiobiotin that can be attached to carboxylate functionalized CNTs. The conjugation of DSB-X biotin hydrazide to the carbon nanotube membrane was carried out in 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer, pH 6.5. An amount of 20 mg of CNT membrane was added to 4 mL of MES buffer containing 0.015 mg of DSB-X biotin hydrazide, and 10 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC); the latter was added to activate the carboxylic acid groups present on the membrane. The CNT membrane was incubated in this reaction mixture at 4 °C on a shaker overnight. The CNT membrane was washed three times with MES buffer to remove any unreacted DSB-X biotin hydrazide. The membranes were characterized by Fourier transform (FT)IR to confirm the conjugation of DSB-X biotin hydrazide. An amount of 2.0 mg of CNT membrane was dried in a vacuum oven, crushed and mixed with 70 mg of KBr to form a pellet. FTIR spectra of the KBr pellet were recorded with a ThermoNicolet Nexus 4700 IR instrument.

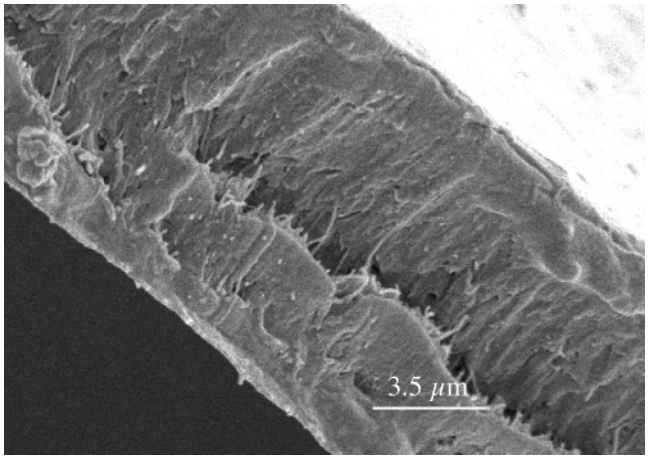

Figure 1. SEM image of aligned multiwalled CNT membrane. Because of the cleaving process, some of the CNTs are protruding away from the cleaved surface. Bar denotes a distance of 3.5 μm.

Streptavidin Binding. DSB-X biotin hydrazide functionalized CNT membranes were incubated in a streptavidin solution prepared by dissolving 1.6 mg of streptavidin in 4 mL of Tris buffer (50 mM tris(hydroxymethyl)aminomethane, 50 mM NaCl, pH 8.0). The reaction was carried out at 4 °C overnight on a shaker. The CNT membranes were washed with Tris buffer three times to remove the unreacted streptavidin.

Flux Measurements. Ionic flux measurements through the CNT membrane were carried out using a simple U-tube cell consisting of two chambers separated by the nanotube membrane. The exposed membrane area was 0.3 cm^2. The feed chamber was filled with 7.2 mL of 5 mM aqueous mixture of methyl viologen (MV^{2+}) and tris-(2,2′-bipyridyl)dichlororuthenium ($Ru(bpy)_3^{2+}$), and the permeation chamber was filled with 1.4 mL of deionized water. Care was taken to avoid any pressure-induced transport by ensuring that solution levels were at the same height in the feed and permeation chambers. No change in the liquid height was observed throughout the experiments, indicating that the effect of osmotic pressure was negligible. The entire permeated solution was periodically assayed using a HP 8543 UV−vis spectrophotometer. Calibration plots of each marker molecule (MV^{2+} or $Ru(bpy)_3^{2+}$) in the range 1×10^{-4} to 1×10^{-7} M (six solutions) were used to quantify the amounts of MV^{2+} and $Ru(bpy)_3^{2+}$ in the permeate solution. The absorbance maximum of MV^{2+} is at 260 nm, and $Ru(bpy)_3^{2+}$ has two peaks at 286 and 452 nm. There is no interference from MV^{2+} at 452 nm, so this wavelength was used to quantify the amount of $Ru(bpy)_3^{2+}$. The MV^{2+} absorbance at 260 nm overlaps with the absorbance from $Ru(bpy)_3^{2+}$. With known $Ru(bpy)_3^{2+}$ concentration (from the 452-nm peak), the overlap can be subtracted allowing calculation of the MV^{2+} concentration. The permeate solution remained at less than 2 orders of magnitude of feed solution concentration, thus the concentration gradient remained nearly constant during the diffusion experiment.

Fluorescence Studies. For streptavidin binding, fluorescence studies were performed to quantify the amount of streptavidin bound to the membrane surface. First, the functionalized nanotube membrane was incubated in 4 mL of a fluorescein-isothiocyanate-labeled streptavidin (streptavidin−FITC) solution for 6−8 h. The streptavidin−FITC solution was prepared by dissolving 1 mg of

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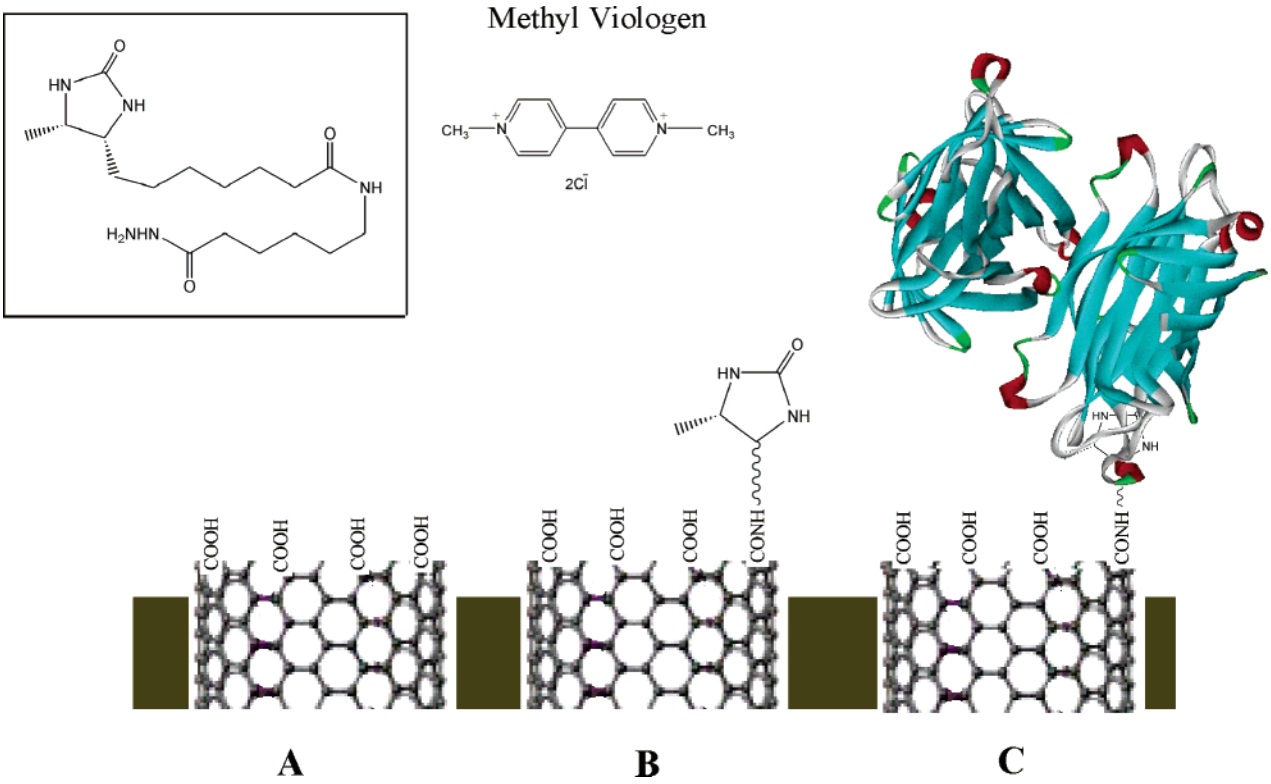

Figure 2. Schematic demonstrating the binding of streptavidin to DSB-functionalized tips of carbon nanotubes at the surface of the CNT−polystyrene composite membrane. (A) Open tips of CNT with carboxylic end groups, (B) DSB-X biotin hydrazide functionalized tip of CNT, and (C) streptavidin bound to the functionalized membrane. The structures of DSB-X biotin hydrazide and methyl viologen are shown an an inset.

streptavidin−FITC in 4 mL of Tris buffer. Fluorescence measurements were performed on a Fluorolog-2 spectrofluorometer (Jobin YVON-SPEX, Edison, NJ), equipped with a 450-W xenon arc lamp using quartz cuvettes. The excitation wavelength was set at 498 nm, and emission was detected at 518 nm. For quantifying the amount of streptavidin−FITC, a calibration plot in the range 0.01−5 μg/mL (seven solutions) was used.

Results and Discussion

Biological ion channels play a critical role in the transport of fluids and chemicals across cell membranes/walls. Nanopore membranes can be used to mimic ligand-gated ion channels if a selective molecule (ligand), which is placed at the entrance of the CNT, is reversibly coordinated with a bulky receptor. This reversible binding of the ligand/receptor system leads to on/off switching of the pores. The desthiobiotin derivative, DSB-X Biotin, used in this study binds reversibly to streptavidin. DSB-X Biotin hydrazide was reacted with the carboxylate end groups of the CNT membrane using a carbodiimide-mediated reaction. FTIR studies were carried out to confirm the functionalization of the CNT membrane with DSB-X Biotin hydrazide. A peak at 1627 cm^{-1} in the plasma oxidized CNT membrane can be attributed to the C═O stretch of COOH. Expansion of the C═O stretch region for the DSB-functionalized membrane showed that the peak had broadened with the maximum still at 1627 cm^{-1} and a shoulder at 1603 cm^{-1}, which can be assigned to the $\gamma_{C=O}$ amide (I) and δ_{NH} amide (II) bands.[20]

The model system depicting binding of streptavidin to DSB-X biotin hydrazide functionalized CNT membrane is schematically shown in Figure 2. Binding can be reversed in the presence of biotin at neutral pH, thus restoring the flux of the marker ions across the channel. The affinity of streptavidin for desthiobiotin is several orders of magnitude lower than that for biotin.[24] Streptavidin binds to the desthiobiotin-functionalized membrane and blocks the pore. The bound streptavidin was released by incubating the membrane in a 1 mM solution of biotin in 50 mM Tris, 50 mM NaCl, pH 8.0, buffer. After releasing the bound streptavidin, the CNT membrane was washed with 50 mM Tris, 50 mM NaCl, pH 8.0, buffer solution three times, before evaluating its transport properties.

The opening and closing of the pores was detected by monitoring the transport of MV^{2+} through the CNT cores across the membrane as seen in Figure 3. Before the binding of streptavidin to the DSB-functionalized CNT membrane, a flux of 4.8 nmol cm^{-2} h^{-1} was observed for MV^{2+}. A flux of 0.2 nmol cm^{-2} h^{-1} was observed after streptavidin binding. After incubating the streptavidin-coordinated membrane with biotin, streptavidin was released from the membrane, and a flux of 4.4 nmol cm^{-2} h^{-1} was restored. Thus, the binding of streptavidin is reversible leading to a regulated transport through the membrane. Additionally, the transport of $Ru(bpy)_3^{2+}$ through the CNT cores across the membrane was also monitored. The ratio of the $MV^{2+}/Ru(bpy)_3^{2+}$ fluxes (was found to be 1.5) through the DSB-functionalized CNT membrane is equal to the ratio of the corresponding diffusion coefficients in aqueous solution.[25] This indicates that the pore size is large enough to not hinder diffusion through the

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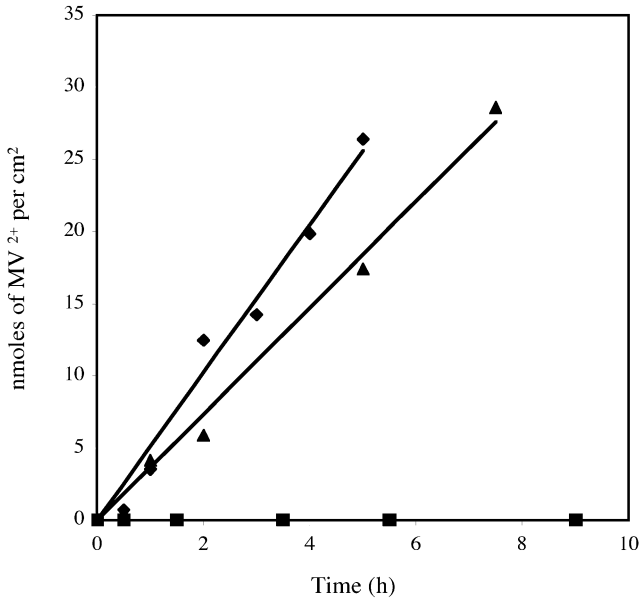

Figure 3. Flux data of methyl viologen through the pores of CNT membrane (the concentration of MV^{2+} in the feed solution was 5 mM). (▲) DSB-functionalized CNT membrane, (▲) streptavidin bound to DSB−CNT membrane, and (▲) after biotin incubation, to release streptavidin from the CNT pore entrance.

Table 1. Experimental and Calculated Values of Streptavidin Bound to DSB-X-Functionalized CNT Membrane (Fluorescence Studies)

membrane surface area	3.2 cm^2
CNT areal density	6.0×10^{10} CNT/cm^2
amt streptavidin	0.267 ± 0.017 μg
streptavidin molecules/pore	16.3 ± 0.8

membrane. Indeed, the size of the DSB molecule is very small (∼1 nm) leaving at least a ∼5-nm diameter at the entrance of the CNT core.

Fluorescence measurements were carried out to determine any amount of FITC-labeled streptavidin that remained bound to the nanotube membrane after exposure to a biotin-containing solution. Estimates of the number of streptavidin molecules per pore and the area of membrane covered by streptavidin are shown in Table 1. An amount of 0.267 ± 0.017 ($n = 3$) μg of streptavidin was bound to the CNT membrane having a surface area of 3.2 cm^2. This corresponds to approximately 16 streptavidin molecules bound per pore using an estimated areal density of 6×10^{10} CNT/cm^2.[21] This can be expected since the nanotubes are multiwalled suggesting more than one binding site at each pore. Additionally, because the tips of the CNTs extend beyond the membrane surface, sidewall modification by the plasma oxidation and subsequent DSB functionalization is possible. To support this hypothesis, the number of reactive sites onto which streptavidin could bind was estimated by decorating thiol-functionalized CNTs with easily observed (by TEM) gold nanocrystals. For this, the polystyrene matrix of an as-prepared membrane was dissolved in toluene, and the multiwalled CNTs were modified with a reagent containing both a thiol and an amine (cystamine) instead of desthiobiotin, using the same carbodiimide reaction. These sites were then coordinated to nanocrystalline gold (5 nm diameter, similar to streptavidin volume[26]). Of 16 multiwalled CNT tips analyzed by TEM, an average of 45 gold coordination sites per CNT were observed,[27] which is consistent with the number of streptavidin molecules found to bind per DSB-functionalized CNT membrane. Given the structural differences of the two molecules used to modify the CNTs (DSB vs cystamine), this difference in the number of reactive sites is not unreasonable, considering the associated uncertainty in the areal density and efficiency of coupling.

Studies were also carried out to investigate whether there is physical adsorption of streptavidin onto the unfunctionalized CNT membrane, which could lead to blockage of the pores. For this, a CNT membrane was incubated in a FITC-labeled streptavidin solution (1 mg/4 mL of Tris buffer) and shaken in a rotary shaker. The membrane was washed three times with the buffer, and flux measurements of MV^{2+} and $Ru(bpy)_3^{2+}$ were carried out before and after incubation with streptavidin. The membrane maintained 95% transport flux for MV^{2+} and 88% for $Ru(bpy)_3^{2+}$, indicating the absence of simple chemi-/physisorption leading to pore blockage. This observation is also implicit in the reversibility data shown in Figure 3.

Another control experiment in support of ionic flux taking place through the cores of CNTs was also carried out because it has been recently reported under other multiwalled CNT growth conditions[28] that Fe nanocrystals could block multiwalled CNT cores in silicon nitride membranes. Specifically, we performed the same membrane fabrication shown here on a multiwalled CNT sample from an Fe-rich region of the deposition reactor, where nanotubes are blocked by Fe nanocrystals. The resultant composite membrane was the same as the working membranes described above (in terms of polymer thickness and processing steps), except for the quality of multiwalled CNT cores, which were blocked. No flux of $Ru(bpy)_3^{2+}$ nor MV^{2+} was seen after 32 h, indicating that diffusion through the polymer or other gross defects is insignificant. The conclusion that the observed flux in working membranes is through CNT cores is also consistent with the ability to gate transport with nanometer-scale biological molecules and N_2 desorption measurements of pore size. The quality of the initial multiwalled CNTs is an important issue that does affect overall flux, and further research to optimize the CNT fabrication process is on going.

Conclusions

Aligned carbon nanotube membranes can mimic biological ion channels by reversibly and selectively opening/closing pores in the presence of bioactive molecules. This approach can be broadly applied to other receptor/ligand pairs that can selectively block CNT core entrances. An important constraint is that the molecule bound to the CNT pore should be small enough (<∼3 nm) to not block the core by itself. In this study, the pores of nanotube membranes open and close using the reversible binding of desthiobiotin to streptavidin. On/off states are obtained by binding and releasing of streptavidin from the DSB-functionalized tips of the CNT in the membrane. This is confirmed by ionic transport through the CNT core.

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