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Irreversible blocking of ion channels using functionalized single-walled carbon nanotubes

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Abstract
We demonstrate irreversible blocking of ion channels using single-wall carbon nanotubes (SWNTs) functionalized with 2-aminoethylmethane thiosulfonate (MTSET). In contrast, as-produced and purified SWNTs exhibit reversible blocking, indicating that the MTSET molecule attached to SWNTs chemically interacts with the cysteine groups in the ion channels. Functionalization of SWNTs with MTSET is inferred from Fourier transform infrared (FTIR) spectroscopy, which clearly shows absorptions due to –CH stretching and deformation modes, and decrease in intensity of the –COOH line due to reaction of acid functionalized SWNTs with the basic N–CH_3 end of the MTSET molecule.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

The utilization of nanomaterials in probing of biological systems has recently received significant interest. The possibilities of probing nanoscopic inter- and intra-cellular features as well as for cell generation appear to be areas of promise for nanomaterials. For biological applications, the nanomaterials must seek out the specific target feature without interacting with the local environment. The nanomaterials must also be inert to the environment in the biological system. Single-walled carbon nanotubes (SWNTs) meet some of these criteria and therefore have received significant interest for use in biological applications. SWNTs are chemically inert, biocompatible and can be dissolved in different solvents (including physiological solutions) via functionalization. The ability to attach specific chemicals to the SWNTs allows them to be used as probes with specific detection capabilities.

The biological applications of carbon nanotubes have focused on studying interactions with DNA, peptides and proteins [1–4]. The aim of these studies has been to self-assemble macro-molecular structures and electronic devices as well as to separate semiconducting SWNTs from metallic ones. Active devices such as biological sensors with SWNTs functionalized with antibodies have also been demonstrated [5]. Recently, functionalized multi-walled carbon nanotubes (MWNTs) have been used as substrates for the growth of cultured neurons [6]. The ability to purify, functionalize and increase the solubility of SWNTs in physiological solutions has made it possible to consider them for a broad range of biological applications.

Recently, we reported that it was possible to reversibly block ion channels using SWNTs [7]. Specifically, it was demonstrated that SWNTs blocked subunits of K^+ channels in a dose dependent manner. In addition to SWNTs, we also tested the C_{60} fullerene molecules, MWNTs and hyperfullerenes (i.e. carbon 'onions') and found that the

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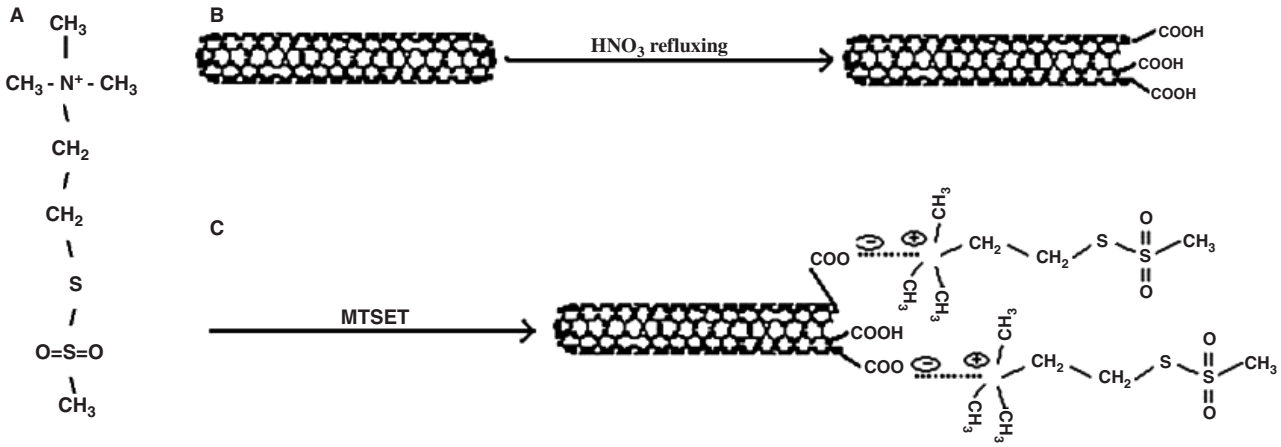

Figure 1. (A) Schematic diagram of the MTSET molecule. (B) Schematic diagram of opening of SWNT ends and functionalization with carboxyl group through ultra-sonication in hot HNO_3. (C) Schematic diagram of attachment of MTSET to SWNTs.

blocking characteristics were dependent on both diameter and the shape of the carbon nanomaterial, with SWNTs being the more effective blocker. Larger carbon nanomaterials such as the MWNTs and carbon onions did not block the ion channel. The blocking with the C_{60} and SWNTs was however found to be reversible. That is, by performing a wash, ion conduction through the channel could be restored. Our results indicated that no chemical interaction between the ion channel and the SWNTs takes place.

Ion channels are proteins that catalyse passive diffusion of ions across the plasma membrane. In doing so, they generate electrical signals that constitute the bases for biological excitability. Thus, the regulation of ion fluxes across the channel is of central importance for cellular functions since they play an important role in regulating the manner in which cells convey information. External agents such as toxins and synthetic molecules can disturb the regulation of ion fluxes through establishing chemical bonds with the channel that could lead to the destruction of the cell. Many agents, such as MTSET, have been used for blocking ion channels because of their small dimensions, which allows the probing of the ion channel atomic structure. However, these smaller molecules cannot provide information regarding some of the nanostructural features of the channels. The possibility of using MTS-functionalized nanotubes represents a novel step in this direction because, given the controlled geometries and overall dimension of SWNTs, it will allow us for the first time to probe macrodomains of channel proteins such as the pore region. Specific interactions between SWNTs and various chemical groups in the ion channel can be tailored through functionalization of the SWNTs. SWNTs are unique in comparison to simple blocking molecules because they allow for attachment of various other molecules, which would target specific sites in the ion channels, making them more versatile molecules for future investigations. The ability to exert control over blocking or opening ion channels could lead to functional information as well as development of therapeutic drugs. In this paper, we report our initial results on SWNTs functionalized with MTSET which can specifically interact with a desired chemical group (in this case the cysteine group) in the ion channel.

We report that by functionalizing SWNTs with 2-trimethylammoniumethylmethane thiosulfonate (MTSET) it is possible to irreversibly block hyperpolarization and cyclic-nucleotide activated (HCN) channels heterologously expressed in Chinese hamster ovary (CHO) cells. We used HCN2 subunits because they contain cysteinyl groups that can be covalently modified by MTSET molecules applied from the external side of the plasma membrane (figure 1(A)). Unmodified SWNTs also block the ion channels but via a reversible process, as the channels can be unblocked upon wash-out, similar to the results from our previous work [7]. The functionalization of SWNTs was inferred by Fourier transform infrared spectroscopy (FTIR), which clearly shows –CH stretchings, deformation modes and decrease in the –COOH due to reaction with the N–CH_3 end of the MTSET molecule.

2. Experimental details

SWNTs prepared by the high pressure CO process (obtained from Carbon Nanotechnologies) were used in this study. The SWNTs were thoroughly purified and dispersed using standard procedures [8]. Briefly, the SWNTs were initially refluxed in nitric acid for 45 h in order to remove any amorphous carbon and catalyst particles. Following the acid reflux, the suspension was centrifuged and the supernatant was decanted. The sediment was re-centrifuged and decanted several times in order to obtain high purity SWNTs. The purified SWNTs were then dispersed by ultra-sonication for 1 h in an aqueous suspension containing 1% Triton X-100 surfactant. The SWNTs were kept in suspension for storage. Prior to all experiments reported here, the Triton X-100 (which is toxic for biological systems) was removed by thorough washing and annealing in Ar at 400 °C for 2 h. The SWNTs were then re-dispersed to form an aqueous suspension using 3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS) as the surfactant. Controlled experiments in our previous study showed that ion channels are not influenced by CHAPS [7].

The functionalization process was carried out by first removing the ends of purified SWNTs via ultra-sonication in nitric acid for 2 h at a bath temperature of 80 °C [9]. After

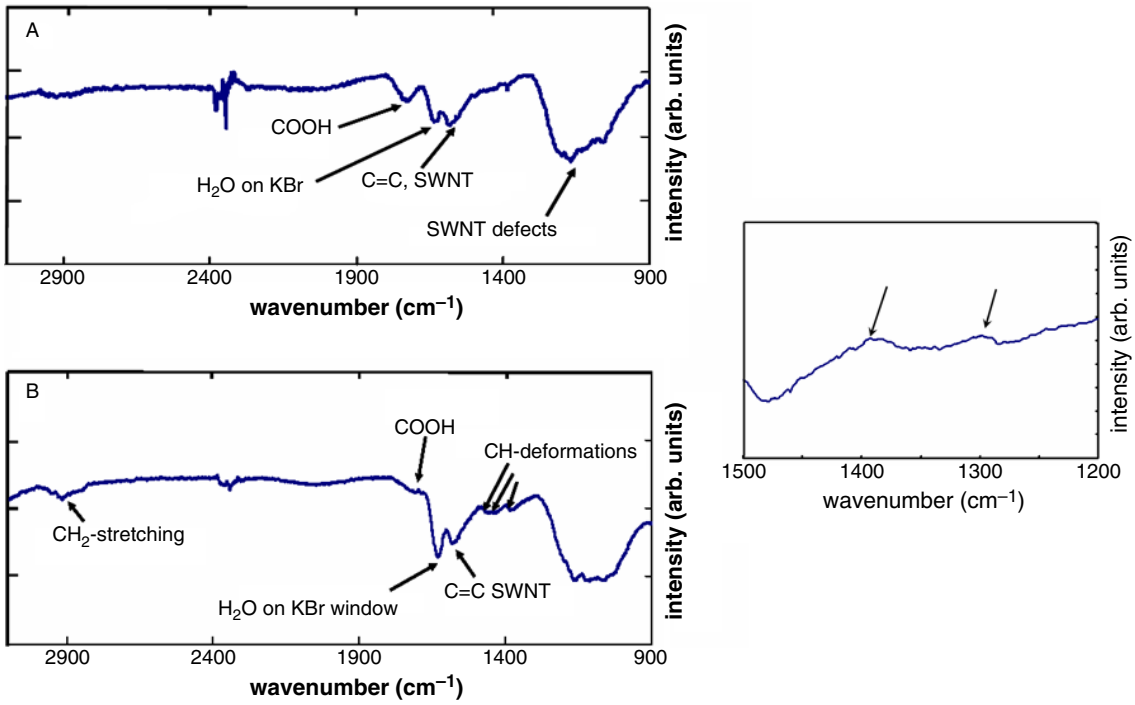

Figure 2. (A) FTIR spectrum of SWNTs after purification in nitric acid. The peak at 1717 cm^{-1} is from the attachment of the –COOH groups at the ends of the nanotubes. Defect modes and peaks from carbon double bonds are also present. Note that the C–H modes are absent. (B) FTIR spectrum of MTSET functionalized SWNTs. The –COOH mode is significantly suppressed and C–H modes appear. The inset on the right shows the expanded region between 1500 and 1200 cm^{-1}. The arrows in the inset indicate the bands responsible for the C–H stretchings.

the acid treatment the SWNT solution was diluted using de-ionized water. The removal of the SWNT end caps and dilution with water leads to functionalization with carboxyl (–COOH) groups, as shown in figure 1(B). The carboxyl terminated SWNTs were then placed in an aqueous solution containing 10 wt% MTSET and the mixture was ultra-sonicated for 2 h. The MTSET is sensitive to light and therefore care was taken to limit the amount of exposure during processing. Subsequent to ultra-sonication, the SWNTs were thoroughly washed with de-ionized water to remove any excess unbonded MTSET.

3. Results and discussion

The attachment of the MTSET was inferred by FTIR spectroscopy. Three milligrams of the sample were uniformly mixed with high purity KBr and compressed under vacuum into a pellet. FTIR spectra of the pellets were then recorded using a Perkin-Elmer FTIR spectrometer. The FTIR spectra of purified SWNTs and SWNTs functionalized with MTSET are shown in figures 2(A) and (B), respectively. The acid purified SWNT starting material shows typical IR features: –COOH group due to acidification at 1717 cm^{-1}; defect induced C=C stretching mode line from SWNTs at around 1580 cm^{-1} along with a line at 1626 cm^{-1} due to traces of water in the KBr windows. The IR spectrum of functionalized SWNTs also shows the defect and C=C modes but is significantly different in other respects. Specifically, C–H deformation modes between 1368–1448 cm^{-1} have appeared and the –COOH (1705 cm^{-1}) is less intense after the reaction. The attachment occurs via an interaction between the negative oxygen ion of the –COO group of the SWNTs and the positive nitrogen ion in the N–CH_3 end group of the MTSET molecule, as shown schematically in figure 1(C). Such interaction should lead to the suppression of the –COOH mode intensity and the appearance of C–H stretching modes in the FTIR spectra. This is clearly observed in figure 2(B). However, FTIR analysis alone is not sufficient to assess the bond between the MTSET and SWNTs. Attempts to further characterize SWNT functionalization with MTSET are hindered by the fact that MTSET is unstable in light. Therefore, in the absence of additional analysis, the mechanism for MTSET attachment to SWNTs shown in figure 1(C) can only be classified as speculative. For example, the ion pair formation shown in figure 1(C) may not be able to survive in an aqueous environment with large ionic strength.

The electrophysiological experiments were carried out using standard patch-clamp apparatus consisting of a patch-clamp amplifier, a dedicated computer and a perfusion chamber for quick change of test solutions. The patch-clamp technique is a powerful and versatile method for studying the properties of ion channels. A gigaohm 'seal' is initially formed between a test cell and a glass pipette filled with a saline solution operated by a micro-manipulator. By applying a small negative pressure, the seal is broken and electrical contact is established between the pipette and the cell cytoplasm (whole-cell configuration, panel A in figure 3). An electrode in the patch-pipette connected to a patch amplifier (an I–V converter) clamps the cell to any potential and records the current flowing across the channels in the membrane. Opportune perfusion systems allow us to quickly apply/wash out (exchange time ∼5 s) the desired test compounds (figures 3(B) and (C)) during electrical recording. The concentration of SWNTs to MTSET

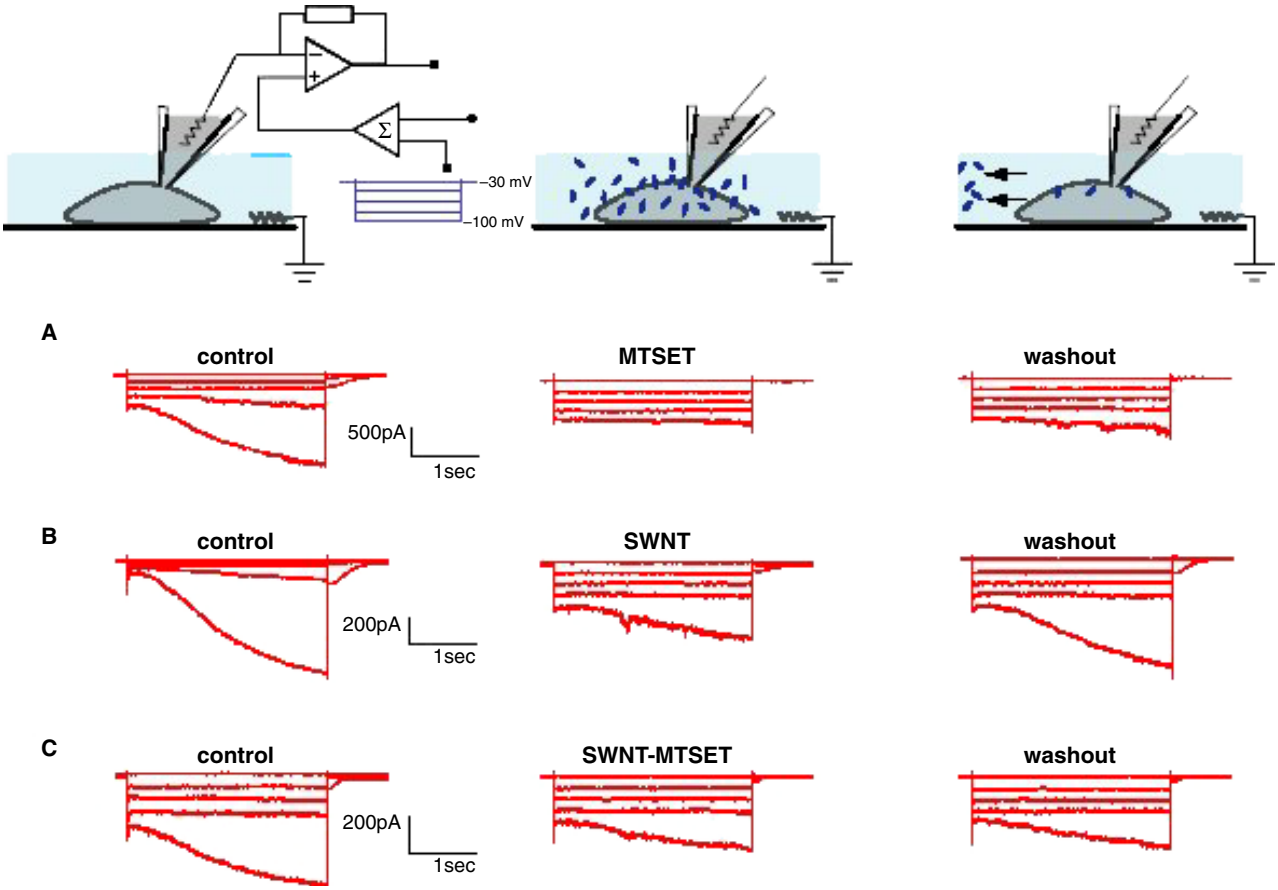

Figure 3. The upper panel shows a cartoon of the three types of electrophysiological experiments carried out. (A) HCN2 currents in the absence (left) or presence (centre) of 5 mM MTSET in the bath. Blocking persisted after wash-out of MTSET (right). (B) As in (A) with purified nanotubes (∼0.1 mg ml^{-1} in the bath). Blocking is relieved upon wash-out (right), indicating reversible interactions. (C) As in (A) and (B) with MTSET functionalized nanotubes. With these compounds inhibition is irreversible (right), indicating that it proceeds through MTSET mediated interactions. Cells were transiently transfected with HCN2 cDNA ligated into pCI-neo using a Superfect kit (Qiagen) and studied after 24–36 h. Data were recorded with an Axopatch 200B (Axon), a PC (Dell) and Clampex software (Axon), filtered at 1.0 kHz and sampled at 2.5 kHz. Bath solution was (in mM) 100 KCl, 5 NaCl, 10 Hepes (pH = 7.5 with KOH), 1.8 $CaCl_2$ and 1.0 $MgCl_2$. Pipette solution: 100 KCl, 10 Hepes (pH = 7.5 with KOH), 1.0 $MgCl_2$, 1.0 $CaCl_2$, 10 EGTA (pH = 7.5 with KOH). Prior to electrophysiological measurements fresh test solutions were prepared by diluting stocks in bath solution. Voltage protocol: 3 s voltage jumps from −30 to −110 mV in −20 mV increments. Holding voltage −80 mV for all experiments and interpulse interval 1 s.

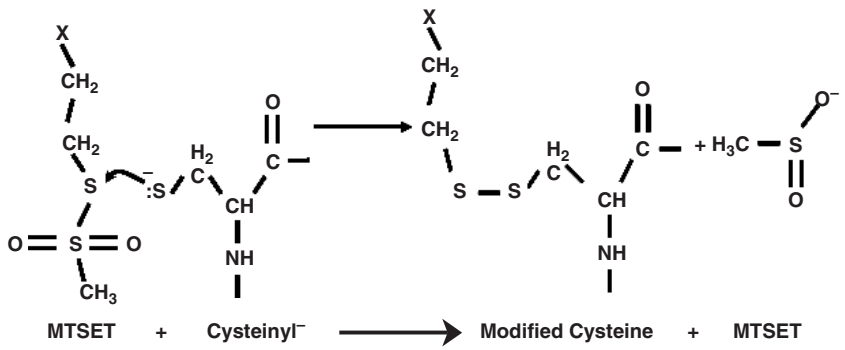

Figure 4. Schematic diagram showing the formation of the disulfide bridge which allows the MTSET reagent to modify the cysteines in ion channels. The 'X' in the figure represents the N–CH_3 group of the MTSET molecule (see figure 1(A)).

to de-ionized water was kept constant at 10 μg to 1 mg to 10 ml for all experiments reported here.

In order to assay whether MTSET functionalized nanotubes can act to modify ion channels through MTSET mediated interactions, we used HCN2 subunits that have endogenous cysteine that can be accesses and modified by externally applied MTSET reagents [10]. Thus, in CHO cells transfected with HCN2 cDNA, negative voltages elicited

robust inward currents that exhibited slow activation kinetics characteristics of HCN2 channels (figure 3(A)). Moreover, mock transfected cells did not produce any current, a result largely expected since endogenous K^+ channels are not present in CHO cells. MTSET in the bath solution (5 mM) inhibited HCN2 currents by $63 \pm 13\%$ ($n = 3$ cells). MTSET blocking was not removed upon wash-out, indicating that MTSET chemically (either through covalent or ionic bonds) modifies the channels (figure 3(A)). When HCN2 subunits were exposed to non-functionalized but purified SWNTs, they were reversibly blocked by $34 \pm 9\%$ ($n = 4$) (figure 3(B)), confirming our earlier work [7]. Although not functionalized with specific groups, the purified SWNTs do indeed have the carboxyl groups attached at the ends. Thus, carboxyl functionalized SWNTs reversibly block the ion channels. MTSET functionalized nanotubes also blocked the channels ($40 \pm 11\%$ $n = 3$) but with one significant difference: that the inhibition was irreversible (figure 3(C)), indicating that a nanotube-mediated interaction between the channel and the MTSET reagent had taken place. MTSET blocked HCN2 channels approximately twofold more efficiently than functionalized nanotubes. This result is reasonable, since it is the SWNT and not the bonded MTSET that dictates the interaction with the channel. Comparatively small molecules such as MTSET are expected to reach target cysteines more easily than roughly tenfold larger objects such as nanotubes. Although the exact nature of the interaction between the MTSET functionalized SWNTs and the ion channel is unclear, cysteine modification is thought to occur via a disulfide bridge between the alkylthio group of the MTSET reagent and the thioalkyl moiety as the sulfonate moiety leaves, as shown in figure 4 [11]. Further analysis of this interaction is needed before any conclusive mechanisms can be stated.

One key concern is that the blocking results from free dissociated MTSET molecules rather than the interaction with functionalized SWNTs. This is unlikely to be the case, since stock solutions were abundantly washed in water in order to get rid of residual MTSET. MTSET is completely is soluble in water and a negligible number of free molecules, if any, should be present in the SWNT solution. However, in order to completely eliminate the possibility of free MTSET molecules blocking the ion channels, we have carried out an additional control experiment. In the control experiment, MTSET was dissolved in the purified SWNT solution and ultra-sonicated for 2 h. The motivation of this task was not to functionalize the SWNTs but solely to introduce MTSET in the SWNT solution. After the ultra-sonication, we washed the non-functionalized SWNTs thoroughly to remove MTSET molecules. Our electro-physiological experiments with the non-functionalized but MTSET exposed SWNTs revealed reversible blocking, indicating that all the MTSET molecules are completely removed after washing. Thus, we can confidently argue that the irreversible blocking of ion channels is mediated by MTSET functionalized SWNTs and not from free MTSET molecules.

4. Conclusions

In summary, we demonstrate that the functionalization of SWNTs with 2-trimethylammoniumethylmethane thiosulfonate (MTSET) is possible. The FTIR data show the presence of C–H stretchings and the suppression of the –COOH mode after functionalization. The blocking of the channel is found to be reversible with unmodified SWNTs while chemical interaction between the ion channel and functionalized SWNTs is inferred through irreversible blocking. Our results indicate that it may be possible to tailor the interactions of SWNTs with specific types of ion channels and studies are currently underway to elucidate blocking mechanisms of functionalized nanotubes in conjunction with cysteine engineered HCN mutant channels.

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