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Facile functionalization by π-stacking of macroscopic substrates made of vertically aligned carbon nanotubes: Tracing reactive groups by electrochemiluminescence

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ABSTRACT

We report a simple, fast and reliable non-covalent route of functionalization of macroscopic carbon nanotubes (CNTs) surfaces based on the π-stacking of CNTs sidewall with fluorescein derivatives (i.e., amino- and isothiocyanate-). The electrochemiluminescent emission of $Ru(bpy)_3{}^{2+}$ labels bearing –COOH and $–NH_2$ side groups coupled with colorimetric and XPS measurements allowed to estimate the quantity of $–NH_2$ and –N=C=S functions obtained. The evaluation of reactivity suggests that functionalized CNTs substrates, in particular those carrying –N=C=S groups, are suitable to covalently bind probe molecules such as proteins and oligonucleotides, thus opening up the possibility of future application in genomics and proteomics fields.

1. Introduction

The functionalization of multi-walled carbon nanotubes (MWCNTs) electrodes with biological probes such as proteins and oligonucleotides is a hot topic in molecular diagnostics [1–3]. The ability to measure extremely small amounts of specific biomarkers is, in fact, a highly desirable feature in bio-assay and CNTs, due to their remarkable electronic properties and high specific surface area, seem to be ideal candidates for this kind of applications [4].

We show here a simple, fast and reliable non-covalent route of functionalization of macroscopic CNTs substrates avoiding compromising their electronic properties. In fact, the covalent functionalization requires often drastic conditions, such as chemical oxidation of the nanotubes with concentrated HNO_3 or sulphonitric mixture to obtain –COOH and –OH reactive groups on the surface [5].

Our purpose is exploiting π-stacking interactions between the CNTs sidewall p orbital and polyaromatic molecules (fluorescein) [6–9], bearing the necessary reactive group to obtain free $–NH_2$ and –N=C=S functions on the substrate. To the best of our knowledge, until now π-stacking interactions have been exploited almost exclusively at molecular level to prepare nanohybrids from CNTs bundles especially for drug delivery purposes [6–9]. In the present work, we investigate the feasibility of such a "soft" functionalization method on macroscopic planar CNT substrates, by estimating the amount of reactive groups obtained and their stability by means of electrochemiluminescent $Ru(bpy)_3$-based labels [10–14]. The fluorescein compounds were simply selected as models, in order to demonstrate that the π-stacking based CNT functionalization could be obtained on macroscopic surfaces, i.e., by planar reaction instead of tridimensional reaction as that of

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nanohybrids preparation in solution. The functionalized substrates here obtained are suitable to covalently bind different kinds of biological probes such as proteins and oligonucleotides. The target molecule (i.e., antigen, antibody or DNA sequence) can be then easily detected by different techniques such as photoluminescence, chemiluminescence and electrochemiluminescence (ECL). The success could open the way to the utilization of pi-stacking functionalization in real CNTs-based substrates for biological assay.

2. Experimental

2.1. Starting materials

Aminofluorescein (Fluor-NH_2), fluorescein isothiocyanate (FITC), $Ru(bpy)_3(ClO_4)_2$, 1-ethyl-3-(3dimethylaminopropyl)carbodiimide (EDC) and sulfo N-hydroxysuccinimide (s-NHS) were obtained from Sigma–Aldrich. [Ru(4-(4′-methyl-2,2′-bipyridin-4-yl)butan-1-aminium)(2,2′-bipyridine)$_2$]$(ClO_4)_3$ ($Ru(bpy)_3$–NH_2) and [Bis(2,2′-bipyridine)-[4-(4′-methyl-2,2′-bipyridin-4-yl)butanoic acid] ruthenium]$(ClO_4)_2$ ($Ru(bpy)_3$–COOH) were obtained from Cyanagen S.r.l. (Italy). The sulfosuccinimidyl-4-[2-(4,4′-dimethoxytrityl)]butyrate (sSDTB) reagent was purchased from Apollo Scientific Ltd. (UK). Dimethylformamide (DMF) and perchloric acid 60% (v/v) were purchased from Fluka (Switzerland). Other chemicals were purchased from Sigma–Aldrich and all the employed reagents were used as received, without further purification.

2.2. Deposition and growth of carbon nanotubes

P-type silicon substrates (resistivity 40–100 Ω cm) were patterned through optical lithography. A Ti (5 nm)/Cu (100 nm) bi-layer was deposited by thermal evaporation under high vacuum conditions ($\sim 10^{-7}$ mbar), with the Ti acting as adhesion layer. A lift-off procedure allowed the pattern definition, selectively removing the metal layer from the desired areas. Subsequently, CNTs deposition was performed through thermal chemical vapor deposition (CVD) in a horizontal furnace at a temperature of 850 °C, as shown in supplementary data (see Fig. S1).

The growth procedure is based on the thermal pyrolysis of commercial camphor as a carbon precursor and subsequent CNTs synthesis catalyzed by iron nanoparticles provided by the cracking of ferrocene (98% purity in weight). Solid precursors were heated at their boiling point and carried in the reaction chamber using N_2 as a gas carrier [15,16]. This technique allows growing massive bundles of vertically aligned MWCNTs on bare silicon substrates. In the present case, the relatively thick metal layer locally hinders the CNTs formation [17], allowing a selective growth that took place only on uncoated circular spots of the substrate, as shown in Fig. S2. This allowed us to grow isolated CNT pillars (height from 0.5 to 2 mm) exposing a disk of 2 mm diameter and producing bundles of different diameters, as shown in the SEM image of Fig. 1.

2.3. Encapsulation and contacting of CNTs electrodes

The procedure illustrated in Fig. 1a shows how to build a manageable and mechanically stable electrode, with the correct electrical contact. After the growth of a cylindrical pillar of vertically aligned CNTs on the silicon surface (step a), as described in the previous section, a drop of conductive glue is deposited on the top of the column (step b), then a metal wire is dipped into the glue until it hardens (step c). Now the electrical connection is done, but the electrode is still too fragile and the glue could be exposed to/in contact with the external solution (e.g., the chemical solution to be tested). To avoid this situation, a hollow structure is positioned all around the CNTs bundle (step d); epoxy resin is poured into the mould and left to harden (step e) in order to obtain a complete covering of the whole CNTs column and its connection (step f).

Finally, once the mould is removed, the whole structure is mechanically detached from the silicon surface, thus exposing only a disk of the initial cylindrical CNTs pillar (step g), as illustrated in Fig. 1b.

2.4. Functionalization of CNTs electrodes

2.4.1. π-Stacking functionalization of CNTs electrodes with fluorescein derivatives

MWCNT electrodes were incubated with 5.0 ml of a 1.0 mM solution of fluorescein derivatives in 0.2 M carbonate/bicarbonate buffer (CB) at pH 9 for 1 h, at ambient temperature, under orbital shaking at 60 rpm. Then, the sample surfaces were thoroughly rinsed with CB and distilled water (both 3 × 30 s) in order to remove the free fluorescein molecules. Being the used fluorescein derivatives aminofluorescein (Fluor-NH_2) or fluorescein isothiocyanate (FITC), from now onwards the obtained samples are named MWCNT-NH_2 and MWCNT-FITC, respectively.

2.4.2. Non-specific adsorption of ruthenium complex

In order to estimate the non-specific contribution from the ruthenium probe, previously functionalized MWCNT electrodes were incubated with solutions of $Ru(bpy)_3{}^{2+}$ lacking moieties able to bind them. MWCNT-NH_2 electrodes were incubated with 5.0 ml of a 0.1 mM solution of $Ru(bpy)_3$–COOH in 0.1 M Phosphate buffer (PB) at pH 7.5. MWCNT-FITC were treated with 5.0 ml of a 0.1 mM solution of $Ru(bpy)_3{}^{2+}$ in CB (0.2 M, pH 9). Both the solutions were incubated for 4 h, at ambient temperature, under orbital shaking at 60 rpm. Finally, the samples were cleaned with the respective buffers (3 × 30 s) and water (3 × 30 s).

2.4.3. $Ru(bpy)_3$–COOH and $Ru(bpy)_3$–NH_2 based labeling methods

After the first series of ECL measurements, samples obtained in the previous step were incubated in order to covalently bind Ru complexes. MWCNT-NH_2 electrodes were treated with a solution of $Ru(bpy)_3$–COOH in order to obtain an amide bond with fluor-NH_2, as shown in Fig. 2. The reaction is activated by the coupling reagents EDC and s-NHS. Therefore MWCNT-NH_2 electrodes were incubated with 5.0 ml of PB containing 0.4 mM EDC, 0.1 mM sulfo-NHS and 0.1 mM $Ru(bpy)_3$–COOH. The reaction was carried on overnight at room temperature. Finally, samples were rinsed with phosphate buffers and water (both 3 × 30 s).

MWCNT-FITC can directly react with $Ru(bpy)_3$–NH_2 to form a thiourea derivative (see Fig. 2), so these electrodes were incubated with CB solution of $Ru(bpy)_3$–NH_2 0.1 mM, overnight at room temperature. Rinsing was carried as for $Ru(bpy)_3$–COOH.

2.5. Colorimetric method for amine groups quantification

A colorimetric method [18,19] based on the reaction with (sSDTB) was applied for the determination of amine group density. Aminated substrates were incubated with a sSDTB freshly prepared solution (0.1 mM carbonate/bicarbonate buffer at pH 8.5) for 30 min at room temperature using an orbital shaker at 100 rpm. After incubation, the substrates were vigorously washed three times with ultrapure water, and then the samples were treated with a 37.5% perchloric acid solution to release 4,4′-dimethoxytrityl cation from the substrates. The absorption value at 498 nm ($\varepsilon_{498} = 70{,}000\ M^{-1}\ cm^{-1}$), measured by means of a V-550 (Jasco Corp., Japan) UV/Vis spectrophotometer, was utilized to quantify the amine group density.