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Tailored carbon nanotube immunosensors for the detection of microbial contamination

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ABSTRACT

The use of carbon nanotubes (CNTs) as building blocks in the design of electrochemical biosensors has been attracting attention over the last few years, mainly due to their high electrical conductivity and large surface area. Here, we present two approaches based on tailored single-walled CNTs (SWCNTs) architectures to develop immunosensors for the bacteriophage MS2, a virus often detected in sewage-impacted water supplies. In the first approach, SWCNTs were used in the bottom-up design of sensors as antibody immobilization support. Carboxy-functionalised SWCNTs were covalently tethered onto gold electrodes via carbodiimide coupling to cysteamine-modified gold electrodes. These SWCNTs were hydrazide functionalized by electrochemical grafting of diazonium salts. Site-oriented immobilization of antibodies was then carried out through hydrazone bond formation. Results showed microarray electrode behavior, greatly improving the signal-to-noise ratio. Excellent sensitivity and limit of detection (9.3 pfu/mL and 9.8 pfu/mL in buffer and in river water, respectively) were achieved, due to the combination of the SWCNTs′ ability to promote electron transfer reactions with electroactive species at low overpotentials and their high surface-to-volume ratio providing a favorable environment to immobilize biomolecules. In the second approach, SWCNTs were decorated with iron oxide nanoparticles. Diazonium salts were electrochemically grafted on iron-oxide-nanoparticle-decorated SWCNTs to functionalize them with hydrazide groups that facilitate site-directed immobilization of antibodies via hydrazone coupling. These magnetic immunocarriers facilitated MS2 separation and concentration on an electrode surface. This approach minimized non-specific adsorptions and matrix effects and allowed low limits of detection (12 pfu/mL and 39 pfu/mL in buffer and in river water, respectively) that could be further decreased by incubating the magnetic immunocarriers with larger volumes of sample. Significantly, both approaches permitted the detection of MS2 to levels regularly encountered in sewage-impacted environments.

1. Introduction

CNTs have been used as promising nanomaterials in electrochemical biosensor development due to their unique chemical, electronic and mechanical properties (Feng et al., 2008). Incorporation of CNTs into electrode design has been achieved by several methods taking advantage of the strength, size, chemical stability and, more importantly, good electronic conductivity of CNTs (Katz and Willner, 2004). CNT-modified electrodes enable the catalysis of redox reactions by promoting the electron transfer at low overpotentials between electroactive species in solution and the electrode (Merkoçi et al., 2005). This role to mediate electron transfer reactions helps to minimize electrode fouling effects (Thomas et al., 2013). Moreover, as a result of the enhanced electron transfer and the large surface area provided by CNTs, CNT-based sensors are usually characterized by high sensitivity values (Wang, 2004). CNTs are typically functionalized *via* the carboxyl group on their tips to immobilize various bioreceptors, such as antibodies or enzymes (Liu et al., 2005; Yu et al., 2006).

Several approaches have been used to develop electrochemical biosensors aiming to incorporate the electrical properties and biocompatible nanostructure of CNTs. CNT-coated electrodes, CNT-composite electrodes, CNT electrodes based on layer-by-layer protocols and vertically aligned CNT-modified electrodes have been prepared. CNT-based electrodes can be easily prepared by drop coating electrodes with CNT suspensions or by electrostatically assembling CNTs on a positively charged surface. However,

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the ability of CNTs to promote electron transfer strongly depends on the ability to obtain stable CNT suspensions and, thus, homogeneous coatings and CNT multilayer films, respectively (Wang et al., 2003; Kumar et al., 2012). CNT-composite electrodes also rely on the homogeneous dispersion of CNTs within the used binder, as well as on the ability to avoid impairing of the electrocatalytic properties of CNTs by the interaction with the binder (Valentini et al., 2003). Vertically aligned CNT-modified electrodes have received much attention over the last few years (Constantopoulos et al., 2010). The small diameter, high aspect ratio and good conductivity of CNTs are responsible for the high sensitivity achieved (Gooding et al., 2003; Yu et al., 2006), as well as for the excellent performance of electrochemical devices based on vertically-aligned CNTs. This is due to a faster charge transfer rate (*e.g.,* between CNTs and redox centers of immobilized molecules) compared to randomly dispersed CNTs. Vertically-aligned CNTs can be prepared by a physical method, based on direct growth of CNTs using template-assisted or template-free synthesis (Huang et al., 2007); or by a chemical method (Diao and Liu, 2010), based on the assembly of modified CNTs on certain substrates. Chemical assembly has been performed on different substrates *via* covalent bonds (Shearer et al., 2010), metal-assisted chelation and electrostatic interactions (Chattopadhyay et al., 2001).

Magnetic particles have been widely used in the development of biosensors (Zhang and Zhou, 2012). They show a large biocompatible surface area for biomolecule immobilization, providing a large number of binding sites for biorecognition events. Moreover, they can be dispersed in solution, increasing the efficiency of the interactions and lowering the analysis time. Their magnetic properties make them useful for analyte separation, purification and concentration from complex samples, avoiding the need for sample pretreatment. Their capture using magnets facilitates and improves the washing protocol, minimizing matrix effects. These features result in improvements in the sensitivity and limit of detection (Xu and Wang, 2012). As an additional advantage of their use, regeneration of the biosensor surface is easily performed by switching off the magnet used to capture the modified magnetic particles.

Incorporation of nanobiotechnology in the design of novel biosensors is essential to maximize their analytical performance (Campàs et al., 2012). Here we present two assemblies of SWCNTs in 3D platforms aiming to detect the bacteriophage MS2 as an indicator of microbiological contamination in water supplies. Both immunoplatforms were used to detect MS2 phage through differential pulse voltammetric measurements after performing an indirect sandwich immunoassay. The use of MS2 as a proof of concept is particularly relevant as it can be used not only as a surrogate for enteric viruses but also as an indicator of the presence of *Escherichia coli*, its natural host. MS2 has been successfully used as a surrogate to examine the effectiveness of water treatment processes as it shows characteristics that are highly similar to enteric viruses (Brehant et al., 2010) and its detection can be performed by simple and inexpensive methods (Leclerc et al., 2000; Petty et al., 2007). In addition, its presence has been well documented in situations where *E. coli* (the host) occurs in significant numbers, such as raw sewage and water courses impacted by raw sewage (Keegan et al., 2009; Li et al., 2012). Therefore, we have chosen the F-RNA coliphage MS2 to develop a proof-of-concept biosensor that could be further tailored to detect other viruses and bacteria or immunoreactive analyte species in general. Based on our previous work (Prieto-Simón et al., 2014), we aim at exploiting the advantages of the incorporation of SWCNTs in sensing devices as a means to improve the sensitivity and limit of detection, while minimizing non-specific adsorption events. SWCNTs are expected to work as conductive linkers between gold electrodes and the antibodies used as bioreceptors. Site-directed chemistry to control the orientation of the immobilized antibodies onto the biosensor surface has a strong impact on the final analytical performance (Trilling et al., 2013). Therefore, antibodies have been immobilized through their Fc moieties as a way to maximize the access to their binding sites.

Initially SWCNTs were covalently anchored on gold electrodes via amide bond formation aiming to improve the electron-transfer rate along the tubes. SWCNTs were immobilized through the formation of amide bonds between the amine-modified surface of gold electrodes and the carboxylic groups at the ends and sidewall defects of the nanotubes (Shearer et al., 2010). Hydrazide groups were then introduced onto the immobilized SWCNTs by diazonium salt electrografting. Site-oriented immobilization of anti-MS2 antibodies was performed through formation of hydrazone bonds.

Additionally, we investigated iron-oxide-decorated SWCNTs as dual magnetic particles and bioreceptor immobilization platforms. Superparamagnetic iron oxide nanoparticles were grafted onto SWCNTs. The hybrid nanomaterials were successfully labeled with anti-MS2 antibodies through hydrazone bond formation with a previously electrografted diazonium salt. The resulting immunocarriers were dispersed and used to capture MS2 bacteriophage. Finally, the magnetic immunocarriers were captured on an electrode to carry out electrochemical MS2 detection. The possibility to rapidly and easily separate the target analyte from the sample by using an external magnetic field allows the pre-concentration of samples. Additionally, the electrode surface can be easily renewed after each measurement by simply switching off the magnetic field, being ready for the next measurement.

Both immunosensors were successfully applied to the sensitive and accurate determination of MS2 in spiked river water samples. These new SWCNT-based platforms offer the possibility to tune the features of the sensing devices accordingly to the requirements of the final application. Significantly, the sensitivity of detection was sufficient to identify the presence of MS2 to levels regularly encountered in sewage-impacted environments. This versatility should translate into broad interest in new uses of CNTs for biosensor development.

2. Material and methods

2.1. Reagents

All reagents were used as received. Cysteamine hydrochloride, mercaptoethanol, 4-aminobenzoic hydrazide (ABH), 4-aminophenol (APh), sodium nitrite, sodium periodate, potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), α-naphtyl phosphate (α-NP), anti-mouse IgG-alkaline phosphatase (ALP), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), diethanolamine (DEA), 2-(N-morpholino)-ethanesulfonic acid (MES), iron (III) chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$) and components of buffers were purchased from Sigma-Aldrich. Sulfuric acid (98% AR grade) and nitric acid (70% AR grade) were purchased from Merck. SWCNTs (pristine, P-2) were purchased from Carbon Solutions, Inc. (USA).

Monoclonal antibody (developed in mouse) and polyclonal antibody (developed in rabbit) against MS2 bacteriophage were obtained from Galahad Sales (Australia). MS2 bacteriophage was kindly supplied by the Australian Water Quality Center, SA Water Corporation. All solutions were prepared using Milli-Q water.

Screen-printed gold electrodes were purchased from DropSens (three-electrode system, ref. 250BT, 4 mm diameter, including a platinum counter electrode and a silver reference electrode).

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