Biosensors and Bioelectronics 43 (2013) 274–280

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Improving immunosensor performance through oriented immobilization of antibodies on carbon nanotube composite surfaces

Sara Puertas [a,1], María de Gracia Villa [b,1], Ernest Mendoza [c], Cecilia Jiménez-Jorquera [b], Jesús M. de la Fuente [a], César Fernández-Sánchez [b,*], Valeria Grazú [a,**]

[a] Biofunctionalization of Nanoparticles and Surfaces (BioNanoSurf), Instituto de Nanociencia de Aragón (INA), Universidad de Zaragoza. Campus Río Ebro, Edificio I+D, Mariano Esquillor, s/n, 50018 Zaragoza, Spain
[b] Instituto de Microelectrónica de Barcelona, IMB-CNM (CSIC), Campus UAB, 08193 Bellaterra, Barcelona, Spain
[c] Grup de Nanomaterials Aplicats, Centre de Recerca en Nanoenginyeria (CRNE), Universitat Politècnica de Catalunya, c/Pascual i Vila 15, 08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 18 September 2012
Received in revised form
23 November 2012
Accepted 4 December 2012
Available online 11 December 2012

Keywords:
Carbon nanotubes
Antibody
Oriented immobilization
Immunoelectrochemical sensor
Carbodiimide chemistry

ABSTRACT

We report the straightforward oriented covalent attachment of antibodies (Abs) on the surface of carboxylated multiwalled carbon nanotube-polystyrene (MWCNT-PS) materials. The combination of this composite material, applied as a robust electrochemical transducer platform, and its covalent functionalization with Abs in a controlled way by means of a two-step process, could contribute to the development of highly sensitive immunosensor devices. Using the simple and versatile carbodiimide chemistry, Abs were attached to the carboxylic groups of the MWCNT-PS composite surfaces via their superficial amine groups. By taking into account the Ab isoelectric point and the net charge of the composite surface, we engineered an immobilization process to achieve the oriented binding of the Ab molecules by favoring an ionic pre-adsorption step before covalent binding occurred. Thus, the antigen binding capacity of the attached Abs was enhanced by up to 10 times with respect to the capacity estimated for a random spatial distribution of these molecules. The proposed strategy would also serve as a model for the efficient biofunctionalization of other carboxylated carbon-based polymer composite materials with potential applications in the biosensor field.

1. Introduction

Since their discovery in 1991 (Iijima, 1991), carbon nanotubes (CNTs) have been widely explored for the development of analytical tools in the fields of clinical diagnosis, environmental analysis and food industry (Bhattacharya et al., 2011; Buch and Rishpon, 2008; Pérez and Fàbregas, 2008; Villamizar et al., 2008). CNTs can be modified with antibodies (Abs) to develop immunosensors exhibiting relatively low detection limits and high sensitivity (Ajayan and Tour, 2007; Britto et al., 1999; Dresselhaus et al., 2003; Ionescu et al., 2004). Due to its simplicity and versatility, one of the most widely applied immobilization protocols for Abs on CNT surfaces consists of their non-covalent binding by hydrophobic adsorption (Lee et al., 2011; O'Connor et al., 2004). However, the application of this approach may give rise to a non-stable modified substrate as a result of Ab desorption caused by the non-specific binding of other proteins when complex samples (e.g. blood, urine, saliva, etc.) are analyzed (Mendoza et al., 2008). Moreover, another drawback of this methodology is that it can produce a significant decrease in the Ab biological activity as a result of extensive denaturation (Butler et al., 1992) of the protein and steric hindrance of the antigen binding regions (Vermeer et al., 2001).

In order to increase the sensitivity of the CNT-based immunosensors, researchers have put considerable effort to immobilize the Ab in an oriented and stable manner. However, most of the methods developed so far require complicated procedures involving the chemical modification of the Ab (via chemical thiolation, carbohydrate oxidation, disulfide reduction, etc.) (Liu et al., 2009; McDevitt et al., 2007) or the use of adapter proteins with the capacity to interact specifically with the constant region of the Ab (Fc fragment) (Villamizar et al., 2011). While the former strategy involves several steps and could give rise to the partial loss of Ab biological activity, the latter adds an extra-step, which involves

* Corresponding author. Tel.: +34 93 5947700; fax: +34 93 580 1496.
** Corresponding author. Tel.: +34 876 555361; fax: +34 976 762776.
E-mail addresses: selmer@unizar.es (S. Puertas), graciavilla@gmail.com (M. de Gracia Villa), ernest.mendoza@upc.edu (E. Mendoza), cecilia.jimenez@csic.es (C. Jiménez-Jorquera), jmfuente@unizar.es (J.M. de la Fuente), cesar.fernandez@imb-cnm.csic.es (C. Fernández-Sánchez), vgrazu@unizar.es (V. Grazú).
[1] These authors contributed equally.

0956-5663/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2012.12.010

using another biomolecule, this being costly and time-consuming. In terms of popularity, the covalent coupling of the superficial amine groups of the Ab with carboxylic moieties present on the CNT via carbodiimide chemistry is by far the most widely used covalent coupling methodology (Lynam et al., 2009; Marches et al., 2009; Venturelli et al., 2011). However, despite the advantage that this approach does not require the chemical modification of the Ab, the oriented attachment of this molecule is compromised.

We recently demonstrated that a rational design of Ab incubation conditions favors its ionic pre-adsorption followed by its covalent attachment rather than its direct covalent binding through its more reactive amine groups. This approach was optimized using magnetic nanoparticles (MNPs) and Abs with large differences between their isoelectric points (pIs) (Puertas et al., 2011). In order to demonstrate the versatility of this simple oriented immobilization methodology, this paper extends the methodology to the orientation of Abs on the surface of carboxylated multi-walled carbon nanotube-polystyrene (MWCNT-PS) substrates. The combined use of this composite material with the oriented immobilization of Abs would allow the straightforward and cost-effective development of robust and highly sensitive immunosensor devices. However, given the marked hydrophobicity of these composite substrates (Hong and Kang, 2011; Werder et al., 2002), the successful implementation of this oriented conjugation strategy on these materials is more complex than in the case of MNPs. This marked hydrophobicity introduces a new immobilization mechanism that promote a non-oriented spatial distribution of the immobilized Ab: a first rapid hydrophobic adsorption of the Ab followed by a further covalent attachment step. Therefore, unlike what happens in the case of MNPs, once the carboxylated groups of the nanomaterial are activated *via* carbodiimide chemistry two mechanisms that favor the non-oriented immobilization of the antibody instead of one, compete with the oriented immobilization strategy. Thus, in this paper, we show how the introduction of an adequate density of carboxylic groups (*via* plasma treatment of the nanocomposite) together with a rationally design of the experimental incubation conditions could favor this oriented immobilization strategy over the non-oriented competing mechanisms (hydrophobic pre-adsorption plus direct covalent amine binding).

2. Material and methods

2.1. Chemicals and reagents

Carboxylated multiwalled carbon nanotubes (COOH-MWCNTs) (NC-3101) produced by chemical vapor deposition were purchased from Nanocyl S. A. (Belgium) and used without further purification. The MWCNTs contain ca. 4% of carbon in the form of carboxylic groups and they are around 10 nm in diameter.

Polystyrene (PS), N-(3-dimethylamino propyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysulfosuccinimide sodium salt (SNHS), 4-Morpholineethanesulfonic acid (MES), Tween-20, polyclonal antibody Anti-Horseradish Peroxidase developed in rabbit (Ab), type I horseradish peroxidase (HRP) 50–150 units/g solid, bovine serum albumin (BSA), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide, Chloramine T and tyrosine were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1 mCi of sodium ^{125}iodide ($Na^{125}I$) in 0.1 N sodium hidroxide was supplied by Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). Coomasie (Bradford) protein assay kit and BSA as protein standard were purchased from Thermo Scientific (Rockford, IL, USA). Sephadex G-25 gel filtration columns were from GE Healthcare Life Sciences (Uppsala, Sweden). Buffers were prepared according to the standard laboratory procedures. Other chemicals were reagent grade and used as received.

2.2. Preparation of carboxylated MWCNT-PS composite substrates

The preparation of the MWCNT-PS composite material and its deposition on gold substrates and gold electrode surfaces was carried out as reported elsewhere (Villa et al., 2011). More details are given in the supporting information.

Both the MWCNT-PS modified substrates and electrodes, when required, underwent an O_2 plasma etching process, which was carried out using a Microwave Gas Plasma System 210. The treatment conditions were: 50 mTorr chamber pressure, 50 sccm O_2, 200 W RF power during 60 s.

2.3. Contact angle measurements

Hydrophobicity analysis was carried out by placing a water drop on different parts of each substrate and measuring the contact angle between the drop and the substrate. An EasyDrop standard equipment (Krüss, Hamburg) was used.

2.4. Covalent immobilization of the antibody on MWCNT-PS composite substrates

2.4.1. Carboxylic groups activation step

The substrates were washed three times in 10 mM MES pH 5. They were incubated for 30 min at 37 °C in 300 μL of 10 mM MES pH 5 containing 15 μmols of EDC and 22.5 μmols of SNHS. Finally, they were washed three times with 10 mM MES buffer.

2.4.2. Antibody immobilization step

- *Protocol A: Immobilization via the more reactive amine groups of the antibody (direct covalent binding):* Immediately after the activation step the substrates were incubated during 2 h at 37 °C with 100 μL of 10 μg/mL Ab solution prepared in 10 mM sodium bicarbonate buffer pH 8 containing 2% Tween 20.
- *Protocol B: Immobilization via the hydrophobic zone of the antibody (hydrophobic pre-adsorption):* Following the activation step, the substrates were incubated during 2 h at 37 °C with 100 μL of 0.1 M phosphate buffer pH 7.2 solution containing 10 μg/mL Ab.
- *Protocol C: Immobilization via the positive charged richest zone of the antibody (oriented approach):* The activated substrates were incubated during 2 h at 37 °C with 100 μL of 10 mM MES pH 5 buffer solution containing 10 μg/mL Ab.

2.4.3. Covalent binding evaluation

After the antibody incubation step, the substrates were washed with different buffer solutions in order to desorb antibodies that were non covalently bound to the substrate (desorption buffers) (Supporting information).

2.4.4. Blocking process of the remaining activated carboxylic-groups

The remaining activated carboxylic groups were blocked by incubation with 100 μL of 10 mM MES pH 5 buffer solution containing 1% BSA at 4 °C for 16 h. Finally the substrates were washed with 10 mM MES pH 5 and stored in 10 mM sodium phosphate pH 7.0 at 4 °C, until use.

2.4.5. Determination of the amount of immobilized antibody by radioactivity (Boni-Mitake et al., 2006; Melkko et al., 1996; Shimura et al., 2002)

10 μL of 2 mg/mL anti-HRP solution prepared in 0.1 M sodium phosphate pH 7.5 were incubated for 15 s at 25 °C with 50 μL of 0.5 M sodium phosphate pH 7.5 containing 60 μCi of $Na^{125}I$ and 264.0 nmols of Chloramine T as oxidizing agent. The amount of antibody was previously determined by Bradford assay using BSA as protein standard (Bradford, 1976). The reaction was immediately terminated by the addition of 60 μL of 0.5 M sodium phosphate pH 7.5 containing 148.0 nmols of tyrosine and 3.6 μmols of potassium iodide. Then, the radiolabeled antibody was purified by Sephadex G-25 gel filtration column equilibrated with 20 mM Tris pH 7.5 150 mM NaCl and dialyzed against the buffer that was to be used for the immobilization process, for 12 h at 4 °C. This labeled antibody was immobilized following the protocols described above. The gamma emission of the functionalized substrates was measured for 60 s in a LKB Wallac 1282 Compugamma Universal Gamma Counter.

2.5. Determination of the biological activity of the anti-HRP-MWCNT-PS

The functionalized substrates were incubated with 100 μL of 6 μg/mL HRP solution prepared in 10 mM sodium phosphate pH 7.0 for 30 min at 37 °C. They were washed several times with 10 mM sodium phosphate buffer pH 7.0. As a control of unspecific interactions between the HRP and the MWCNT-PS, substrates without antibody but with activated carboxyl groups, were used. After the blocking step and the incubation with the HRP, colorimetric and electrochemical measurements were carried out as follows.

2.5.1. Colorimetric measurements

The substrates were incubated with 300 μL of 50 mM sodium phosphate buffer pH 6.0 containing 1 mM ABTS and 1 mM H_2O_2. Using a filter based microplate reader (Biotek Elx800) the increase in absorbance with time at 414 nm was monitored (Pütter and Becker, 1983) (Supporting information).

2.5.2. Electrochemical measurements

A standard three-electrode electrochemical cell that included a Pt counter electrode, a Ag/AgCl/10% (w/v) KNO_3 reference electrode (Orion) and the MWCNT-PS composite based biosensor was used for all the electrochemical experiments. These were performed at room temperature using a type III μ-Autolab potentiostat (Ecochemie), controlled with GPES 4.7 (general purpose electrochemical system) software package. The electrochemical cell was filled with 25 mL of a 50 mM sodium phosphate buffer pH 6.0 containing 1 mM ABTS. Amperometric measurements were recorded at a set potential of +200 mV vs. Ag/AgCl reference electrode, applied for 150 s. At a set time of 50 s, 250 μL of a 100 mM H_2O_2 stock solution in DI water were added to the electrochemical cell.

3. Results and discussion

We had previously shown that the initial oxygen plasma treatment applied to the MWCNT-PS composites substrates, created a high density of oxygen-containing functional groups at the surface of both CNT and PS components, this being mainly carboxylic groups (Fernández-Sánchez et al., 2009). Measurements of contact angle shows that although this treatment significantly reduced the marked hydrophobicity of these composite substrates from 103.4 ± 2.2° of the intact substrates to 65.7 ± 3.5° following the plasma treatment, they cannot be considered as hydrophilic (Xu et al., 2009).

Thus, upon activation of the carboxylic groups of the treated substrates with EDC/SNHS, Ab covalent immobilization can occur by three distinct mechanisms that compete with each other (Fig. 1). First, the direct covalent reaction between the most reactive amine moieties of the Ab and the activated carboxylic groups. Additionally,

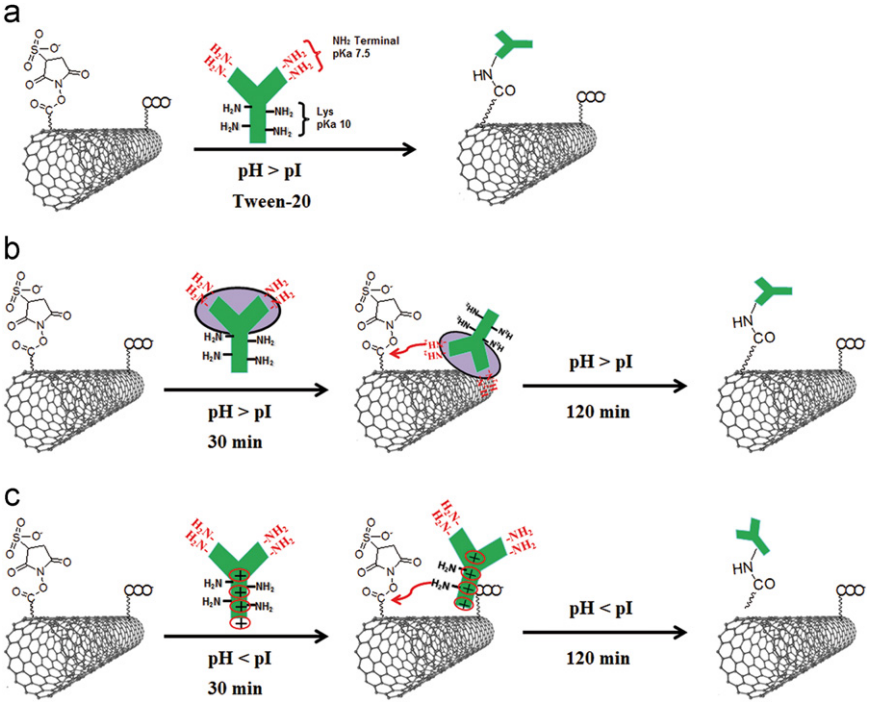

Fig. 1. Antibody immobilization mechanisms after carboxylic groups activation process. (a) Immobilization via the more reactive amine groups of the antibody (direct covalent binding; (b) Immobilization via the hydrophobic zone of the antibody (hydrophobic pre-adsorption); (c) Immobilization via the positive charged richest zone of the antibody (oriented approach). The most hydrophobic regions of the Ab are marked in purple. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

two conjugation mechanisms may also arise. These are favored by the presence of negative charges on the surfaces of CNTs coupled with the intrinsic hydrophobicity of these structures and the kinetic differences between physical adsorption processes and covalent reactions. Briefly, both mechanisms involve a two-step process in which the first step is the rapid physical adsorption of the Ab driven by hydrophobic or ionic interactions. Subsequently, the site-specific irreversible covalent binding of the Ab would occur only through its amine-groups that face the activated carboxylic moieties of the support surface.

This work shows that despite using carboxylated surfaces activated with the same chemistry, it is possible to favor one mechanism of immobilization over another. In this context, three different immobilization protocols were rationally designed using anti-HRP as a model IgG since its biological activity could be determined in a straightforward way by measuring either colorimetrically or electrochemically the enzymatic activity of its specific antigen (peroxidase) based on the use of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as redox mediator.

In *Protocol A*, the immobilization was carried out at pH 8.0, at which the Ab had a net negative charge (pI 5.85–6.55), thereby preventing ionic interactions between the substrate and the protein. Moreover, 2.0% Tween 20 was added to the incubation buffer in order to also prevent hydrophobic interactions. In this context, Ab conjugation may occur mainly through direct covalent binding between the most reactive amine groups of the Ab and the activated carboxylic moieties of the composite substrate. As it could be observed in Fig. 2, using this binding protocol the antigen binding efficiency of the attached Ab was over 2-fold higher if the carboxylic groups of the substrates were previously activated with EDC/SNHS. Moreover, only in the case of activated substrates, the biological activity of the functionalized composites remained constant after the sequential washing steps that were carried out to ensure the desorption of Ab molecules non covalently attached by means of electrostatic or hydrophobic interactions. Therefore, these results are consistent with a mechanism of immobilization via direct covalent binding through the most reactive amine groups of the Ab.

In *Protocol B*, Ab ionic pre-adsorption was also avoided as the immobilization was carried out at pH 7.2. However, an initial hydrophobic adsorption step was favored as no surfactant was added to the incubation solution. In this case, the antigen binding efficiency of the bound Ab was similar when using composites substrates activated or not with EDC/SNHS. This result suggests that strong hydrophobic adsorption drives the Ab immobilization. As hydrophobic interactions are reversible processes, the adsorbed Ab molecules could be removed by adding surfactants. By contrast, if the Ab is covalently attached to the substrate, the bond would be stable regardless the surfactant content added. However, in this case, even after increasing up to 2-fold the amount of surfactant used in *Protocol A* during the washing steps, no difference in the amount of active bound Ab was observed between activated and non-activated substrates. For desorption to occur, all contacts between protein and surface must be simultaneously broken. Thus the failure to find suitable conditions to desorb reversibly bound Abs is a common observation when a strong hydrophobic interaction occurred. In these conditions, protein adsorption is largely irreversible because of the difficulty or improbability of simultaneous disruption of all contacts (Grazu et al., 2006; Savov et al., 1997; Xu et al. 2009). Therefore, although these results does not make possible to ensure that a covalent Ab binding took place when using activated surfaces, it reinforces the idea that using this immobilization conditions a hydrophobic pre-adsorption of the Ab takes place.

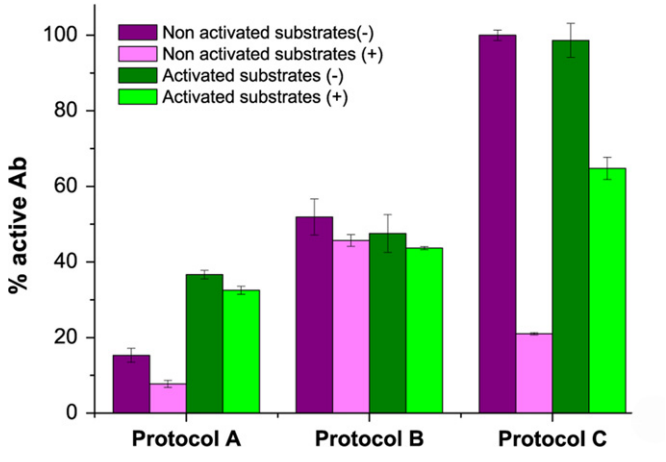

Fig. 2. Evaluation of Ab covalent attachment via the main mechanism proposed in each of the protocols studied. The biological activity was colorimetrically measured, (−) before and (+) after the washing steps were carried out. The immobilization was carried out using composites substrates activated or not with EDC/SNHS. The highest signal obtained was considered the 100% of active Ab (composite substrates functionalized with the *Protocol C*) and the results are normalized to this value. Error bars represent the standard deviation of at least three replicates.

Finally, in *Protocol C* the Ab immobilization was carried out at low ionic strength and pH 5.0 so the Ab ionic pre-adsorption was promoted as the Ab had a net positive charge at this incubation conditions. The biological activity of the functionalized composite substrates previously activated or not with EDC/SNHS was similar before the washing steps. However, after increasing the pH from 5 to 8 during the washing steps, the activated substrates retained more than 60% of the Ab biological activity while only less than 20% of the active Ab remained attached to the non-activated ones. Only if Ab molecules are attached by ionic adsorption could they be eluted by changing the net charge of the Ab due to a shift in the pH of the media. Therefore, such significant differences between the activated and non-activated substrates confirm that the Ab incubation conditions used in *Protocol C* favor the covalent attachment of the Ab driven by an initial ionic pre-adsorption step.

Then, the use of activated and non-activated composite surfaces together with washing steps with sequential changes of pH, ionic strength and use of detergents, allowed us to demonstrate that different incubation conditions favor different antibody immobilization mechanisms. As a result, the antigen binding efficiency of the functionalized substrates was significantly different depending on the mechanism responsible for the immobilization. This could be clearly seen when normalizing the biological activity of MWCNT-PS composite electrodes functionalized with *Protocols B* and *C* with respect to those functionalized using *Protocol A*. Fig. 3 shows the results of the amperometric analyses carried out with the resulting electrochemical immunosensors fabricated using the different protocols.

The maximum peroxidase binding capacity of the Abs attached using *Protocol C* was 10 and 2.5 times higher when compared to those attached using *Protocols A* and *B*, respectively (Fig. 3d). It is important to point out that *Protocol B* was previously used by our group for the detection of rabbit Immunoglobulin *G* target analyte to concentration levels down to 3 ng/mL (Fernández-Sánchez et al., 2009). These significant difference in the antigen binding capacity may be due to different yields of immobilization and/or different spatial orientations of bound Abs. However, the total amount of bound protein determined by radioactivity resulted to be the same regardless the applied immobilization protocol (ca. 1.8 ng of Ab per mm^2) (Supporting information). Therefore, these large differences in

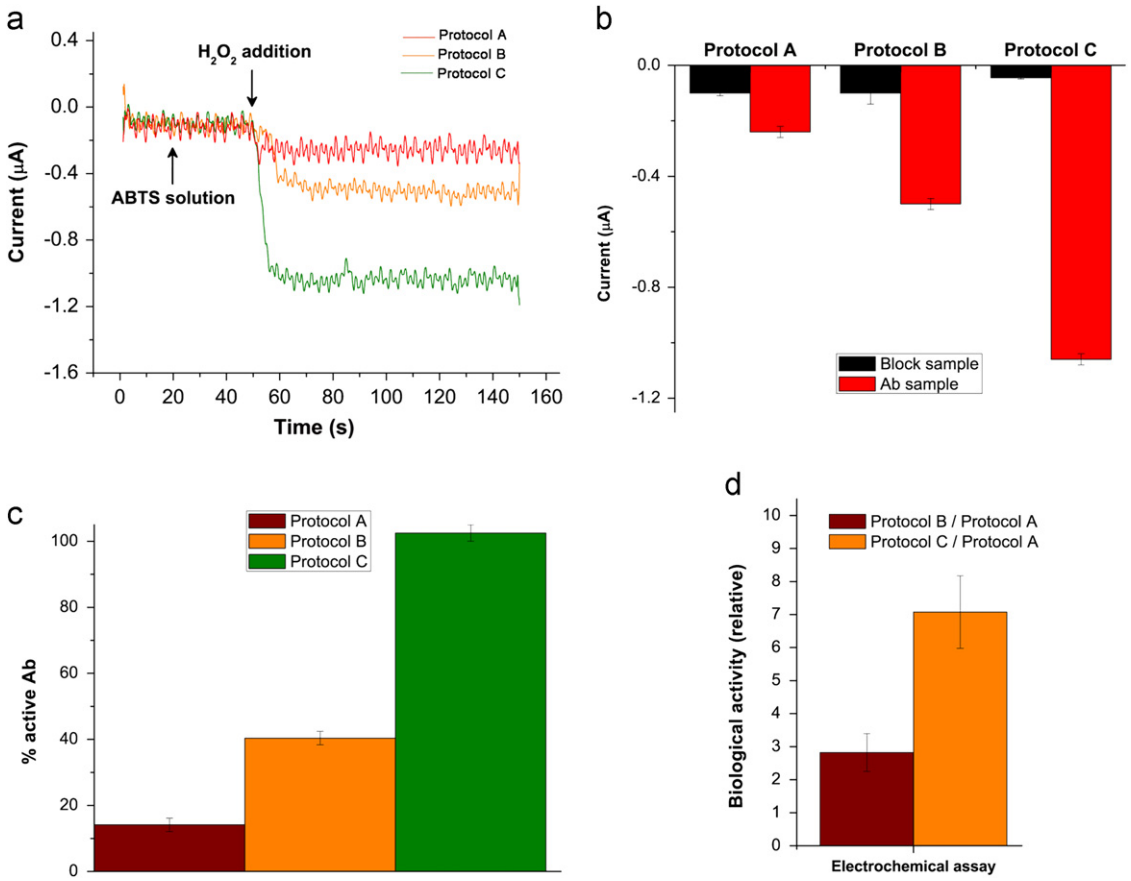

Fig. 3. (a) Amperograms recorded with MWCNT-PS composite electrodes modified following the three different protocols. Amperometric measurements were recorded at a set potential of +200 mV vs. Ag/AgCl reference electrode, applied for 150 s. (b) Bar graph depicting the current values extracted from the amperometric measurements of the Ab-modified electrodes and those prepared to test the effectiveness of the blocking process. (c) Transformation in percentage of active Ab attached after normalized data by subtracting the current related to the blocking process from the current of the corresponding Ab-modified electrode. The activity of the oriented methodology was taken as the 100%. (d) Relative biological activity electrochemically measured of MWCNT-PS modified gold electrodes functionalized with anti-HRP using *Protocol A* after the sequence of washing steps. The results are normalized to the biological activity of substrates functionalized using *Protocols B* and *C*. Error bars represent the standard deviation of at least three replicates.

biological activity must be a consequence of different molecular orientations adopted by the immobilized Ab.

In this regard, the lower antigen binding efficiencies obtained when using *Protocols A* and *B* are because most of the Ab molecules adopt molecular spatial orientations with reduced antigen binding capacity as a result of steric hindrance of their antigen binding sites (variable domain or Fv). Particularly in the case of *Protocol A*, this is because immobilization mainly occurs through direct covalent binding of the most reactive amine groups of the antibody. These are the terminal amine moieties of the four Ab polypeptide chains (pK_a around 7–8), which are all unfortunately located in the Fv area (Sesay, 2003). In the case of *Protocol B*, the hydrophobic pre-adsorption step also promotes a decrease in the antigen binding capacity, but in this case not only by steric hindrance due to preferential adsorption of the Ab through its more hydrophobic surface areas (Fab fragments), but also by extensive Ab denaturation (Butler et al., 1992). Therefore, when EDC is selected as the Ab linking chemistry if either of these two protocols is carried out, the resulting random orientation would limit the sensitivity of the final device for the detection of very low concentrations of analyte (Puertas et al., 2010; Rao et al., 1998). However, if the immobilization protocol favors an ionic pre-adsorption step (*Protocol C*), a better antigen binding capacity of the covalently bound Ab is ensured. This is because only in this case most Ab molecules adopts an oriented spatial disposition and thus the Fv area is not involved during the covalent binding. The latter is a result of being the plane involving the Ab four protein subunits the region with the greatest number of charges (Puertas et al., 2011). Our finding thus confirms that the oriented covalent immobilization of Abs on MWCNT-PS composites can be tailored using carbodiimide chemistry provided that a correct choice of the incubation conditions is carried out.

However, to promote the Ab ionic pre-adsorption, not only the suitable incubation conditions are important but also the density of negatively charged groups on the MWCNT-PS composite substrates. It is well known that ionic adsorption processes require the simultaneous interaction among several groups of the Ab and several groups of the support (Fuentes et al., 2004; Mateo et al., 2003; Pessela et al., 2006). As already mentioned, although carboxylated MWCNTs were used to prepare the supports, it has been previously shown that the oxygen plasma preconditioning step generates a higher density of carboxylic groups on the MWCNT-PS composite surface (Fernández-Sánchez et al., 2009). Therefore, to check the importance of this treatment in order to promote the ionic adsorption of the Ab, substrates with (P) or without plasma treatment (SP) were functionalized using the three different Ab immobilization protocols. The amount of bound/active Ab was evaluated by radioactivity and by quantification of the antigen binding efficiency of the functionalized substrates. As it could be observed in Fig. 4, the higher hydrophobicity and lower number of

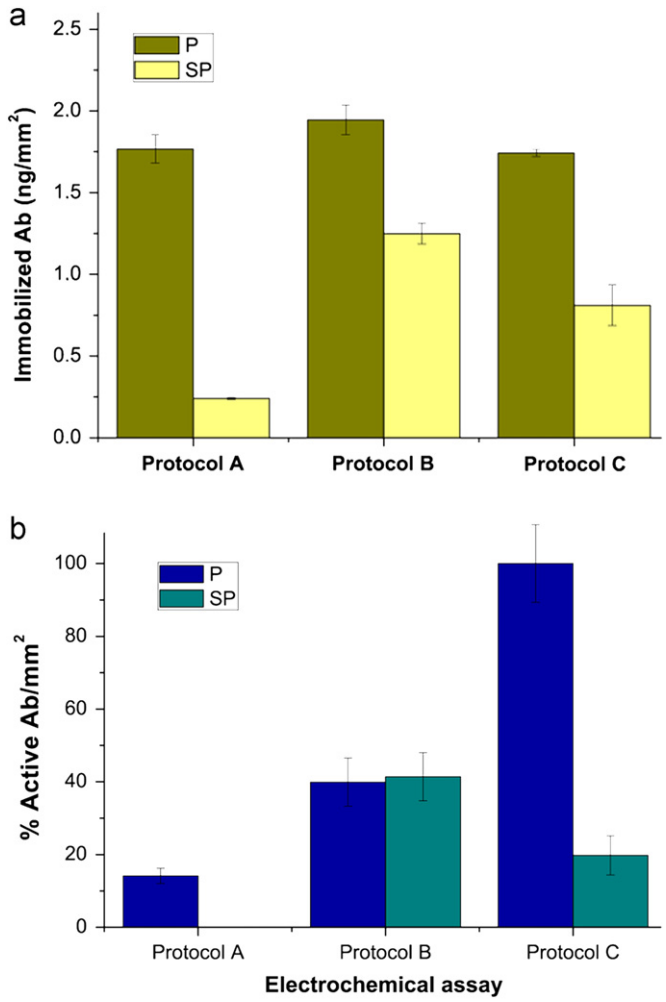

Fig. 4. (a) Amount of attached Ab per mm^2 of MWCNT-PS composite substrates determined by radioactivity measurements. (b) Percentage of active Ab per mm^2 of MWCNT-PS composite substrate. SP means substrates without plasma treatment and P with this treatment. The highest signal of enzymatic measurements obtained was considered the 100% of active Ab and the results are normalized to this value. Error bars represent the error of at least three replicates. For SP samples functionalized using *Protocol A*, no significant activity values were obtained.

carboxylic groups of the substrates that were not treated with plasma favored hydrophobic interactions. Indeed, the highest immobilization yields with the non-treated composite substrates were obtained when using *Protocol B*. However, when increasing the number of negative charges by the plasma treatment, the immobilization yields increased more drastically when using *Protocols A* and *C*. Both protocols need the presence of carboxylic groups in order to: (i) promote the direct covalent binding of the amine groups of the Ab (*Protocol A*) or (ii) the Ab ionic pre-adsorption step (*Protocol C*). Thus, these results highlight the importance of the plasma treatment in promoting the oriented immobilization of Abs driven by their ionic pre-adsorption, thus producing MWCNT-PS composite substrates with higher biological activity.

4. Conclusions

This work demonstrated that using a rational design of incubation conditions, Abs can be selectively attached and oriented on the surface of a MWCNT-PS composite using the simple and well-known carbodiimide chemistry. This proper spatial orientation significantly improved the antigen binding capacity of the attached Ab, thereby resulting in an increase of the analytical signal of an amperometric biosensor that used MWCNT-PS based electrodes as electrochemical transducers. The signal increased up to 10 times when compared to the random orientation driven by direct covalent attachment of the Ab. Moreover, the signal increased up to 2.5 times compared with the random covalent orientation driven by a hydrophobic pre-adsorption step. Remarkably, the methodology described here is straightforward and does not require any initial chemical modification of the Ab molecule or the use of other adapter biomolecules, such as protein A or G. This functionalization approach could also serve as a model to improve the biological activity of other biofunctionalized carboxylated carbon-based polymer composites that have been previously applied or have the potential to be applied in the biosensor field.

Acknowledgments

This work has been funded by the CONSOLIDER CSD2006-12 and TEC2010-17274. S. Puertas thanks DGA, and J.M. de la Fuente thanks ARAID for financial support. We thank Iñigo Echaniz for technical support and other BioNanoSurf members for their contributions to the paper. We also thank to Jesús Puertas for technical support in radioactivity measurements.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.12.010.

References

Ajayan, P.M., Tour, J.M., 2007. Nature 447, 1066–1068.
Bhattacharya, M., Hong, S., Lee, D., Cui, T., Goyal, S.M., 2011. Sensors and Actuators, B 155, 67–74.
Boni-Mitake, M., Costa, H., Vassilieff, V.S., Rogero, J.R., 2006. Toxicon 48, 550–555.
Bradford, M.M., 1976. Analytical Biochemistry 72, 248–254.
Britto, P.J., Santhanam, K.S.V., Rubio, A., Alonso, J.A., Ajayan, P.M., 1999. Advanced Materials 11, 154–157.
Buch, M., Rishpon, J., 2008. Electroanalysis 20, 2592–2594.
Butler, J.E., Ni, L., Nessler, R., Joshi, K.S., Suter, M., Rosenberg, B., Chang, J., Brown, W.R., Cantarero, L.A., 1992. Journal of Immunological Methods 150, 77–90.
Dresselhaus, M.S., Lin, Y.M., Rabin, O., Jorio, A., Souza Filho, A.G., Pimenta, M.A., Saito, R., Samsonidze, G.G., Dresselhaus, G., 2003. Materials Science and Engineering C 23, 129–140.
Fernández-Sánchez, C., Pellicer, E., Orozco, J., Jiménez-Jorquera, C., Lechuga, L.M., Mendoza, E., 2009. Nanotechnology 20, 335501.
Fuentes, M., Pessela, B.C.C., Mateo, C., Munilla, R., Guisan, J.M., Fernandez-Lafuente, R., 2004. Journal of Chromatography A 1059, 89–94.
Grazu, V., Betancor, L., Montes, T., López-Gallego, F., Guisan, J.M., Fernández-Lafuente, R., 2006. Enzyme and Microbial Technology 38, 960–966.
Hong, J., Kang, S.W., 2011. Colloids and Surfaces A 374, 54–57.
Iijima, S., 1991. Nature 354, 56–58.
Ionescu, R.E., Gondran, C., Gheber, L.A, Cosnier, S., Marks, R.S., 2004. Analytical Chemistry 76, 6808–6813.
Lee, D., Chander, Y., Goyal, S.M., Cui, T., 2011. Biosenors and Bioelectronics 26, 3482–3487.
Liu, Z., Tabakman, S.M., Chen, Z., Dai, H., 2009. Nature Protocols 4, 1372–1382.
Lynam, C., Gilmartin, N., Minett, A.I., O'Kennedy, R., Wallace, G., 2009. Carbon 47, 2337–2343.
Marches, R., Chakravarty, P., Musselman, I.H., Bajaj, P., Azad, R.N., Pantano, P., Draper, R.K., Vitetta, E.S., 2009. International Journal of Cancer 15, 2970–2977.
Mateo, C., Torres, R., Férnandez-Lorente, G., Ortiz, C., Fuentes, M., Hidalgo, O., López-Gallego, F., Abian, O., Palomo, J.M., Betancor, L., Pessela, B., Guisan, J.M., Fernandez-Lafuente, R., 2003. Biomacromolecules 4, 772–777.
McDevitt, M.R., Chattopadhyay, D., Kappel, B.J., Jaggi, J.S., Schiffman, S.R., Antczak, C., Njardarson, J.T., Brentjens, R., Scheinberg, D.A., 2007. Journal of Nuclear Medicine 48, 1180–1189.
Melkko, J., Kauppila, S., Niemi, S., Risteli, L., Haukipuro, K., Jukkola, A., Risteli, J., 1996. Clinical Chemistry 42, 947–954.

Mendoza, E., Orozco, J., Jiménez-Jorquera, C., González-Guerrero, A.B., Calle, A., Lechuga, L.M., Fernández-Sánchez, C., 2008. Nanotechnology 19, 075102.
O'Connor, M., Kim, S.N., Killard, A.J., Forster, R.J., Smyth, M.R., Papadimitrakopoulos, F., Rusling, J.F., 2004. Analyst 129, 1176–1180.
Pérez, S., Fàbregas, E., 2008. Analyst 137, 3854–3861.
Pessela, B.C.C., Fuentes, M., Mateo, C., Munilla, R., Carrascosa, A.V., Fernández-Lafuente, R., Guisan, J.M., 2006. Enzyme and Microbial Technology 39, 909–915.
Puertas, S., Moros, M., Fernández-Pacheco, R., Ibarra, M.R., Grazú, V., de la Fuente, J.M., 2010. Journal of Physics D: Applied Physics 43, 474012.
Puertas, S., Batalla, P., Moros, M., Polo, E., del Pino, P., Guisan, J.M., Grazú, V., de la Fuente, J.M., 2011. ACS Nano 5, 4521–4528.
Pütter, J., Becker, R., 1983. Methods of Enzymatic Analysis. VerlagChemie, Weinheim.
Rao, S.V., Anderson, K.W., Bachas, L.G., 1998. Mikrochimica Acta 128, 127–143.
Savov, L., Heller, H.-P., Janke, D., 1997. Metallurgy 51, 475–485.
Sesay, M.A., 2003. Biopharm International 16, 32–39.
Shimura, M., Sogawa, Y., Kawakita, Y., Ikekita, M., Yamazaki, N., Kojima, S., 2002. Nuclear Medicine and Biology 29, 491–496.
Vermeer, A.W.P., Giacomelli, C.E., Norde, W., 2001. Biochimica et Biophysica Acta 1526, 61–69.
Villa, M., de, G., Jiménez-Jorquera, C., Haro, I., Gomara, M.J., Sanmartí, R., Férnandez-Sánchez, C., Mendoza, E., 2011. Biosensors and Bioelectronics 27, 113–118.
Villamizar, R.A, Maroto, A., Rius, F.X., Inza, I., Figueras, M.J., 2008. Biosensors and Bioelectronics 24, 279–283.
Villamizar, R.A, Maroto, A., Rius, F.X., 2011. Analytical and Bioanalytical Chemistry 399, 119–126.
Venturelli, E., Fabbro, C., Chaloin, O., Ménard-Moyon, C., Smulski, C.R., Ros, T.D., Kostarelos, K., Prato, M., Bianco, A., 2011. Small 7, 2179–2187.
Werder, T., Walther, J.H., Koumoutsakos, P., 2002. International Conference on Computational Nanoscience and Nanotechnology, pp 490–493.
Xu, Z.-K., Iuang, X.-J., Wan, L.-S., 2009. In: Chu, L.-Y. (Ed.), Smart Membrane Materials and Systems. Zhejiang University Press, Hangzhou and Springer-Verlag, GmbH Berlin Heidelberg, pp. 54.