Covalent Immobilization of Antibodies on Finally Inert Support Surfaces through their Surface Regions Having the Highest Densities in Carboxyl Groups

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The correct immobilization of antibodies is one of the most critical steps in the preparation of immunosensors and immunochromatography matrices. In addition, the final support has to be chemical and physically inert to avoid the unspecific adsorption of proteins that can reduce the sensitivity of the biosensor or the purification achieved by the chromatography. The solution to both problems is one of the major challenges in the field. Here, we have presented two different novel and simple alternatives to have the unmodified antibody anionically exchanged to a support, further covalently immobilized with more than 90% of the antibodies bonded to the support by the four subunits, retaining a high functionality and giving a final "inert" surface. The first solution was the use of supports having a low superficial density of amino groups activated with glutaraldehyde. Here, the inertness was achieved by the use of a very low density of amino groups, unable to adsorb proteins at 100 mM sodium phosphate, while immobilization proceeds mainly via a first adsorption of the antibody and a further reaction with the glutaraldehyde groups. The second solution implies the design of a novel support (amino-epoxy). This support again produces a first ionic exchange of the antibody on the support and a further reaction with the epoxy groups, but because the epoxy groups can be finally blocked with aspartic groups (annulling the charge), the initial density of amino-epoxy groups can be as high as possible. Both systems permitted the correct and oriented immobilization of IgG. The immobilized antibody showed high-functionality (65−75%) and a final inert support surface. This immobilized antibody (antiperoxidase) was able to capture fully specifically HRP contaminating a protein crude extract from *E. coli*.

Introduction

The correct immobilization of antibodies is one of the most critical steps in the preparation of an immuno-biosensor or a chromatographic immuno-affinity column.[1–4]

The final matrix should be chemical and physically inert to prevent undesired adsorptions of other components of the medium, the report molecules (e.g., a labeled second antibody), and so on. However, the support must be initially reactive because it should be able to immobilize the antibody. Moreover, it is necessary to prevent distortions of the recognition place of the antibody to maintain its functionality. Finally, if the analyte is a macromolecule (e.g., other protein), the antibody needs to be properly oriented regarding the support surface to be accessible for the interaction with these large molecules.

However, there is not only one adequate orientation when immobilizing antibodies, because the correct orientation of the antibody could depend on the method to determinate the analyte. At first glance, an antibody immobilized with the Fab regions far from the support should be the most suitable: the capture of the analyte may be very rapid; it should be possible to use a second marked antibody to determine the amount of captured analyte (ELISA) and so on. However, in some cases, other orientations may also have interest and may even be more adequate than this one if the immobilized antibody retains a reasonable percentage of the recognition capacity.

That may be the case for the use of immobilized antibodies on carbon nanotubes when the captured antibody is going to be detected via the nanotube field effect transistors technique.[5,6] Carbon nanotubes are good electrical conductors, but the intensity of the electric current may strongly be altered if any molecule interacts with its surface and these alterations in the electrical current may be used to determine the amount of captured antigen. In this case, it may be interesting to have the Fab regions very near to the nanotubes surface. The rate of adsorption of the antigen by the immobilized antibody could be reduced, but the adsorption of the analyte very close to the carbon nanotubes surface would be the key to get an intense nanotube field effect transistors. Otherwise, an apparent "correct" orientation of the antibody, with the Fab region very far from the surface, will convert the antibody in a spacer arm and, hence, a minimal interaction of the antigen with the nanotube surface would occur.

There are some techniques to immobilize the antibodies by the Fc region, leaving free and far from the support surface the Fab regions: using immobilized protein A or G[7,8] via covalent immobilization through the oxidized sugar chains of the antibody,[9–14] previous site-directed biotinylation of the Fc region to get site direct immobilization on avidin or streptavidin supports,[15–17] and so on. All these protocols are more or less sophisticated and, in many cases, involve the antibody modification.

However, there are no studies to immobilize the antibodies with the Fab regions near to the support surface but still able to recognize the antigen. Figure 1 shows that the terminal amino

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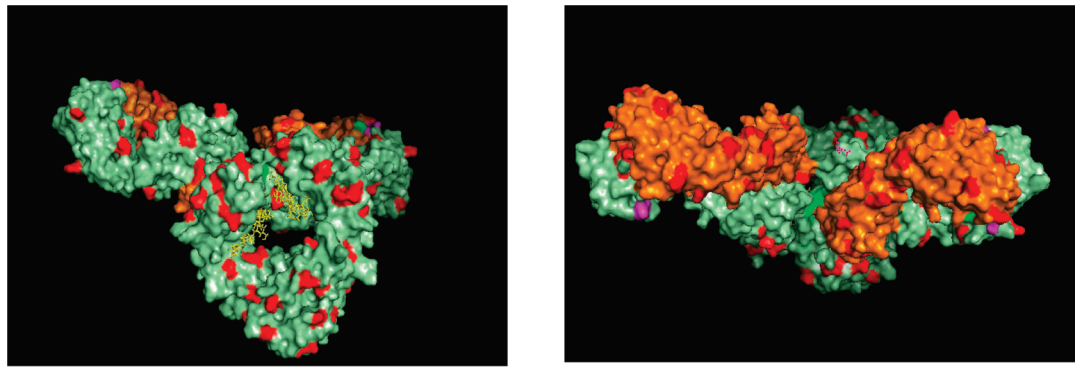

Figure 1. 3D structure of Ig G from rabbit (Anti-HRP). The structure was obtained from the Protein Data Bank (PDB) and visualized using PyMol v0.99. The PDB entry for the antibody was 1IGY. Picture A, lower view; picture B, upper view. The heavy chains are represented in green, the light ones in brown. In red are represented the carboxyl groups and in purple the amino terminal groups.

groups are placed near to the recognition place, therefore, the immobilization of the antibody via the most reactive amino group could immobilize the antibody with the Fab regions very close to the support surface, but very likely involved in the immobilization, strongly reducing the adsorption capacity of the immobilized antibody.[18–20]

It is possible to observe that there is a very high density of Asp and Glu all along the antibody surface, while the region Fab, where the immobilization is not desirable, is not much richer in these groups than other areas of the antibody. As the adsorption of the proteins on supports via ionic exchange occurs via a multipunctual process,[21–24] it may be expected that the antibodies may immobilize on the support by the position where the highest possibilities of getting this multipoint adsorption exists. In this case, this orientation could imply the antibody has immobilized, implying the largest area able to interact with the support; that is, a plane involving the four subunits in the immobilization. If this was the case, antibodies anionically exchanged will have the two Fab very near to the support surface, but the recognition places could be not involved in the immobilization, preserving its adsorption capacity.

However, the inertness of the final support surface after the antibody immobilization remains as an important feature of the system. Thus, a simple ionic exchange may not be valid to immobilize the antibody on the support. If that ionic exchange is strong enough to keep immobilized the antibody during use, the support will very likely be able to adsorb many other proteins. Thus, the driving force of the immobilization should be the ionic exchange, but after the antibody immobilization, this property of the support should be reduced or annulated. Bearing this in mind, after the first antibody immobilization by anionic exchange, it seems to be convenient to covalently bind the antibody to the support (preventing its release to the reaction medium) and to alter the support surface (to reduce its adsorption capacity).

There are some techniques that immobilize proteins following a two-step immobilization mechanism, that is, a first anionically exchange of the proteins on the support followed by a support-protein covalent attachment: (i) The most popular one is the aminated supports activated with glutaraldehyde. Using these supports at low ionic strength, the first step in the protein immobilization is mainly an anionic exchange of the protein[25] by the areas of the protein having the richest density in negatively-charged groups (much more rapid than the direct covalent attachment) and a further covalent reaction among the nearby residues of the protein and the glutaraldehyde groups in the support. The glutaraldehyde activated supports may release the protein if exposed to high concentrations of nucleophiles or react with other molecules. Therefore, to produce immobilized antibodies to be used as a biosensor or in immuno-affinity seems to be convenient to perform a final reduction step. However, we will finally have positively-charged groups. When this protocol was used, the only solution to have a very low adsorption of proteins in the support is introducing as few amino groups as possible to prevent the ionic exchange of proteins under certain conditions,[24] while being able to adsorb the antibody under milder conditions. (ii) Another possibility is to build amino-epoxy groups on the support surface. Epoxy supports can hardly directly immobilize soluble proteins due to the low reactivity of the epoxy groups with soluble proteins (much lower than the glutaraldehyde).[26,27]

This has permitted the development of a new generation of heterofunctional epoxy supports,[28,29] among them, amino-epoxy supports.[30–32] They should be similar to the glutaraldehyde, giving similar antibody orientation and having a strong ionic exchanger capacity. However, due to the strong bond between the antibody and the support (thio-ether, ether, secondary amino),[33] it is possible after the antibody immobilization to modify the remainder epoxy groups with negatively-charged groups (e.g., aspartic acid) to alter the final properties of the support by generating a neutral surface by combining positively- and negatively-charged groups.[23]

In this paper, the immobilization of nonmodified antibodies via a first anionic exchange followed by a covalent reaction (using epoxy or glutaraldehyde chemistry) has been intended, the orientation of the antibody on the support analyzed, and the adsorption capability of the immobilized antibodies investigated. Finally, some strategies to get a final inert surface (after the ionic exchange) have been presented.

Materials and Methods

Materials. Sodium periodate was from Merck (Darmstadt, Germany). Ethylendiamine (EDA), sodium borohydride, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI), β-mercaptoethanol, aspartic acid, 1,4-butanediol diglycidyl ether, glutaraldehyde solution 25%, horseradish peroxidase (HRP), and polyclonal antihorseradish peroxidase (anti-HRP; developed in rabbit) were supplied by SIGMA Chem. Co. (St. Louis). Coomassie (Bradford) protein assay kit was purchased from Pierce (U.S.A.). LMW-SDS Marker Kit (14 kDa-97 KDa) was from Pharmacia (Uppsala, Sweden). Acrylamide/bis 30% solution was supplied by BioRad (Hercules, U.S.A.). All other reagents were of analytical grade. Agarose (4% cross-linked beads) and carboxymethyl

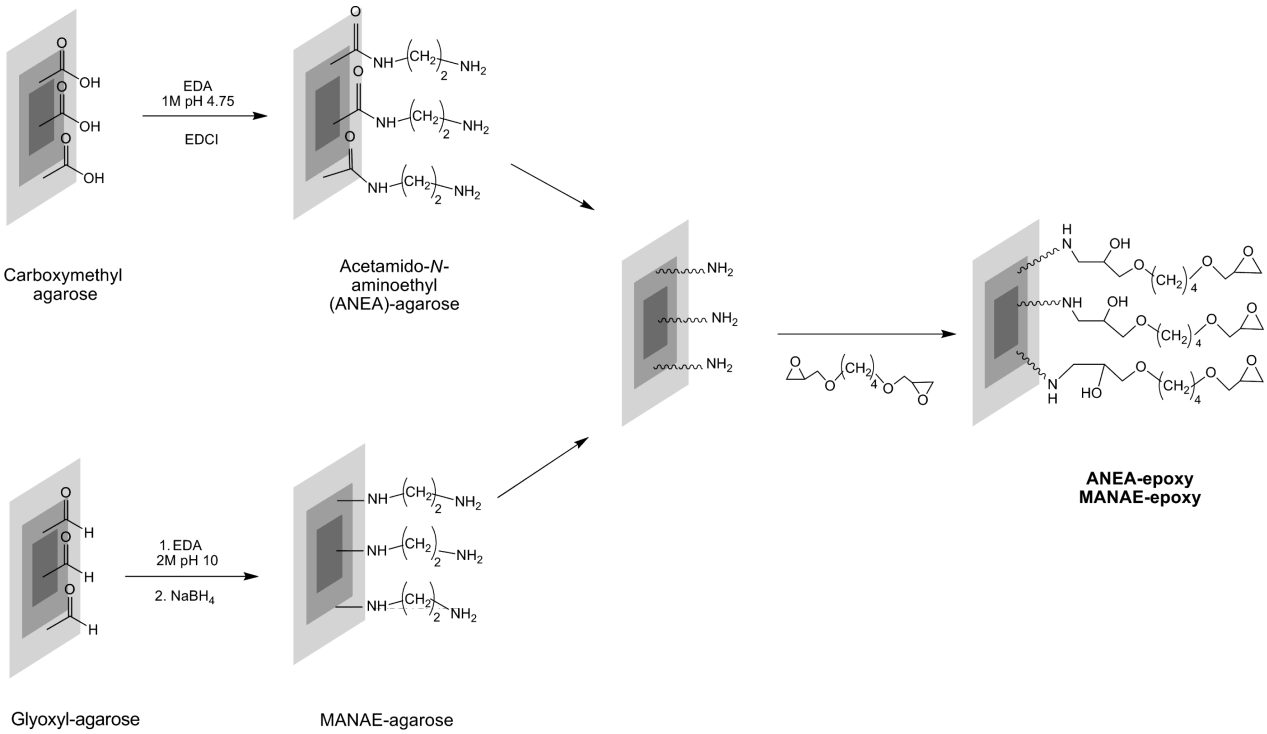

Figure 2. Preparation of amino-epoxy activated supports (MANAE-epoxy and ANEA-epoxy).

(CM) agarose 4BCL were supplied by Pharmacia (Uppsala, Sweden). Monoaminoethyl-*N*-ethyl (MANAE)-agarose beads with different degrees of activation were prepared as previously described.[34] Starting from glyoxyl-agarose with the desired activation degree and EDA, the final support has a secondary amine bond and a primary group per MANAE moiety. Acetamido-*N*-aminoethyl (ANEA)-agarose was prepared from CM-agarose using ECDI and EDA.[34,35] The final support has EDA attached to the support via an amide bond and a free primary amino group (Figure 2).

Methods. The experiments were carried out at least by triplicate, being the experimental error was lower than 5%.

Determination of HRP Activity. HRP activity was determined using H_2O_2 as the oxidizing substrate and ABTS as the reducing substrate. Activity was followed spectrophotometrically by recording the increase in absorbance at 430 nm promoted by the ABTS oxidation product. Experimental conditions were 1 mM ABTS and 1 mM H_2O_2 in 50 mM sodium phosphate at pH 6 and 25 °C.

The activities of enzymes are given in μmol of oxidized substrate/min/mg under the described conditions.

Oxidation of Anti-HRP IgG. A total of 1 mg/mL anti-HRP IgG was incubated with 10 mM sodium periodate at 4 °C. After 2 h, the oxidized antibodies were dialyzed against distilled water at 4 °C and immediately used.[20,36,37]

Preparation of Glutaraldehyde Supports. A total of 10 mL of amino-agarose was suspended in 15% (v/v) glutaraldehyde in 200 mM phosphate buffer pH 7.0 for 16 h at 20 °C.[25] The suspension was kept under mild stirring, and after that, the activated support was filtered and washed exhaustively with distilled water.

In some instances, the support was incubated with 10 mg $NaBH_4$ per mL of suspension to ensure the reduction of any chemically reactive group in this support.

Preparation of Amino-Epoxy Supports. A total of 15 mL of aminated supports (MANAE or ANEA) with different degrees of amination,[34,35] were suspended in 160 mL of sodium bicarbonate buffer, 100 mM, pH 9. Then 24 mL of acetone and 40 mL of 1,4-butanediol diglycidyl ether were added. The final pH value of the mixture was adjusted at 9. The suspension was incubated over a period of 12 h and, after this period, was washed with an excess of distilled water.

To block the epoxy groups, the amino-epoxy activated agarose (10 mL) could be incubated in 90 mL of different compounds for 12 h and pH 8.5 (5% v/v mercaptoethanol, 3 M aspartic acid). The suspension was incubated over a period of 12 h and then washed with a large excess of distilled water.

Immobilization of Sodium Periodate Oxidized Antibodies in Aminated Supports. A total of 1 g of the different supports was incubated with 1 mL of the oxidized anti-HRP solutions (see above) diluted with 8 mL of the desired buffer and maintained under very gentle stirring. During the immobilization, protein concentration of the supernatant (following Bradford's method [38]) was determined.

Adsorption of Proteins on the Different Supports. A total of 1 wet g of the indicated support was incubated in a solution of 5 mg/mL protein. Adsorption of the proteins was followed by Bradford's method[38] and confirmed by SDS-PGE as described below. HRP adsorption was also followed by activity, using the assay described above.

SDS-PAGE. Samples of the different soluble and immobilized preparations were analyzed by SDS-PAGE, as described by Laemmli.[39] Using immobilized preparations, the supports containing the proteins were boiled in the presence of 20% SDS and 10% mercapto-ethanol for 10 min (to release all noncovalently bond protein present in the immobilized protein preparation[40]) and the supernatants obtained were used in the SDS-PAGE analysis. The equipment was a SE 250-Mightt small II electrophoretic unit (Hoefer Co.), using gels of 15% polyacrylamide in a separation zone of 9 × 6 cm and a concentration zone of 5% polyacrylamide. Gels were stained by silver [41,42] and coomasie.[43] Low molecular weight markers from Pharmacia were used (14−94 kDa).

Results

1. Anti-HRP IgG Immobilized on Glutaraldehyde-Activated Supports. *1.1. Adsorption of Proteins on Gutaraldehyde-Activated Supports.* To have a system useful to utilize immobilized antibodies as biosensors or immuno-affinity columns, as stated in the Introduction, it is necessary to have one where the nonspecific adsorption of other proteins that were presented in the sample may be negligible. In this case, the ionic exchange is desired in the antibody immobilization, but this can be performed at very low ionic strength, while the use of the

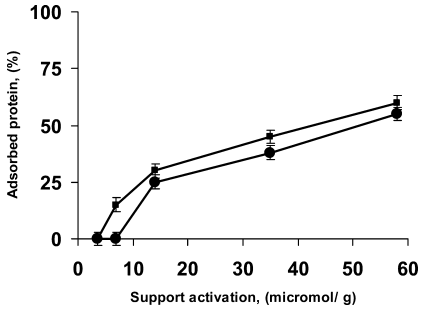

Figure 3. Effect of the activation degree in the support on the adsorption of proteins from a crude extract from *E. coli* on MANAE-support (●) and MANAE-glutaraldehyde reduced support (■). The adsorption was performed at pH 7.0 and 25 °C in 100 mM sodium phosphate.

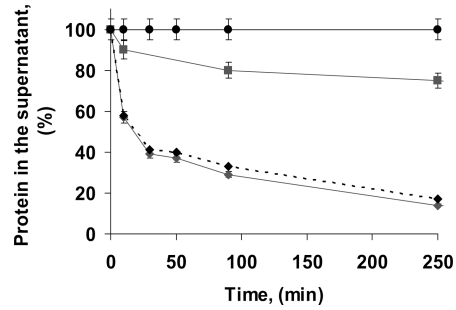

Figure 4. Immobilization courses of anti-HRP on different glutaraldehyde activated supports under different conditions. The supports were activated with 3.5 μmol/g. Immobilization in 5 mM sodium phosphate, pH 7 with glutaraldehyde support (full rhombus) or reduced glutaraldehyde (full rhombus and dash line). Immobilization in 250 mM sodium phosphate pH 7 with nonreduced glutaraldehyde (squares) or reduced glutaraldehyde (circles).

immobilized antibody may be performed at a moderate, but higher, ionic strength (e.g., 100 mM sodium phosphate pH 7).

Figure 3 shows the adsorption of proteins from a crude extract from *E. coli* on MANAE-support and MANAE-glutaraldehyde reduced by reaction with sodium borohydride (unable to give covalent reaction) with different activation degrees. The supports activated at the maximum activation degree with MANAE (58 μmol/wet g of support) adsorbed around 60% of the protein of a protein crude extract from *E. coli* in 100 mM sodium phosphate, pH 7. Decreasing the activation of the support, the percentage of adsorbed protein decreased, with no adsorption of proteins when using an activation of 7 μmol MANAE/wet g of support. However, the groups modified with reduced glutaraldehyde seemed to be able to adsorb proteins at lower activation degrees of the support. The support having only 7 μmol MANAE/wet g of support did not adsorb proteins at 100 mM sodium phosphate at pH 7, while under those conditions the support modified with reduced glutaraldehyde was able to adsorb about 15% of the proteins. However, using an activation of 3.5 mmol/g, this support did not adsorb proteins under those conditions.

Using anti-HPR IgG, it was found that this lowly activated support still adsorbed the antibody at 5 mM sodium phosphate, pH 7, while was unable to adsorb the antibody using 100 mM sodium phosphate, pH 7. That is, at low ionic strength, the antibody may be ionically adsorbed on the support but at 100 mM sodium phosphate the adsorption of proteins may be fully prevented.

1.2. Immobilization of Anti-HRP IgG on Glutaraldehyde Supports. Anti-HRP IgG was offered to glutaraldehyde support reduced by incubation with sodium borohydride and reactive glutaraldehyde support, both activated with 3.5 μmol MANAE/g at 5 mM sodium phosphate pH 7. The immobilization rate was almost identical using nonreactive and reactive glutaraldehyde supports (Figure 4). On the other hand, the immobilization on glutaraldehyde-reactive supports in 250 mM sodium phosphate, where the antibody could not be ionically adsorbed, was around 5-fold slower than in 5 mM sodium phosphate (Figure 4). Both results suggested that the ionic exchange of the antibody on the support surface was the main first step in the immobilization of the antibody under the selected conditions, that is, the antibody was being oriented by the area permitting an anionic exchange on this lowly activated supports.

After 12 h, the anti-HRP IgG immobilized at low ionic strength in both nonreactive and reactive glutaraldehyde supports were incubated in 500 mM sodium phosphate, pH 7. While the nonreactive support released all the adsorbed antibody, when using the support with reactive glutaraldehyde groups, the amount of released anti-HRP at high ionic strength was negligible, confirming the covalent attachment of the antibody to the support AFTER the first ionic exchange. In any case, to ensure that only covalently attached antibody molecules was present in the final preparation, all the immobilized antibodies were washed at high ionic strength before their use. These immobilized antibody preparations were reduced to eliminate any remaining reactive glutaraldehyde group.

1.3. Evaluation of Functionality of Immobilized Anti-HRP IgG onto Glutaraldehyde-Activated Support. The immobilized antibody prepared above was offered to a solution of a crude protein extracted from *E. coli* containing a known amount of HRP. Compared to the soluble antibody, the immobilized one expressed around 65 ± 5% of the expected capacity to capture HRP.

The second feature of the system should be an inert surface. The adsorption of proteins on the immobilized antibody preparations was not detected by following the concentration of proteins in the supernatant.

A more sensitive way to determine the adsorption of proteins on the support is directly to desorb any adsorbed protein from the support. Thus, after the incubation of the immobilized antibody with the crude protein extract containing peroxidase, the immobilized antibody preparation was washed with water and boiled in the presence of SDS and mercaptoethanol, and the supernatant obtained was used in a SDS-PAGE experiment. Figure 5 shows the SDS-PAGE gel, where the only visible proteins bands correspond to that of the HRP and that from the antibody. Comparing the antibody bands from the immobilized antibody and that of the equivalent soluble antibody, the immobilization seems to involve more than 90% of the antibody subunits, with slightly less light chain involved in the immobilization. This result suggested that the antibody could be immobilized in an orientation that permits the immobilization of all the antibody subunits, as it was intended. Very interestingly, this orientation still permitted a good adsorption of the HRP by the immobilized antibody.

2. Anti-HRP IgG Immobilized on Amino-Epoxy Activated Supports. *2.1. Immobilization of Anti-HRP on MANAE-Epoxy-Activated Supports.* The glutaraldehyde strategy described above permitted to have an antibody with the Fab regions quite near to the support surface, but still preserving a high functionality, with a support surface having a quite final inert surface, but the immobilization rate was not very high due to the low activation of the support, which is necessary to give a relatively inert surface. The use of amino-epoxy supports may be

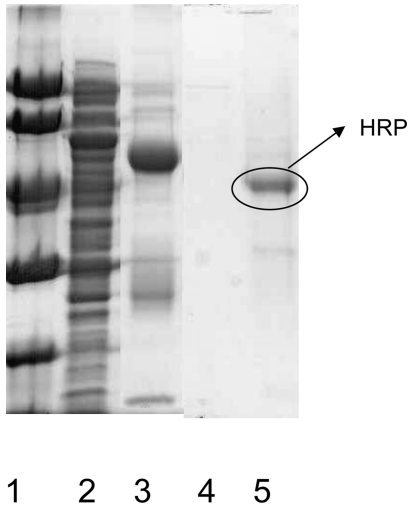

Figure 5. SDS-PAGE analysis of the proteins adsorbed on antibody-glutaraldehyde supports. The antibody was immobilized on glutaraldehyde supports having 3.5 μmol MANAE groups per wet g of supports in 5 mM sodium phosphate pH 7 at 25 °C. Samples were prepared as described in Materials and Methods. Lane 1, weight markers; lane 2, crude extract from *E. coli* containing HRP; lane 3, commercial preparation of anti-HRP; lane 4, reduced glutaraldehyde support with a crude extract of *E. coli* containing HRP; lane 5, immobilized anti-HRP incubated with a crude extract from *E. coli* containing HRP. This sample was boiled in absence of mercaptoethanol.

advantageous: they can be finally blocked with negatively-charged groups, therefore, they can be activated at the maximum amount of amino groups. Figure 6 shows the immobilization courses of the anti-HRP IgG in 5 mM sodium phosphate, pH 7, on different aminated supports. The immobilization rate was almost identical using MANAE-agarose, amino-epoxy agarose, or amino-epoxy-activated agarose blocked with mercaptoethanol. At 1 M sodium phosphate, pH 7, the immobilization of the antibody was negligible on all the supports, confirming that amino-epoxy agarose beads are not able to directly immobilize the soluble proteins.[31,32]

The immobilized preparations were leave to react for different times and then incubated in 1 M sodium phosphate, pH 7, to study the desorption of the immobilized antibody from the different supports. During the first time intervals, almost all the anti-HRP IgG could be desorbed from all the supports, showing that the covalent attachment was much slower than the ionic adsorption in the case of epoxy-amine. The incubation for longer times reduced the percentage of antibodies that could be released from amino-epoxy agarose; after 24 h, there is almost no antibody released from the support, while using mercaptoethanol blocked amino-epoxy or MANAE-epoxy support most of the antibody molecules could be released from the support (in these supports only ionic exchange can occur). Thus, after 24 h, most anti-HRP molecules were covalently attached to the support onto amino-epoxy support.

In summary, the amino-epoxy supports are able to covalently immobilize antibodies based on a previous anionic exchange.

Next, the inertness of the surface was studied. The amino-epoxy support, even after blocking the epoxy groups with mercaptoethanol, presented an adsorption capacity near to the MANAE-support (see Figure 3). Thus, these supports seemed to not be inert enough to be suitable for immunobiosensors or immunochromatography purposes.

It has been reported that a support having similar concentration of positive and negative groups was almost unable to adsorb proteins at moderate ionic strength (although at low ionic strength they were still able to adsorb some proteins).[35]

Thus, the support (AFTER the antibody immobilization) was blocked with aspartic acid. This treatment reduced the adsorption capacity, but still a significant adsorption of proteins at 100 mM could be detected (around 20%). The reason for this could be that the system presented three amino groups and two carboxylic ones per epoxy group, and although the p*K* of one of the amino groups is low, apparently, this excess of amino groups is still able to produce the adsorption of proteins.

2.2. Immobilization of Anti-HRP IgG onto ANEA-Activated Supports. To overcome this problem, we modified the strategy to produce the amino-epoxy supports and, instead of using MANAE-supports as starting activated matrix, we modified carboxy-methyl agarose with EDA via the carbodiimide route having a support with just one primary amino group (ANEA-agarose; Figure 2)[34,44–46] (see Materials and Methods). This new amino-epoxy support, after blocking with aspartic acid and having two secondary amino groups and two carboxylic groups, was unable to fully adsorb proteins at 100 mM sodium phosphate and pH 7.

After immobilizing the antibody, this preparation was boiled in the presence of SDS and mercaptoethanol, and the supernatants obtained were analyzed by SDS-PAGE (Figure 7). Although it was possible to detect both heavy and light chains of the antibody, the intensity of the bands compared to the equivalent soluble antibody revealed that more of 95% of the antibodies chains were bound to the support. If the immobilized antibody was incubated at pH 9 for 24 h, the intensity of the band corresponding to the heavy chain was not detected. This suggested that the incubation under alkaline conditions permitted the multipoint and multisubunit attachment of the antibody to the support.[47]

That way, the antibody Fab regions should be in close contact with the support surface.

2.3. Evaluation of Functionality of Immobilized Anti-HRP IgG onto ANEA-Epoxy-Activated Support. Anti-HRP IgG was immobilized on ANEA-epoxy agarose, incubated 24 h at pH 9 to ensure the covalent attachment of the antibody, and finally incubated in 3 M aspartic acid to modify the epoxy groups and obtain a neutral surface. This new immobilized preparation was incubated in the presence of a crude extract from *E. coli* containing HRP. The functionality of this antibody was even slightly higher than the previous one (about 75 $\pm$ 5% of that corresponding to the soluble antibody). This slight higher functionality could be due to the fact that now there is not a significant percentage of antibody molecules immobilized directly via covalent attachment, but in all cases, this occurs after the ionic exchange. When the lowly activated glutaraldehyde support is used and when the immobilization rates using glutaraldehyde and glutaraldehyde-reduced supports is compared, around 15% of the antibody molecules could be directly covalently immobilized via the most reactive amino groups placed on the recognition region.

This preparation was incubated in the presence of a crude extract of *E. coli* contaminated with a known amount of HRP. After washing the immobilized antibody with water, this was boiled in the presence of SDS and the SDS-PAGE analysis of the proteins contained in this preparation, as in the case of glutaraldehyde-immobilized anti-HRP, showed only the HRP and the chains of the antibody, confirming that the final surface was really inert (results not shown).

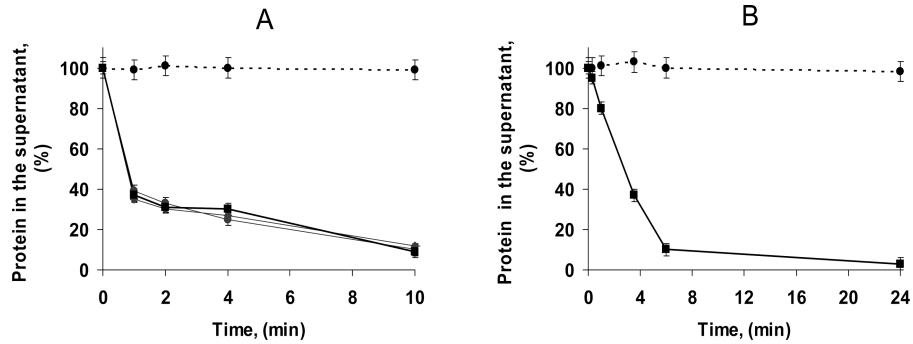

Figure 6. Immobilization courses of the anti-HRP on different aminated supports under different conditions. (A) Adsorption course: anti-HRP in 5 mM sodium phosphate, pH 7, on MANAE-agarose (full circles), amino-epoxy agarose (full squares), or amino-epoxy agarose blocked with mercaptoethanol (rhombus). Sodium phosphate (1 M), pH 7, and amino-epoxy agarose (full circle and dash line). (B) These immobilized preparations were incubated in 1 M sodium phosphate, pH 7, after different immobilization times to study the desorption of the immobilize antibody, to check the covalent link. Amino-epoxy agarose (full squares) and amino-epoxy blocked with mercaptoethanol (full circle and dash line). All the reactions were performed at 25 °C.

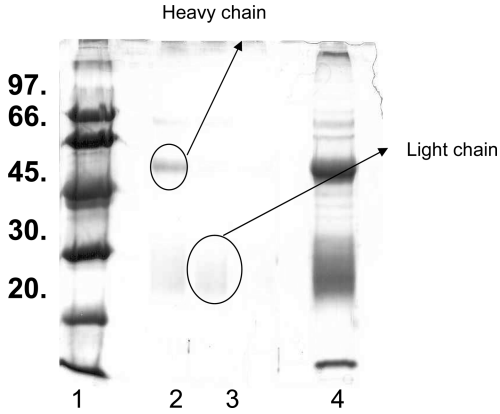

Figure 7. SDS-PAGE analysis of the proteins adsorbed on antibody-epoxy-ANEA-agarose supports. The supernatants were obtained by boiling with SDS and mercaptoethanol the anti-HRP immobilized in 5 mM sodium phosphate, pH 7, on epoxy-ANEA-agarose supports blocked with aspartic acid, at 25 °C, after incubation with a crude of *E. coli*, where HRP was added. Lane 1, weight markers; lane 2, immobilized anti-HRP incubated with a crude extract from *E. coli*, at pH 7; lane 3, immobilized anti-HRP incubated with a crude extract from *E. coli* at pH 9; lane 4, commercial preparation of anti-HRP.

Conclusions

It has been shown that the ionic exchange of anti-HRP IgG permits the recovery of a high functionality of the antibody (around 65−75%), although the antibody immobilization involved the four subunits of the antibody. This suggested that the Fab regions should be quite near to the support surface, and this kind of antibody immobilization could be ideal if the capture of the antigen is going to be determined by interaction between the antigen and the support surface, as in the case of carbon nanotube field effect transistors. The main problem is to design surfaces where the adsorption of proteins after the immobilization of the antibody may be annulated. Unspecific adsorption of any biomacromolecule on the support, being important in any system, may be critical in the case of carbon nanotube field effect transistors, where there is not a second specific antibody to perform the reading of the analysis.

Here we have presented two different alternatives to have the unmodified antibody anionically exchanged to a support, further covalently immobilized with all the subunits in close contact with the support surface, and giving a final inert surface.

The first strategy was the use of aminated supports activated with glutaraldehyde, with a very low superficial density of amino groups in the support surface (to have a moderately inert surface) and finally reduced (to have a chemically inert surface). In these supports, even using lowly activated supports, the most rapid mechanism of anti-HRP immobilization is the ionic adsorption (around 5-fold more rapid), giving that 5 out of 6 antibody molecules should be immobilized by the most negatively-charged regions in the antibody surface. The final functionality of the antibody is about 65%, with the only problem being the relatively slow adsorption of the antibody to these lowly activated supports.

The second alternative is the use of amino-epoxy-supports. The use of this kind of supports makes possible the use of highly activated supports, having a very rapid ionic exchange of the protein on the support surface. The covalent attachment of the antibody via very strong bonds permits the final modification of the support surface with groups having a net negative charge, giving mixed ionic exchangers that cannot adsorb protein under moderate ionic strength. When this strategy is used, the antibodies were very rapidly immobilized and kept about 75% of their functionality.

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