Detection of Polyclonal Antibody Against Any Area of the Protein-Antigen Using Immobilized Protein-Antigens: The Critical Role of the Immobilization Protocol

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Antigens immobilized on solid supports may be used to detect or purify their corresponding antibodies (Ab) from serum. Direct immobilization of antigens on support surfaces (through short spacer arms) may promote interesting stabilizing effects on the immobilized antigen. However, the proximity of the support may prevent the interaction of some fractions of polyclonal Ab with some regions of the antigen (those placed in close contact with the support surface). Horseradish peroxidase (HRP) was immobilized on agarose by different protocols of multipoint covalent immobilization involving different regions of the antigen surface. Glyoxyl-agarose, BrCN-agarose, and glutaraldehyde-agarose were used as activated supports. Each HRP-immobilized preparation was much more stable than the soluble enzyme, but it was only able to adsorb up to 60−70% of a mixture of polyclonal anti-HRP antibodies. On the other hand, HRP was also immobilized on agarose through a very long, flexible, and hydrophilic spacer arm (dextran). This immobilized HRP was hardly stabilized, but it was able to adsorb 100% of the polyclonal anti-HRP. The absence of steric hindrances seems to play a critical role favoring the complete recognition of all classes of polyclonal Ab. Another solution to achieve a complete adsorption of polyclonal Ab on immobilized−stabilized antigens has been also reached by using a mixture of the differently immobilized and stabilized HRP−agarose preparations. In this case, an improved storage and operational stabilities of the immobilized antigens can be combined with the complete adsorption of any class of antibody.

1. Introduction

The detection of antibodies in biological fluids is one of the most widely used techniques in diagnostics.[1−4] Moreover, the purification and identification of antibodies against a determined antigen have also a great interest in many research areas.[1,5−8] Both cases have in common that the antibodies are identified by using immobilized antigens on different supports (e.g., microtiter plates, magnetic particles, etc.).[2−4,7] In many instances, the used antigen is a protein (e.g., derived from the cell wall, etc.).[4]

Therefore, a proper immobilization of the antigen protein may play a critical role in the successful detection of the antibodies. Thus, it is necessary not to dramatically distort the antigen, to keep the affinity of the antibody by the immobilized antigen.[7−12]

Moreover, it should be considered that the immobilized protein antigen may be in a particular orientation against the support surface. In this case, a large percentage of the antigen protein will not be accessible to the antibody and the antibodies against these areas will not be detected. In general, most of the protocols of immobilization may orientate the immobilized proteins.[13−16]

In some cases, the immobilization is by the most reactive amino group of the protein that will usually be the amino terminal if it is exposed (agarose-bromocyanogen, or agarose-glutaraldehyde at high ionic strength).[13] In other cases, the immobilization is produced via a first multipoint interaction between the enzyme and the support. In this case the immobilization rate is exponentially dependent on the concentration of reactive groups on the protein and the support. This means that the protein is mainly immobilized by the area having the highest density of reactive groups in the protein. This occurs for example using glyoxyl-agarose,[13] where immobilization is produced by the area having more Lys (glyoxyl-agarose) or an immobilization via ionic adsorptions,[14,15] that implies the immobilization by the area with the highest charge density (ionic exchanger, ethylendiamine-modified agarose, etc.).

Immobilization of proteins via their sugar moiety may at least partially prevent the steric problems,[16−18] but the chemical changes in the sugar may promote that some antibodies are no longer able to be bound to the antigen.

Here, we propose the use of a very long spacer arm as a solution to this problem. Aldehyde-dextran has been proposed as a long, flexible, and inert spacer arm, used with some success in the immobilization of rennin, protein A, or antigens.[19−22] A very mildly immobilized protein (ideally via just one bond) in this spacer arm will keep almost full accessibility to the interaction against any other biomacromolecule, except in the location where the enzyme has reacted with the support (usually, the terminal amino group).

2. Materials and Methods

2.1. Materials. Agarose (2% cross linked beads, 2 BCL, and 4% cross linked beads, 4 BCL) were supplied by Pharmacia (Uppsala, Sweden). Glyoxyl-agarose was prepared as described by Guisan.[22] MANAE-agarose was prepared as described by Fernandez-Lafuente et al.[23] Glutaraldehyde support was prepared from MANAE groups as previously described.[21] Aldehyde-dextran support was prepared as previously described.[24] Sodium periodate was from Merck (Darmstadt, Germany). Dextran from *Leuconostoc mesenteroides* (20 kDa), ethyl-

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endimine (EDA), sodium borohydride, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), horseradish peroxidase (HRP), and polyclonal antihorseradish peroxidase (anti-HRP) (developed in rabbit) were supplied by SIGMA (St. Louis, MO). Glutaraldehyde and trimethylaminoborane were purchased by Fluka (Buchs, Switzerland). All other reagents were of analytical grade.

2.2. Methods. *2.2.1.Determination of Horseradish Peroxidase Activity.* Horseradish peroxidase (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 buffer at pH 6 and 25 °C.

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

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

2.2.2. Immobilization of HRP onto Different Supports. The supports employed were the following: (i) glyoxyl-agarose,[13] having a dense layer of linear aldehyde groups able to react with primary amino groups in the protein; immobilization requires a multipoint immobilization at pH 10;[13] (ii) MANAE-agarose[23] presenting low p*K* amino (around 7) able to react with periodate oxidized proteins;[20] (iii) glutaraldehyde-agarose,[14] with cyclic aldehydes, able to immobilize enzymes at neutral pH value by the terminal amino group (if immobilization is at high ionic strength); (iv) aldehyde-dextran-agarose,[19] which permits the immobilization of proteins via long, hydrophilic, and flexible spacer arms; (v) cyanogen bromide, a commercial support that immobilizes proteins at neutral pH values via the amino terminal.[13]

2.2.2.1. Immobilization of HRP on Glyoxyl-Agarose 4 BCL. Ten milligrams of HRP was incubated with 10 g of glyoxyl-agarose (4 BCL) for 1 h in sodium carbonate buffer 100 mM pH 10.05 at room temperature. Then, the immobilized preparation was reduced by addition of 1 mg/mL of sodium borohydride.[13] After 30 min the derivatives were washed with distilled water.

2.2.2.2. Immobilization of HRP on Glutaraldehyde-Agarose. Ten milligrams of HRP was incubated with 10 g of glutaraldehyde-agarose in sodium phosphate buffer 200 mM pH 7 for 16 h at 25 °C. Then, the immobilized preparation was reduced by addition of 1 mg/mL of sodium borohydride. After 1 h, the derivatives were washed with distilled water.[14]

2.2.2.3. Immobilization of HRP onto MANAE-Agarose Supports. 2.2.2.3.1. Oxidization of HRP. HRP was incubated with 10 mM sodium periodate at 4 °C. After 2 h, the oxidized HRP was dialyzed against distilled water at 4 °C.[25]

2.2.2.3.2. Immobilization of Oxidized HRP onto MANAE-Agarose. The oxidized HRP (10 mg) in 150 mM sodium phosphate buffer pH 7.5 was added to 10 g of MANAE-agarose (4 BCL) for 16 h at room temperature. The Schiff's bases formed were reduced by addition of 1 mg/mL of sodium borohydride at pH 8.5 at 4 °C for 1 h. Then, the derivatives were washed with abundant water.[16,17]

2.2.2.4. Immobilization of HRP on BrCN Supports. Ten milligrams of HRP was incubated at pH 7.5 in sodium phosphate buffer with 2 mL of cyanogen bromide support as described by the manufacturer (technical documentation Pharmacia (Uppsala, Sweden).

2.2.2.5. Immobilization of HRP on Aldehyde-Dextran Supports. Ten milligrams of HRP was incubated with 10 g of aldehyde-dextran supports[16,19] at pH 7 in 100 mM sodium phosphate buffer during 4 h. Then, the immobilized preparation was reduced by addition of 1 mg/mL of sodium borohydride. After 1 h, the derivatives were washed with abundant water.

In all cases, the immobilized HRP was determined by quantifying the difference in enzyme activity in the supernatant before and after immobilization. The experiments were carried out in triplicate, typically yielding an experimental error lower than 5%.

2.3. Determination anti-HRP via Gel Filtration. HRP (10 mg of pure HRP) was added to anti-HRP solution (maximum 1 mg/mL) in 25 mM sodium phosphate buffer (pH 7) and left to interact for a minimum of 2 h at room temperature. Then, the samples were analyzed using gel filtration. Gel filtration analyses were performed using a glass column packed with agarose 4 BCL (column bed volume, 100 mL). The column was previously equilibrated with 5 vol of elution buffer (100 mM sodium phosphate buffer pH 7). All experiments were carried out at 25 °C with a flow rate of 0.25 mL/min. The samples were divided into 1 mL aliquots, and their HRP activities were assayed using spectrophotometric methods as described above.[27] The M_r of the proteins was estimated from a calibration curve plotted using standard proteins (catalase (from bovine liver) 240 kDa, PGA 90 kDa, BSA 67 kDa, lysozime 14 kDa). The elution volume of the standard proteins was 58, 64, 76, and 90 mL for catalase, PGA, BSA and lysozime, respectively.

2.4. Adsorption of anti-HRP on HRP-Immobilized Preparations. An amount of 0.1 mg of polyclonal anti-HRP IgG was incubated in the presence of 1 g of different HRP derivatives in 150 mM sodium phosphate buffer pH 7 (10 mL) at room temperature.[4] The adsorption of the proteins on the different matrixes was determined by quantifying the difference in protein concentration in the supernatant before and after adsorption, using the method described by Bradford.[26] The exact percentage of anti-HRP adsorbed on the matrixes was determined by measurement of the anti-HRP adsorbed on the different immobilized HRP derivatives as described above, comparing the total activity that eluted as free or anti-HRP−HRP complexes. The experiments were carried out at least in triplicate, typically yielding an experimental error lower than 5%.

2.5. HRP Thermal Stability Studies. To study the thermal stability of HRP derivatives, preparations having only 1 IU/mL were used to prevent diffusion problems. The inactivations were carried out at pH 7 and 40 °C. Samples were withdrawn at different times, and their activity was tested as described previously. The remaining activity was calculated as the ratio between activity at a given time and activity at zero time of incubation.

2.6. SDS−PAGE Analysis. Samples of anti-HRP adsorbed on HRP derivatives were analyzed by SDS−PAGE as described by Laemmli,[28] using an SE 250-Mightt small II electrophoretic unit (Hoefer Co.) using gels of 15% polyacrylamide in a separation zone of 9 cm × 6 cm and a concentration zone 5% polyacrylamide. Gels were stained by silver and/or coomasie. Low molecular weight markers from Pharmacia were used (14−94 kDa).

3. Results and Discussion

3.1. Characterization of the Polyclonal anti-HRP Used. The immobilized antigen is intended to be used to determine the presence of antibodies on organic fluids; thus, we decided to use the IgG commercial preparation without any previous purification. To solve the problem of the determination of the anti-HRP present in the sample, a gel filtration of a mixture of a large excess of peroxidase and IgG was carried out. The peroxidase activity bound to the anti-HRP eluted much more rapidly than the free HRP; this way it was possible to determine the presence or absence of anti-HRP in the studied sample. Figure 1 shows the typical chromatogram achieved by these gel filtration experiments. Thus, this test could be used to determine the amount of antiperoxidase adsorbed on the different HRP-immobilized preparations.

3.2. Adsorption Course of Polyclonal anti-HRP on Different HRP Derivatives. HRP immobilized on different supports was offered to a small amount of polyclonal anti-HRP, accounting only for around 10−15% of the maximum loading that was obtained from offering an excess of IgG to each of the preparations. Figure 2 shows the incorporation of the proteins contained on polyclonal anti-HRP samples on different HRP-immobilized preparations. HRP immobilized via the most

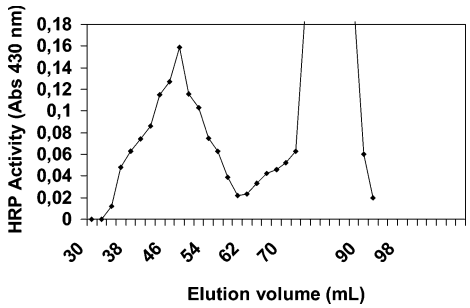

Figure 1. Gel filtration analysis of HRP and anti-HRP mixtures. The anti-HRP/HRP (in a large excess) samples (see the Materials and Methods for the preparation) were injected on the column using 100 mM sodium phosphate buffer pH 7.

reactive amino groups (glutaraldehyde-agarose, BrCN-agarose) or the regions most enriched in Lys residues, (glyoxyl-agarose) adsorbed between 20% and 35% of the proteins contained in anti-HRP preparations in 2 h.

Immobilization of HRP via its oxidized sugar chain on primary amino supports was able to adsorb approximately 50% of the proteins from anti-HRP. Thus, this immobilization technique allows a larger percentage of the antigen protein to be accessible to the antibody. This may suggest that the sugar chain could act as a spacer arm and might permit the antigen−antibody recognition because the protein is away from the support[18−20] and also could avoid the steric hindrance generated by immobilization through short spacer arms, where a large portion of the surface of the immobilized proteins (e.g., 20−30% of the total protein surface) becomes very close to a large support surface.[20]

Gel filtration experiments performed on the supernatants of the different solutions of anti-HRP showed the presence of some anti-HRP, confirming that a certain percentage of the anti-HRP had not been able to recognize the immobilized peroxidase molecules (Figure 2B).

Considering that we add a defect of antibody, and that some of the antibodies that cannot be adsorbed on one immobilized HRP could be adsorbed on another, and that some antibodies desorbed from one HRP preparation could not be adsorbed on another column, we can assume that really some antibodies could not recognize some areas of the proteins, very likely because they presented some steric hindrances to the recognition.

3.3. Adsorption of Polyclonal anti-HRP onto HRP Immobilized on Aldehyde-Dextran Activated Supports. Figure 3 shows that 70% of the proteins from the antipreparation could be rapidly adsorbed when offered to HRP immobilized by aldehyde-dextran. Apparently, this derivative allows a higher exposition of HRP to the interaction with the antibodies against most of the protein surface. In fact, gel filtration experiments of the mixture of HRP and the supernatant of the solution containing anti-HRP did not show any peak corresponding to the antigen−antibody complex. That is, 100% of the anti-IgG was adsorbed on the immobilized peroxidase in this case. This suggests that the 30% of the protein that was not immobilized

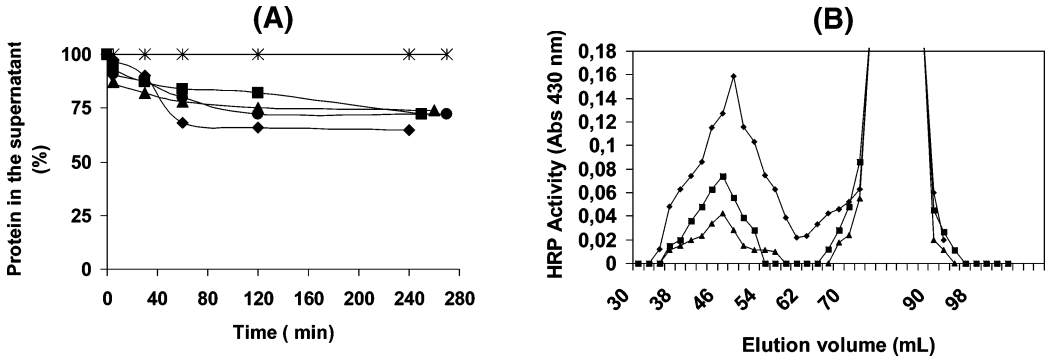

Figure 2. Anti-HRP adsorption on different immobilized HRP preparations. (A) Adsorption course of polyclonal anti-HRP on different HRP-immobilized derivatives. Adsorption experiments were carried out at pH 7 and 4 °C, in 150 mM sodium phosphate buffer. The percentage of polyclonal anti-HRP adsorbed was measured as described in the Materials and Methods. (▲) HRP oxidized immobilized on MANAE-agarose. (◆) HRP-glyoxyl derivative. (■) HRP-glutaraldehyde derivative. (●) HRP-bromocyanogen derivative. (∗) reference with inert agarose. (B) Gel filtration analysis of a mixture of HRP and the supernatant of the anti-HRP supernatant after adsorption on HRP−bromocyanogen-immobilized preparation. (◆) Reference anti-HRP/HRP. (■) Supernatant of HRP−bromocyanogen-immobilized preparation/HRP. (▲) Supernatant of HRPox-MANAE-agarose/HRP.

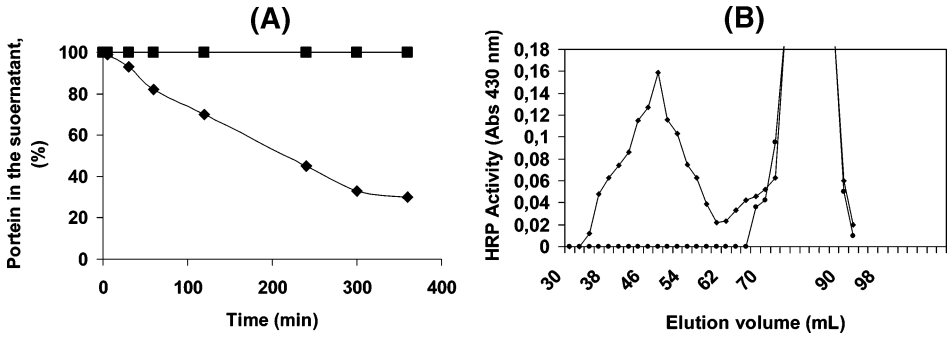

Figure 3. Anti-HRP adsorption on different HRP-dextran-MANAE-agarose preparations. (A) Adsorption course of polyclonal anti-HRP on the HRP-dextran-MANAE-agarose preparation. Adsorption experiments were carried out at pH 7 and 4 °C, in 25 mM sodium phosphate buffer. The percentage of polyclonal anti-HRP adsorbed was measured at pH 7 and 25 °C as described in the Materials and Methods. (◆) HRP-dextran derivative. (■) Reference with inert agarose. (B) Gel filtration analysis of a mixture of HRP and the supernatant of the anti-HRP supernatant after adsorption on HRP-dextran-MANAE-agarose preparation. (◆) Reference anti-HRP/HRP. (●) Supernatant of HRP-dextran derivative and the collection of different immobilized HRP (excluded HRP-dextran derivative)/HRP.

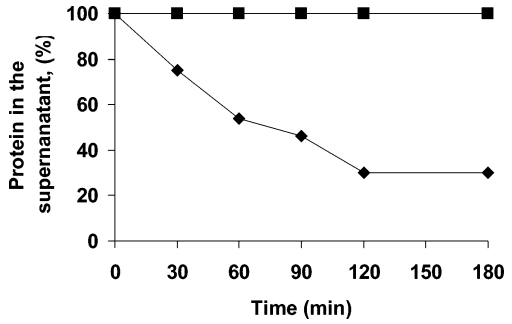

Figure 4. Adsorption course of polyclonal anti-HRP on a mixture of different HRP-immobilized derivatives. Adsorption experiments were carried out at pH 7 and 4 °C, in 150 mM sodium phosphate buffer. The percentage of polyclonal anti-HRP adsorbed was measured as described in the Materials and Methods. (◆) Mixture of HRP derivatives (HRP-bromocyanogen derivatives, HRP-glyoxyl derivative, HRPox-MANAE derivative, HRP-glutaraldehyde derivative). (■) Reference with inert agarose.

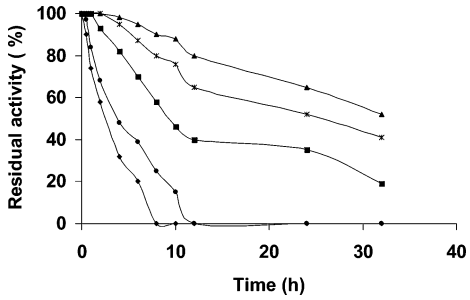

Figure 5. Thermal inactivation courses of different HRP preparations. HRP immobilization was performed on activated supports as described in the Materials and Methods. Inactivation of derivatives was performed at pH 7 and 40 °C. (◆) Soluble HRP. (●) HRP-dextran derivative. (■) HRP-bromocyanogen derivative. (∗) HRP-glutaraldehyde derivative. (▲) HRP-glyoxyl derivative.

is unable to recognize the HRP immobilized on dextran-agarose and is also unable to recognize free peroxidase under similar circumstances, corresponding to other IgGs or BSA.

Furthermore, the previously adsorbed anti-HRP on the other different HRP-immobilized preparations (glyoxyl-agarose, glutaraldehyde-agarose, BrCN-agarose) was eluted by incubation at pH 4. All these different samples could be fully adsorbed on HRP-dextran derivatives (results not shown).

3.4. Adsorption of Polyclonal anti-HRP onto a Mixture of Several HRP Derivatives. When we used a mixture of all HRP derivatives described in point 2, it was possible to adsorb around 70% of the proteins from anti-HRP preparations in a very rapid fashion (Figure 4). This percentage coincides with the percentage of proteins immobilized on the HRP immobilized via aldehyde-dextran technique. Gel filtration experiments using the supernatant of this experiment show again absences of antigen−antibody complexes.

The proteins desorbed by incubation at pH 4 from this collection of HRP preparations could be adsorbed on the HRP immobilized on aldehyde-dextran and vice versa.

However, if these proteins were offered to one of the individual supports, only a certain percentage of proteins became adsorbed on the different supports, maintaining the qualitative relation found using the initial sample but increasing the adsorption values by around a 40%.

These results together suggested that the commercial preparation has 70% anti-HRP while the other 30% of the proteins detected by the method described by Bradford were other kinds of proteins, unable to recognize HRP both in soluble or immobilized form.

These results suggest that a mixture of HRP derivatives (with different regions of the HRP exposed to the medium) permit the adsorption of the whole set of antibodies Another alternative may be the use of the antigen protein via a long and flexible spacer arm like aldehyde-dextran under controlled conditions.

3.5. Stability of HRP Derivatives. An indirect test to check the stability of the different antigens is the maintenance of the biological activity of the antigen. Obviously, it is possible that the capacity of the antigen to recognize the antibody remains when the activity has disappeared, but we can assume that if the activity is maintained, the protein structure may be considered more rigid, and therefore, the recognition of the antibody will be maintained. Figure 5 shows the thermal inactivation of HRP immobilized on different activated supports (MANAE-agarose, BrCN-agarose, aldehyde-dextran-agarose, glyoxyl-agarose, glutaraldehyde-agarose) at 45 °C compared to that of the soluble HRP. The best thermal stability was achieved by using glyoxyl supports and then glutaraldehyde supports (this stabilization may be related to the establishment of a more or less intense multipoint covalent attachment.[20,29] On the other hand, the thermal stability of the HRP-dextran derivatives was similar to that of the soluble HRP, as can be expected from the immobilization strategy used, where we can expect to keep only the properties of the soluble enzyme: the enzyme keeps a very similar freedom to move.

4. Conclusion

This manuscript shows that HPR immobilized following different protocols immobilized different percentages of poly-anti-HRP. With the use of conventional protocols of immobilization, even using very low amounts of IgG (around 10−15% of the amount of IgG that can be adsorbed on the preparations), a certain percentage cannot be adsorbed on each individual support, the sequential adsorption of the antibodies to different columns, and the adsorption of the antibodies desorbed from some of the columns, confirm that some antibodies cannot be adsorbed on certain immobilized HRP but can be adsorbed on another. Thus, it seems that of that most of the conventional immobilization protocols immobilize the proteins via quite specific regions (the most reactive group, the region with the highest density of reactive groups, etc.). This implies that when an antigen protein is immobilized to detect antibodies, a certain percentage of the antibodies addressed against areas near to the support surface may not be detected.

Here, we propose two ways to overcome these problems.

The simplest way is the use of aldehyde-dextran activated supports. Its flexibility will keep the properties of the antigen (e.g., mobility) almost unaltered and will permit an almost full exposition of the antigen surface to the antibody recognition. On the other hand, the stability of the antigen molecule will be kept unaltered when compared to that of the soluble enzyme.

The second will be the joint use of different immobilization protocols; this way we can expect that all areas of the protein may be exposed in one or the other preparation. This strategy may have as its main advantage that the stability of the antigen protein may be improved by using proper immobilization protocols that can promote an intense support−enzyme multipoint attachment.

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