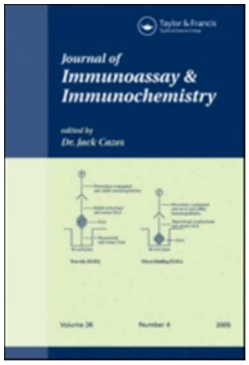

Antibody labeling with Remazol Brilliant Violet 5R, a vinylsulphonic reactive dye

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ABSTRACT

Colloidal gold is the first choice for labeling antibodies to be used in Point Of Care Testing. However, there are some recent reports on a family of textile dyes – named 'reactive dyes'– being suitable for protein labeling. In the present work, protein labeling conditions were optimized for Remazol Brilliant Violet 5R, and the sensitivity of the labeled antibodies was assessed and compared with that of colloidal-gold labeled antibodies. Also, the accelerated stability was explored. Optimal conditions were pH 10.95, dye:Ab molar ratio of 264 and an incubation time of 132 minutes. Labeled antibodies were stable, and could be successfully used in a slot blot assay, detecting as low as 400 ng/ml. Therefore, the present work demonstrates that vinylsulphonic reactive dyes can be successfully used to label antibodies, and are excellent candidates for the construction of a new generation of Point of Care Testing kits.

INTRODUCTION

For several decades, colloidal gold has been the first choice for labeling antibodies to be used in Point Of Care Testing (POCT). Dot blot and immunochromatography (IC) –two of the most common POCT assays–, require adequate sensitivity, simplicity and usually also low cost. In this context, a family of textile dyes –named 'reactive dyes'– has been proposed as a new generation of protein dyes. Reactive dyes differ from each other in their solubility and chemical structure. Highly reactive dyes bear a heterocyclic system –like dichlorotriazones (Procion MX and Cibacron F series) and difluropyrimidine–; dyes with intermediate reactivity bear a characteristic suphatoethylsulphonyl precursor group, like vinylsulphones in the Remazol series; some of these dyes have low reactivity, like aminochlorotriazine (Procion H series) and trichloropyrimidine [1]. All these dyes have very low cost, especially when compared to that of colloidal gold or enzymatic labeling, and are available in a very wide range of colors. Active chemical groups of reactive dyes are known to interact with the OH and NH_2 groups of other molecules, establishing covalent bonds. Also, it has been extensively described that these dyes can establish non-covalent interactions with proteins, which is a property that has been used for pseudo affinity protein purification [2, 3]. These interactions are due to structural similarities between the dyes and some natural ligands of those proteins. Covalent interactions can be controlled, as it is known that the covalent binding occurs at high pH –in most of the cases–. Non-covalent interaction, on the other hand, cannot be controlled and therefore might produce interference when antibodies are labeled with a reactive dye.

Until now, there is a single report on reactive dyes used to label antibodies (Abs) [4]. In that work, antibodies were coupled to Procion Blue MX-7RX and successfully used in an IC assay. Nevertheless, further characterization is needed regarding sensitivity of the IC assay and the stability of the label, as well as a comparison with colloidal gold labeling.

In this work, we have explored the non-covalent binding of reactive dyes to serum proteins, and selected a vinylsulphonic dye, the Remazol Brilliant Violet 5R (RBV5R). Rabbit antibodies were raised against human IgG and labeling conditions with RBV5R were optimized to maximize color intensity in a direct slot blot assay. Rabbit antibodies labeled in optimal conditions were then immunochemically characterized, determining the sensitivity achieved in a capture slot blot assay and their stability in an accelerated stability test. Furthermore, the relative sensitivity achieved with RBV5R was compared to that obtained with colloidal gold.

EXPERIMENTAL

Reactive dyes

Selected dyes were Remazol Brilliant Violet 5R, Procion Blue H-EGN, Procion Red HE-7B, Remazol Yellow 3RS, Orange R-HE and Procion Brown MX-5BR. Dyes were chosen according to their colors and the available information about them. All dyes were provided by Surfactan S.A.

Non-covalent binding of dyes to serum proteins

Non-covalent binding to proteins from human serum was tested for all dyes, in order to choose an adequate label for the further development of a slot blot assay. Direct binding to proteins from a normal human serum was tested for active and alkali-inactivated dyes.

Briefly, two aliquots of each dye were prepared diluting 10 mg in 50 µl of deionized water. One of the aliquots was inactivated by incubation at 100 °C in a water bath during 1 h, in the presence of 50 µl of NaOH 2M. After incubation, carbonate buffer 0.5 M (pH 10) was immediately added, to a final volume of 400 µl. The other aliquot was equally treated but without incubation. Then, 50 µl of normal human serum were added, and all aliquots were incubated 1 h at 37 °C. Protein-dye interaction was assessed by direct observation of a native PAGE. After gels were digitalized, standard Coomasie Blue staining procedure was performed.

Antibody-antigen system

In order to optimize the conditions for antibody labeling, and to study the stability and sensitivity that can be achieved, an antibody-antigen system was selected. This system consisted of rabbit immunoglobulins raised against human IgG, both purified by pseudoaffinity chromatography using a HiTrap protein G column (GE Healthcare).

Optimization of labeling conditions

After the non-covalent interaction assay, Remazol Brilliant Violet 5R was selected for further studies. Labeling conditions –incubation time, the dye:protein

molar ratio and the reaction pH– were optimized, and temperature was fixed at 37 °C. In all cases, labeling was performed in carbonate buffer 0.5 M, incubating 100 µl of the purified rabbit immunoglobulin at a final concentration of 16.5 mg/ml, with an aqueous solution of the dye at different final concentrations.

Free and bound dye were separated by size exclusion chromatography in PBS, using a column (0.5 cm x 20 cm) containing Sephadex G25 (GE Healthcare). Fractions of 0.5 ml were collected, protein content was determined using BCA Protein Kit (Pierce) and the amount of bound dye was calculated by its absorption at 542 nm (ε_{542nm} = 685.72 M^{-1}). Labeled antibody was diluted in PBS before testing its reactivity, so as to normalize the final protein concentration at 0.35 mg/ml.

In order to optimize the three parameters simultaneously, a 'variable size simplex' method was used [5]. This method –although time consuming– is commonly used when complete factorial strategies imply too many combinations to be tested, and has been extensively used elsewhere [6, 7, 8]. Briefly, a 'simplex' is defined as an *n+1* geometrical shape, where *n* is the number of parameters to be optimized. Each vertex of the simplex –considered a step of the method– represents a combination of values for these factors, and the response of the system has to be experimentally tested in those conditions. Then, a response ranking is established, and the vertex with the lowest value is discarded and replaced by a 'reflection vertex', defining a new simplex shape. In the 'variable size' algorithm variant, the reflection can be expanded or contracted, if the new vertex has the best or the worst response when compared with the others, respectively.

The initial conditions were selected according to the reported optimum values for Procion Blue MX-7RX (45.6 min, 188 dye:protein ratio, pH 11.4 and 35.7 °C) and varied until a *plateau* was reached in the response values. The yield of each condition was assessed by means of a direct slot blot test. Nitrocellulose membranes were coated with 100 µg of purified human IgG using a slot blot device (Höeffer PR600), blocked overnight at 4 °C with 3% nonfat milk in PBS, washed three times with PBS-Tween 20 0.05% and then incubated with the labeled antibody during 1 h at room temperature. Membranes were washed and scanned, and the response was determined measuring the intensity of each slot with Scion Image Analyzer (Scion Corporation). Once the optimum values were selected, a final comparison was made between these conditions and those reported for Procion Blue MX-7RX.

Quantitation limit

The minimum amount of human IgG that can be detected was determined in a capture slot blot test. Membranes were coated with 5 µg of purified rabbit-anti-human-IgG immunoglobulins (capture antibody), blocked overnight at 4 °C with 3% non fat milk PBS and washed three times with PBS-Tween 20 0.05%. Antigen (human IgG) was incubated during 1 h at room temperature in several dilutions ranging from 100 µg/ml to 100 ng/ml, followed by three washes. Antibodies labeled in the optimal conditions were incubated at a final protein concentration of 0.35 mg/ml, during 1 h at room temperature. Membranes were washed and scanned, and the intensity of each slot was determined.

Comparison with colloidal-gold

RBV5R labeling was compared with colloidal gold labeling, using the direct slot blot assay. RBV5R labeling was performed in optimum conditions, and colloidal gold labeling was performed according to manufacturer instructions. Briefly, optimum relation of antibody mass and 20 nm colloidal gold particles (Sigma Chemical Co) was determined incubating 250 µl of colloidal gold suspension and 20 µl of antibody in different dilutions during 5 minutes at room temperature. Then, 100 µl of NaCl 10% w/v were added, and the ideal relation was considered to be the one in which no color change is detected after the addition of NaCl. Preparative antibody labeling was performed in that condition, followed by centrifugation at 10000 rpm and resuspension in PBS.

Labeling comparison was conducted performing the direct slot blot assay with both labeled-antibodies in two-fold serial dilutions from a 3 mg/ml antibody solution. Membranes were scanned, and color intensity was analyzed and compared for each dilution.

Accelerated stability assays

Conjugation can affect the structure and stability of proteins, altering their structure and isoelectric point. In order to explore the stability of the labeled antibodies, sodium azide was added at a concentration of 0.2% p/v, and the solution was then sterilized by filtration using a 0.22 µm filter. Aliquots were stored at 4 °C and 37 °C during 1, 2, 3 and 4 days, and the ability to recognize the antigen after these treatments was studied by direct slot blot, as described before.

RESULTS

Non-covalent binding of dyes to serum proteins

Several bands were detected by native PAGE 10% of stained serum proteins, as can be seen in Figure 1A. Direct binding of dyes to serum proteins was demonstrated in all cases, although each dye produced a different staining pattern. Binding after alkaline hydrolysis –which is supposed to inactivate covalent binding– indicates that all dyes have residual non-covalent interaction with serum proteins, but that it varies with the dye. Figure 1B suggests that the main bands in Figure 1A probably correspond to albumin and immunoglobulins, the most abundant proteins in human serum.

Optimization of labeling conditions

Simplex evolved rapidly to alkaline pH and high dye:Ab molar ratios, but incubation time remained relatively short, indicating that the labeling efficiency has a strong dependence on the first two parameters (Table 1). Simplex step number 10 was considered the optimum, since all further steps (11 to 17) yielded lower response values. Therefore, all further labeling was performed at 37 °C, pH 10.95, 264 dye:Ab molar ratio and 131 minutes.

Quantitation limit

Antigen detection limit for the particular slot blot assay that was used in this work, was 400 ng/ml. As shown in Figure 2A and 2B, the linear interval ranged from 400 ng/ml to 25 µg/ml, which represents a 62.5 fold span.

Comparison with colloidal-gold label

RBV5R label resulted in higher color intensities in all antibody dilutions as shown in Figure 3. Although colloidal gold labeling may possibly be improved, this initial comparison indicates that RBV5R label yields intense colors, and is a suitable candidate for replacing colloidal gold.

Accelerated stability assays

The results shown in Figure 4 indicate the titer of the labeled antibody did not decay during the thermal treatment, neither at 4 °C nor at 37 °C, thus suggesting that the label is stable and does not alter the conformation of the antibody. Nevertheless, complete characterization of label stability requires further studies.

DISCUSSION

There are many aspects that define a suitable antibody label. One of them is the ability of the labeling compound to react covalently with the Ab during the labeling process, and not to interact with other substances during the assay. The non-covalent interaction assay performed here demonstrates that all tested dyes react with serum proteins. However, only RBV5R lost almost all its reactivity after alkaline treatment, which is supposed to inactivate its reactive chemical groups. Therefore, it can be deduced that the reactivity of this dye is mainly due to the covalent bonds established by the vinylsulphonic groups, and not to the non-covalent interactions. This feature makes RBV5R an interesting candidate for further testing.

Optimal labeling conditions were pH 10.95, dye:Ab molar ratio of 264 and incubation time of 132 minutes. However, the labeling extent in those conditions was not the highest; this can be explained considering that the response values depend on the intensity of the color, as well as on the residual Ab reactivity. The latter is conditioned by the denaturation the Ab might suffer during the labeling process, and by the probability of the dye reacting in the Ab paratope. Hence, simplex steps 12 and 15 resulted in a high extent of labeling but a low response, probably because the extremely high pH produced Ab denaturation. Simplex step 7 also conduced to extensive labeling, and the low response can be explained considering that the dye might have blocked some of the Ab paratopes.

Further studies demonstrated that labeled antibodies are stable, and can be successfully used in slot blot assays, detecting as low as 400 ng/ml. This sensitivity is comparable with the one that can be achieved with colloidal gold, which is reported to range from ng/ml [9] to µg/ml [10] and yielded even lower color intensities in our Ab-Ag system.

CONCLUSIONS

The present work demonstrates that vinylsulphonic reactive dyes can be successfully used to label antibodies, which remain stable and provide adequate sensitivity in slot blot assays. Also, Remazol Brilliant Violet 5R has shown less non-covalent interaction with serum proteins than all the other reactive dyes tested, indicating that this can be the better choice for Ab labeling. Furthermore, given their

extremely low cost, these results suggest reactive dyes are excellent candidates for the design of a new generation of Point of Care Testing kits.

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TABLES

Table 1. Conditions during simplex optimization of labeling parameters.

§ Response is expressed in arbitrary units of OD.

¥ The extent of labeling was calculated as the molar ratio of bound dye and antibody.

* Extent of labeling could not be determined because the absorbance of the solution at 542 nm was negligible.

FIGURE CAPTIONS

Figure 1. Non-covalent binding to serum proteins.

(This figure should be published in color just in the Web)

(A) Native PAGE 10% of stained serum proteins. (B) Same gel, after standard Coomasie Blue staining. Lanes 1 to 6 correspond to active dyes; lanes 7 to 12 correspond to the dyes previously subjected to alkaline hydrolysis. Dyes are: Remazol Brilliant Violet 5R (lanes 1, 7); Procion Blue H-EGN (lanes 2, 8); Procion Red HE-7B (lanes 3, 9); Remazol Yellow 3RS (lanes 4, 10); Orange R-HE (lanes 5, 11) and Procion Brown MX-5BR (lanes 6, 12).

Figure 2. Quantitation of human IgG by a slot blot assay, using Remazol Violet 5R labeled antibodies.

(A) Digitalized membranes. (B) Analysis of slot intensity. Antigen concentration ranged from 100 ng/ml (left) to 100 µg/ml (right). Each measurement was performed by

triplicate. Error bars represent the standard deviation. * $p < 0.01$ by the Student t test, when compared to blank (dotted line).

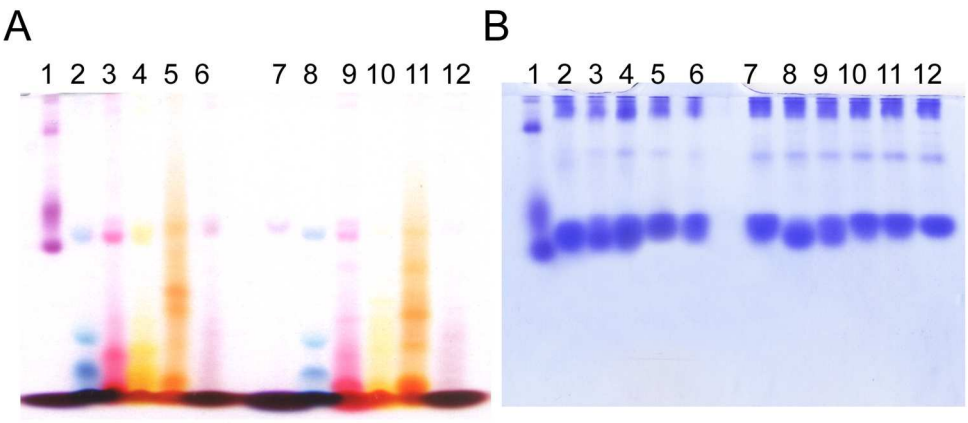
Figure 3. Comparison with colloidal gold.

Band intensity of serial two-fold dilutions of antibodies labeled with either BRV5R or colloidal gold. Results are expressed as a relative value compared to the maximum intensity achieved in the assay.

Figure 4. Accelerated stability assay.

(A) Slot blot for the accelerated stability test. Temperature is indicated above, and the numbers represent the days of incubation. Control slot shows the response of a freshly prepared aliquot. (B) Membranes were scanned and analyzed, and the results are presented as bar graphs.

Simplex step	pH	Incubation time (min)	Dye:Ig molar ratio	Response (AU[§])	Extent of labeling[¥]
1	10.00	30.00	100.00	9.33	***N/A*** *
2	10.00	60.00	250.00	9.01	***N/A***
3	8.50	90.00	200.00	32.67	***N/A***
4	12.10	120.00	200.00	27.82	100
5	10.40	100.00	83.33	0.00	***N/A***
6	10.10	70.00	208.33	54.59	120
7	10.47	156.67	305.56	35.31	429
8	12.08	128.33	256.94	204.81	146
9	12.39	55.56	137.96	37.48	94
10	10.95	131.39	263.66	221.64	207
11	9.99	99.81	285.96	120.00	190
12	11.91	169.69	329.37	197.50	313
13	12.82	219.54	389.89	173.22	163
14	9.81	138.93	329.05	171.36	163
15	11.79	193.53	328.76	154.34	270
16	11.34	170.10	318.06	145.89	109
17	10.44	123.24	296.66	182.62	148



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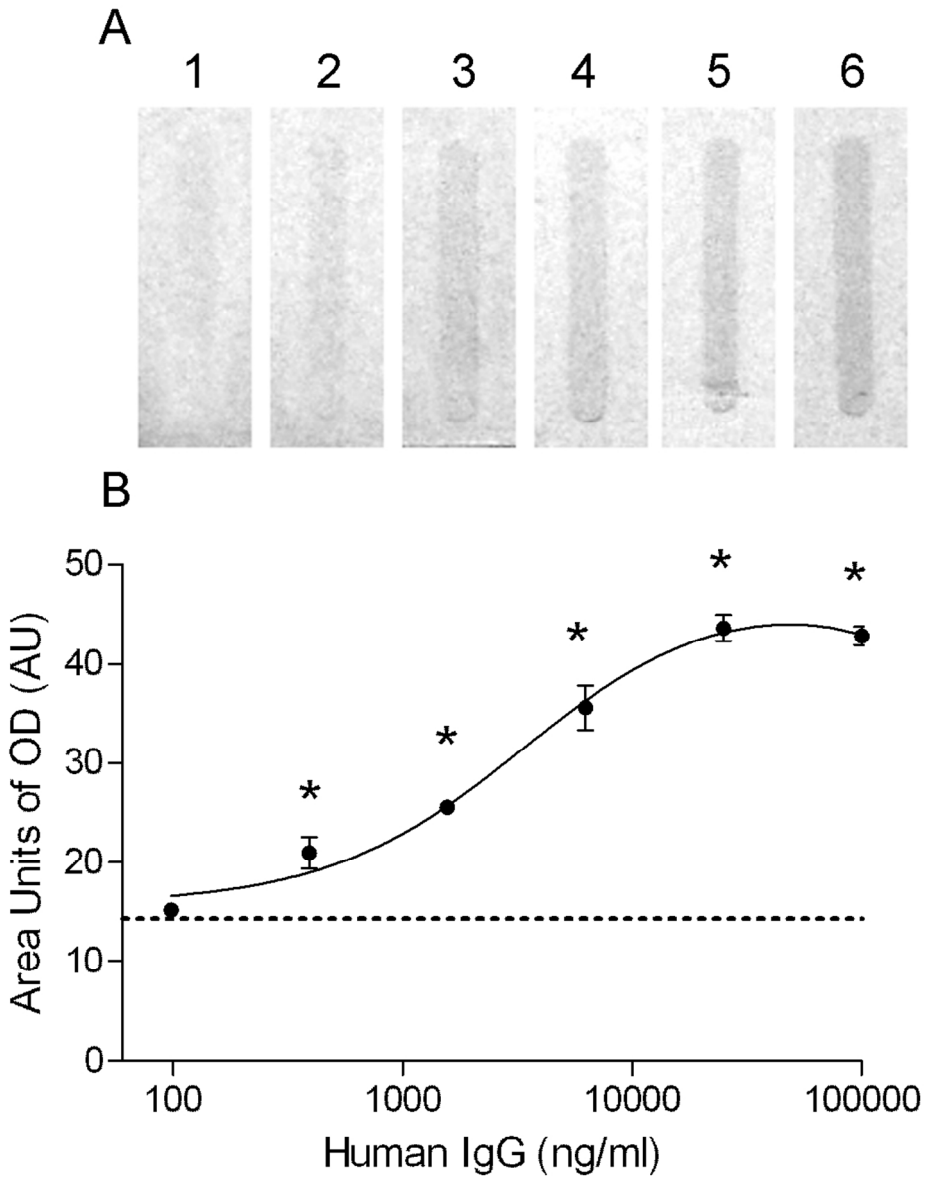

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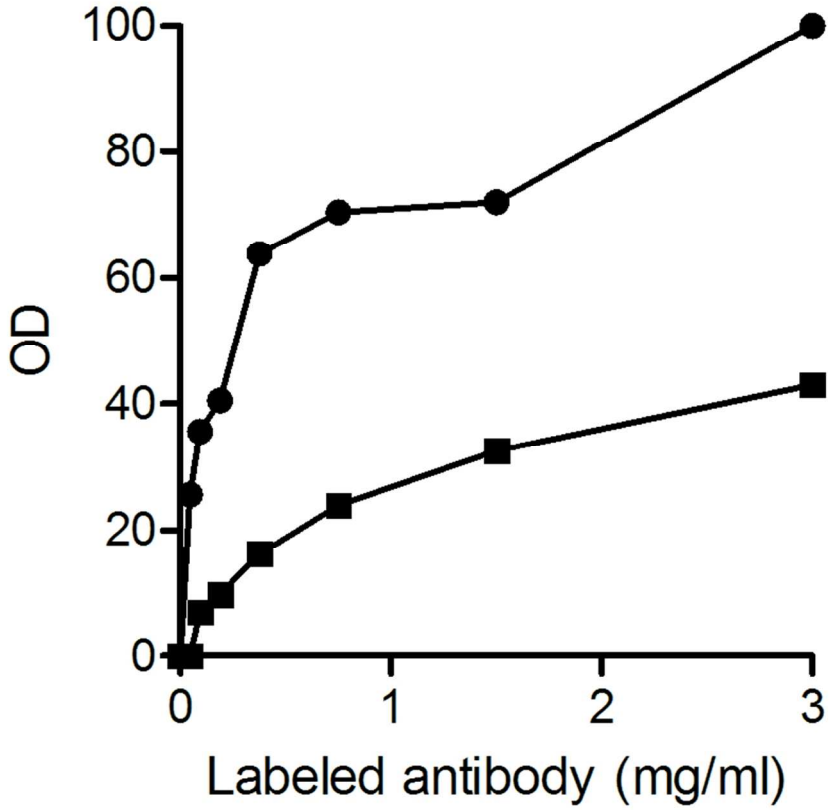

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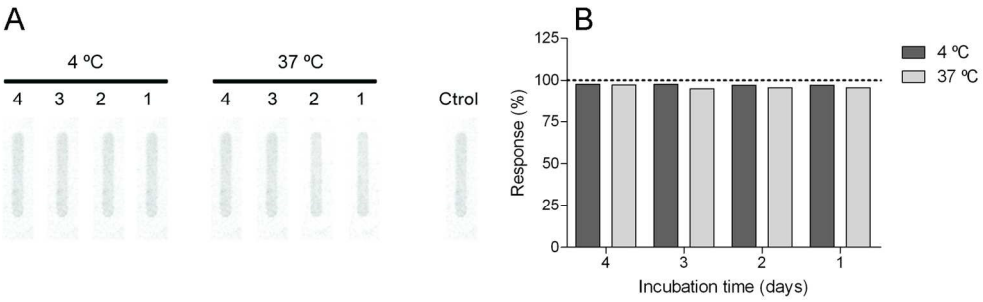

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