Novel Diazonium-Functionalized Support for Immobilization Experiments

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ABSTRACT: A hydrophilic, water-insoluble polymer was prepared, starting from commercial poly(vinyl alcohol) that was crosslinked and functionalized by means of glutaraldehyde and 4-nitrobenzaldehyde. The resulting beads were then reduced and subsequently diazotized, and finally contained diazonium moieties capable of covalently coupling with electron-rich aromatic systems such as histidine and/or tyrosine residues of proteins. The described resin is therefore well suitable for protein immobilization whenever lysine residues (those involved in covalent coupling with several popular immobilization procedures) are not available and/or cannot be used unless the biological activity of the protein is destroyed. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **66:** 1433–1438, 1997

Key words: poly(vinyl alcohol); acetalation; diazotization; immobilization

INTRODUCTION

Poly(vinyl alcohol) (PVA) is a hydrophilic, water-soluble polymer, readily available in a wide range of molecular weights at a very low price.[1–3] The polymer could be profitably used for immobilization experiments,[4–11] provided that the substance is crosslinked and transformed in macroporous beads. To this purpose, the reticulation by means of suitable dialdehydes[12] seems to be the method of choice because several dialdehydes are commercially available at low cost. The crosslinked polymers could be directly used as they are for immobilization experiments or further derivatized by means of suitable functionalized monoaldehydes.[9] This modification could be achieved after the crosslinking step or at the same time under some experimental precautions to prevent undesired side reactions between functionalized aldehydes (e.g., nucleophilic addition of primary amino groups of aminoaldehydes to activated double bonds of oligomeric glutaraldehyde).

In the present work, the preparation of a nitroaromatic derivative of crosslinked PVA is described that in turn was reduced to the corresponding amino aromatic substance and finally diazotized by means of conventional organic chemistry procedures. A macromolecular diazonium salt was obtained that was tested to assess its ability to covalently couple with proteins.

EXPERIMENTAL

Reagents

PVA (average molecular weight 30,000–70,000 Da, fully hydrolyzed), bovine serum albumin (BSA), β-glucosidase (crude, from almonds), and β-D-(4-nitro-phenyl)glucopyranoside (PNPG) were from Sigma Italia (Milan, Italy); 4-nitrobenzaldehyde (99+% purity degree), glutaraldehyde 50% aqueous solution, 4-amino-*N,N*-diethylaniline sulfate, L-histidine, tyramine hydrochloride, and histamine monohydrochloride came from Fluka (Buchs, Switzerland). All these and other reagents were of the best grade available and were used without any

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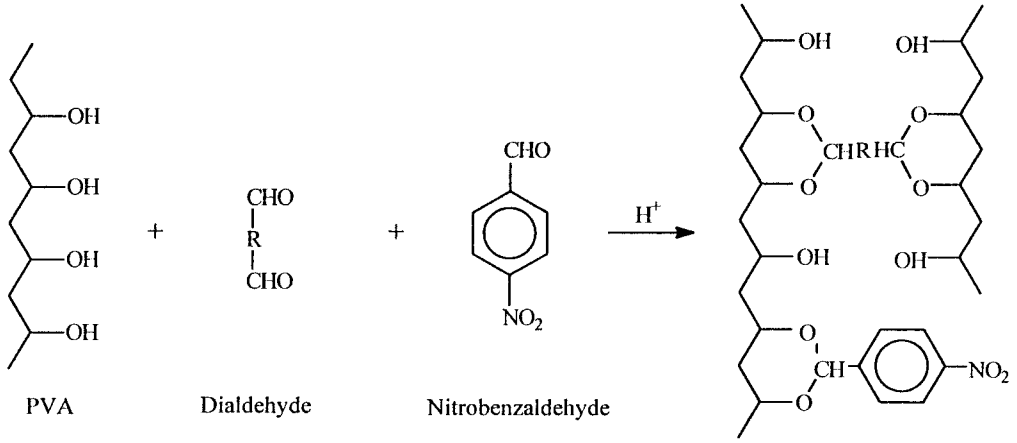


Scheme 1 Grafting of PVA with 4-nitrobenzaldehyde.

further purification. A concentrate Coomassie reagent for protein quantitation was from Bio Rad Italiana (Milan, Italy).

Quantitation of Acetalation Degree

The amount of the acetalation reaction between PVA and 4-nitrobenzaldehyde was determined by means of the reaction between the aldehyde and 4-amino-*N,N*-diethylaniline sulfate,[13] under sharply acidic conditions, to form the colored (λ_{max} = 465 nm) immonium salt. A solution of the amine sulfate (10 mg/mL) was prepared in ethylene glycol monomethylether; 1 mL of this solution was mixed with 1 mL of the aldehyde solution obtained by extracting the solution from the acetylation procedure with dichloromethane. The mixture in stoppered test tubes was heated in a boiling water bath for 10 min, rapidly cooled with tap water to room temperature, and the absorbance finally read. A standard curve was prepared with 4-nitrobenzaldehyde dissolved in dichloromethane.

Quantitation of Azocoupling

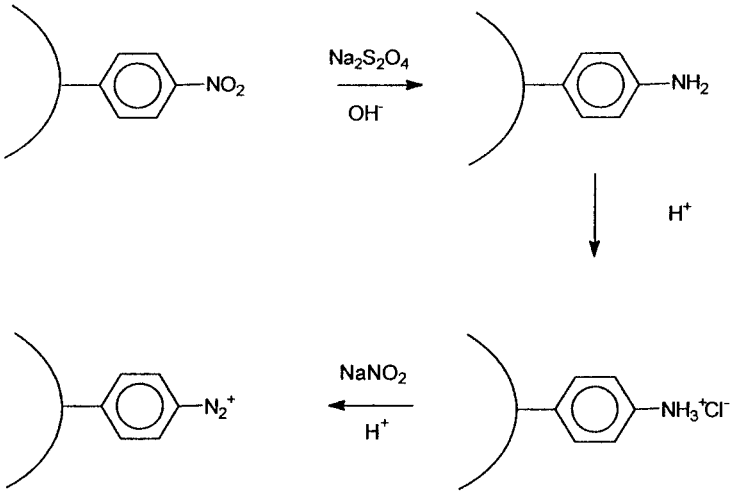
For these measurements a ninhydrin/hydrindantin reagent[14] was used in the case of histidine, histamine, and tyramine. In the case of BSA and β-glucosidase, the method of Bradford[15] was used with a commercial reagent. In all cases, the amount of coupled amine or protein was quantitated by difference, that is, by subtracting the residual substance in the supernatant, plus combined washings, from the total substance before the coupling reaction.

Determination of β-Glucosidase Activity

These measurements were performed as previously described,[16] by using PNPG as the substrate. For immobilized preparations, a test tube whirling device was used.

Preparation of Polymer Beads

Ten grams of PVA were dissolved with warming in 100 mL of distilled water. When a clear solution was obtained, it was cooled to room temperature and treated with 1 mL of glutaraldehyde solution and 1 g of 4-nitrobenzaldehyde previously dissolved in 5 mL of dioxane. The mixture was then dropwise treated under magnetic stirring with 6*M* HCl, which causes the gelation of the entire solution to an elastic, translucent, almost colorless mass. The reaction was allowed to continue overnight, then a certain extent of volume contraction was observed concomitant with liquid expulsion from the crosslinked polymer. The polymer was washed to remove excess HCl and ground with a blender to a coarse slurry. To remove fines this slurry was repeatedly washed with decantation until no Cl^- could be detected in the washings. The polymer was finally lyophilized to a white, coarse, highly hydrophilic powder. The 4-nitrophenyl functionalized polymer was reduced to the corresponding amino derivative by heating in a boiling water bath with a large excess of sodium dithionite for 30 min. The resin was then thoroughly washed on a sintered glass funnel, first with ammonia solution (2% w/v) and then with distilled water, until no ammonia could be detected in the washings. The aminophenyl resin



Scheme 2 Steps to obtaining the polymer.

was then transformed into the sulfate salt by treating it with an excess of 2% sulfuric acid. The resin was washed again with distilled water until no sulfate ions could be detected in the washings, and finally cautiously dried at 50°C under reduced pressure. The obtained product, a white, rather hygroscopic, free-flowing coarse powder, could be immediately used for diazotization or alternatively stored at −20°C.

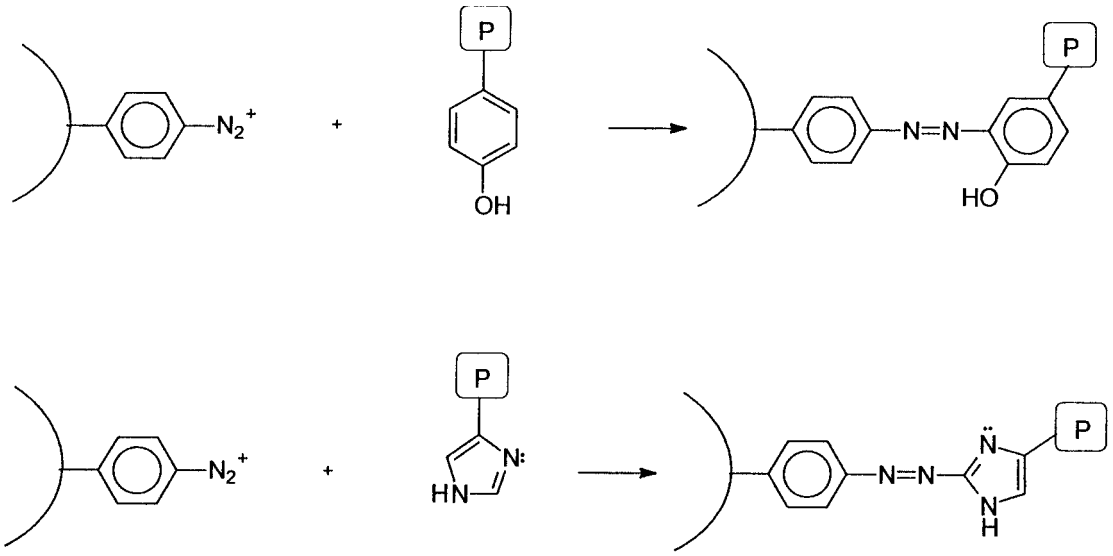
Diazotization of 4-Aminophenyl Resin and Coupling Reactions

Fifty milligrams of $NaNO_2$ were cautiously added under stirring to 100 mg of the resin, suspended in 5 mL of 2*M* HCl and kept in an ice bath until the entire procedure ended. After 10 min the resin was washed with precooled 0.1*M* HCl plus 0.1*M* urea to destroy excess nitrous acid, and subsequently with ice-cold water until no HCl was present in the washings. The cold wet resin was immediately transferred to a glass test tube containing 5 mL of the molecule chosen to be coupled (10 mL in a series of experiments with BSA) that was dissolved in the appropriate buffer containing 0.5*M* NaCl. In the case of the amines and amino acid a 0.15 m*M* solution was used; solutions containing 1, 2, 5, 10, 15, and 20 mg/mL were used for proteins. The buffers used were sodium acetate (pH 4 and 5), potassium phosphate (pH 6 and 7), and sodium diphosphate (pH 8 and 9). All the buffers were 0.1*M* with respect to the buffering species and contained 0.5*M* NaCl. Each mixture was then slowly inverted for 24 h at 4°C, then the mixture was thoroughly washed on a sintered glass funnel with 0.5*M* NaCl to remove the uncoupled substance.

RESULTS AND DISCUSSION

The grafting of PVA with 4-nitrobenzaldehyde proceeded rapidly and almost quantitatively under the described conditions, and it is common knowledge that acetalation of PVA is easily accomplished with most aldehydes because the resulting acetals are usually water insoluble. This is also the case of the crosslinked resin described here, which is obviously completely insoluble in whatever solvent. The acetal is then continuously subtracted from the equilibrium and the formed 1,3-dioxane ring is sterically favored (Scheme 1). Accordingly, the product is quite stable under alkaline, neutral, and moderately acidic conditions. Only prolonged boiling with excess hydrochloric acid leads to a slow hydrolysis, with release of both glutaraldehyde and 4-nitrobenzaldehyde. A similar stability is shown by the corresponding amino resin. In this case, detection of 4-aminobenzaldehyde is impossible with the described method, because the aminoaldehyde rapidly polymerizes under the described conditions.

With regard to glutaraldehyde, it is not really the monomer but a complex mixture of oligomers, the simplest of which bears at least three aldehyde groups.[17] Other experiments with the true monomeric aldehyde, obtained during the acetalation reaction starting from its bishydrogenosulfite adduct, lead to a product that does not show any advantage over the one obtained with the com-



Scheme 3 Tyrosine and histidine residues forming covalent compounds with diazonium salts.

mercial, low-priced, oligomer solution (data not shown).

Other diazotizable, PVA-based polymers have already been described,[18] but their preparation is more complicated and involves the multistep functionalization of a precrosslinked PVA. The polymer described here is easily obtained with a few steps (Scheme 2), starting from very cheap and easily available reagents.

Among the proteic amino acids, only tyrosine and histidine residues could form definite covalent compounds with diazonium salts (Scheme 3) whereas tryptophan is sparingly represented in proteins and moreover is fully buried into the polypeptide chain. The benzene ring of phenylalanine is not electron rich enough to couple with diazonium salts, whereas the coupling products of lysine, like diazoamino compounds, are rather labile and therefore are not useful for stable covalent immobilization. Therefore, it is of some interest to study the influence of pH on the amino acid residues most likely to couple with diazonium salts, for example, tyrosine and histidine. Because tyrosine is almost water insoluble within a wide range of pHs, the use of a similar molecule like tyramine, which is readily soluble in water regardless of pH, could give valuable information with respect to the 4-hydroxyphenyl side chain in a protein. For the reasons of coherence, tyramine was compared to histamine; a third series of experiments was performed using histidine. Inspection of Figure 1 shows that no significant differences could be seen between histidine and histamine behavior toward immobilization as a function of pH, and therefore it appears fully legitimate to suppose a very close resemblance between tyrosine and tyramine.

As expected for azocoupling reactions, the higher the pH, the higher the extent of coupling. However, at neutral pH values a sharp preference for the hydroxyphenyl ring was shown with respect to the imidazole ring. This is most probably due to the pK_a of the imidazolium cation (about

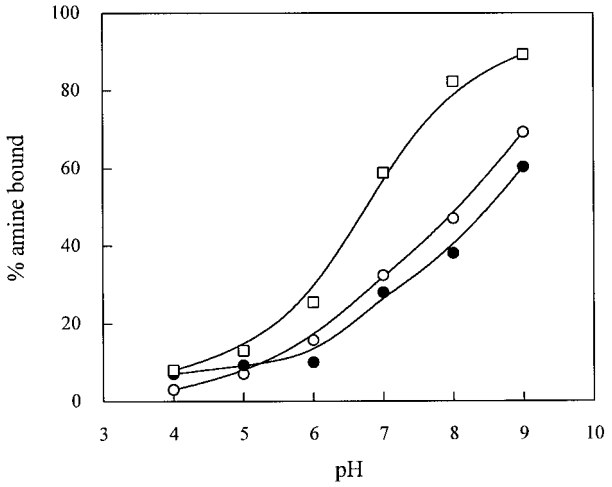


Figure 1 Relative amount of (□) bound tyramine, (○) histamine, and (●) histidine on the diazotized resin as a function of pH; 100 mg of diazotized resin were used for 5 mL of the amine solution.

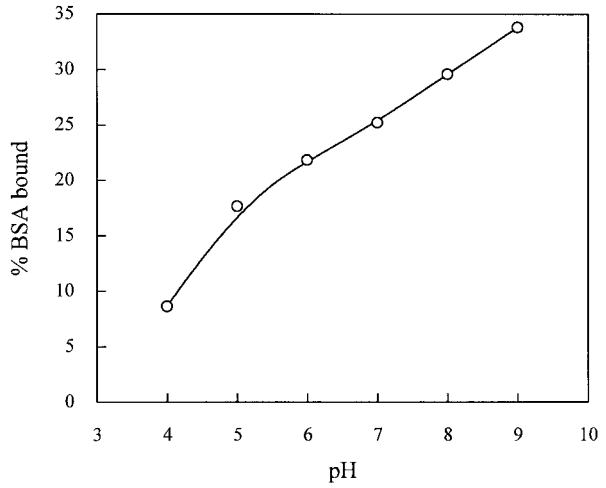


Figure 2 Relative amount of bound BSA as a function of the pH; 100 mg of the diazotized resin were used for 5 mL of BSA solution.

6.5): at low pH values the protonated form, bearing a net positive charge, prevails, and this form is completely unable to react with diazonium salts. On the other hand, the nondissociated form of the hydroxyphenyl ring (pK_a about 10) is not electrically charged and is electron rich enough to allow a slow coupling even at pH 4. Therefore, a relatively low pH could lead to a preferential coupling with tyrosine residues, whereas this specificity in the coupling reaction should be lost at higher values where both residues are prone to react. Above pH 10, tyrosine should again become the best candidate, because it is in its extremely reactive phenoxide anion form. However, this last observation is of only poor significance, at least in regard to protein immobilization, because proteins are usually denatured at such high pHs.

A resin similar to the one described here could be very useful, being readily obtained at very low costs, for immobilization procedures to obtain immobilized enzymes to be employed in industrial processes. Therefore, the ability of the resin to couple with proteins was tested by means of a "typical" protein, BSA; as expected, the extent of coupling increases as pH increases (Figs. 2, 3). The same was noted for the enzyme, β-glucosidase (data not shown). In this case the attention was focused on activity recovery rather than coupling efficiency, and it was found that pH 7 allows for both high coupling efficiency and high activity retention. As a tyrosine residue could conceivably be present within the active site of glycanases and glycosidases (e.g., this is true for lysozyme), the involvement of that specific tyrosine in the covalent coupling should lead to enzyme inactivation. To check this possibility, a parallel series of experiments was performed in the presence of the competitive inhibitor, δ-gluconolactone.[17] Preliminary results show that when the active site is occupied by the specific inhibitor, a significant increase in activity recovery is seen. In particular, as much as 67% immobilized activity was recovered when the immobilization of the enzyme was carried out in the presence of the inhibitor, compared with 52% obtained in the absence of the same.

In conclusion, a very cheap resin was obtained that is highly hydrophilic and therefore well suitable for working on proteins and is capable of fast covalent coupling with tyrosine and/or histidine containing proteins. The pH dependence of coupling was tested with two proteins, and some indications with respect to coupling selectivity toward different amino acidic residues was drawn. The diazo coupling could be highly valuable when lysine residues are not present or also when they are part of the active site of an enzyme. Moreover, some popular immobilization methods, as are those based on cyanogen bromide and trichlorotriazine, are subject to progressive hydrolysis of the chemical bridge between the support and the ligand, leading to ligand loss. On the contrary, the azo bridge is quite stable under the usual operative conditions and no ligand loss could be observed at all. So, the diazo coupling with a resin such as that described here could be the method of choice for most applications.

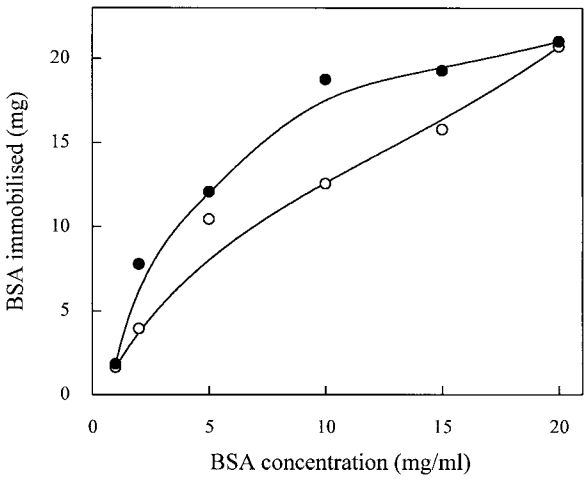


Figure 3 Relative amount of bound BSA as a function of BSA concentration; 100 mg of diazotized resin were used for (○) 5 mL and (●) 10 mL BSA solution in potassium phosphate buffer, pH 7.

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