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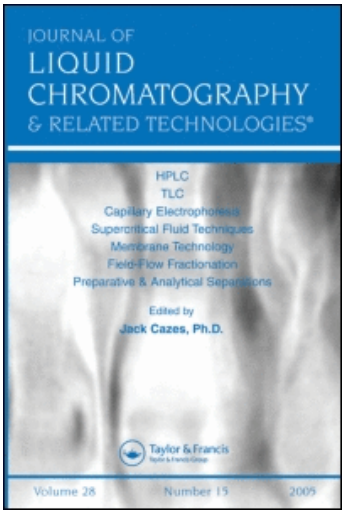

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An Improvement for the Synthesis of a Styrene-Divinylbenzene, Copolymer Based, 6-Aminoquinoline Carbamate Reagent. Applications for Derivatlzation of Amino Acids, Peptides, and Proteins

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AN IMPROVEMENT FOR THE SYNTHESIS OF A STYRENE-DIVINYLBENZENE, COPOLYMER BASED, 6-AMINOQUINOLINE CARBAMATE REAGENT. APPLICATIONS FOR DERIVATIZATION OF AMINO ACIDS, PEPTIDES, AND PROTEINS

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Abstract

An improved method for the synthesis of a styrene-divinylbenzene based 6-aminoquinoline carbamate (6-AQC) reagent with a benzotriazole linkage is reported in this paper. By using 6-aminoquinoline (6-AQ) and triphosgene as the starting materials, the synthesis of the intermediate quinoline-6-isocyanate became much simpler. Directly from quinolyl-6-isocyanate, instead of from the rearrangement of quinolyl acyl azide, a polymeric reagent with high loading and few adsorbed impurities was obtained. This was then used for the derivatization of amino acids (AAs) and peptides. High efficiencies and reproducibilities of the derivatizations make this reagent attractive for the analysis of AAs and small peptides. The reagent was also used for a derivatization study of proteins. This was the first time that any polymeric reagent has been described for the successful, more selective tagging of larger proteins. The effect of surfactants on the reaction was also studied.

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Derivatizations with the solid polymeric reagent and a 6-AQC solution reagent were compared. With the solid phase reagent, fewer tagged products were usually obtained, conditions dependent. Derivatization with the solution reagent can yield a single product, again conditions dependent, corresponding, it is believed, to all amino groups tagged. This precolumn reaction now makes it possible to detect subpicomoles of proteins in reverse phase high performance liquid chromatography (HPLC).

Introduction

Solid phase reagents have been developed for performing derivatizations of numerous organic analytes in various HPLC modes [1]. Such reagents utilize ionic or covalent attachments of various labile tags that possess specific detector enhancement properties; UV, EC, FL, and so forth. The covalently attached moiety has better stability and other desirable properties. Many kinds of polystyrene based covalent reagents have been developed and applied for reaction detection [2]. An immobilized anhydride was prepared on a microporous polystyrene (PS) crosslinked with small amounts of divinylbenzene (DVB) (STY-DVB) support for the analysis of primary and secondary amines [3]. This reagent was based on a 1% crosslinked, DVB chloromethylated polystyrene. The anhydride was capable of rapid conversion of amines in aqueous/organic environments to stable amides, which were chromatographically stable and possessed excellent UV and EC detection properties.

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Gao et al. also prepared an active ester based on a 1-hydroxybenzotriazole leash [4]. The benzotriazole activated ester on a macroporous support proved to be a powerful acylating reagent when labeled with 9-fluorenylmethoxycarbonyl chloroformate (9-FMOC). Urinary polyamines were analyzed successfully using this reagent. The chemical leash, o-nitro-p-carbonyl benzophenol, was prepared on a 4% crosslinked microporous STY/DVB polymer. The polymer, labelled with a 9-FMOC tag proved to be stable for on-line precolumn conversions [5]. Amphetamine spiked in urine has been derivatized and detected via HPLC-UV/FL with this particular reagent.

Although many polystyrene based reagents, with different underlying supports and final analytical tags, have been reported within the last decade, few of them dealt with the derivatization of amino acids (AAs) and peptides. Zhou et al. developed a polymeric reagent containing a 9-fluorenylacetyl (9-FA) tag [6], which was successfully used for the derivatization of AAs and peptides. However, the derivatization efficiencies were not sufficient to perform quantitative determinations of AAs, especially with the less hydrophobic species.

Recently, a polymer tagged 6-AQ, activated carbamate reagent was prepared for derivatization of amines and AAs in HPLC or HPCE [7]. The major advantage of this reagent has been that the hydrolysate(s) of the tag (6-AQ or its urea dimer) usually show(s) little interference(s) with the 6-AQC derivatives by FL detection [8]. The derivatization yields of AAs; however, were still not optimal, due to the low loading of the 6-AQC tag [7]. In the current paper, the method for preparing this polystyrene based 6-AQC reagent has been improved. A reagent with a higher loading of the final tag and less adsorbed impurities was finally obtained. Reasonable derivatization efficiencies of AAs and small peptides have been obtained.

In the case of proteins, in general, solution derivatization reactions tend to be random, indiscriminate, and nonselective, often leading to multiple products [9-10]. Almost all solution reactions tag multiple sites on a protein's surface, and usually cannot select one in particular to form a single derivatized product. The formation of multiple protein products, each having different sites and number of tags present, leads to confusion and complexity in HPLC or HPCE, as well as increased detection limits (DLs) and questionable identification of the original protein or a specific derivative. This is not a trivial problem, and it has become more important in the case of precolumn tagging in HPLC or HPCE, wherein a single, highly tagged single product is the ultimate goal (11-12). Because of this, most work for enhancing the detection of proteins has focused on postcolumn derivatization [13-15], and few articles deal with precolumn derivatizations [16], especially in reverse phase HPLC [17].

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There exists a real need in the field of protein reaction chemistry to develop reactions that will form fewer and more selective or controlled products [9-10]. At times, a single, well defined product should be the goal for improved (lowered) HPLC/HPCE applications. Improved DLs will result from fewer products formed in the precolumn derivatization mode. Careful control of the conformations present, and limiting the protein reactions to terminal and/or exposed lysine (Lys) groups should result in improved reproducibility and fewer products for all protein tagging. Aside from HPLC/HPCE purposes, there is a general, long-standing need to be able to modify proteins in more specific and selective ways, such as when adding polyethylene glycol (PEG) side chains, carbohydrate side chains, protecting groups, and other chemical (tagging) modifications at specific amino acid sites [9-10]. Almost all prior and current work has involved solution chemical reactions on proteins.

Solution reactions can be affected by the nature and concentration of surfactants added along with the reagent and buffers, though this has not been studied very

extensively. Altering the mixture and/or nature of products formed, such as by the addition of surfactants in solution chemistry, presents an alternative control in protein reactions to the use of polymeric reagents. Similarly, the use of surfactants to affect altered products derived from proteins has not been studied with any polymeric reagents.

We have investigated here the solution and solid phase reagent reactions of some typical proteins, partly to demonstrate the differences that result from immobilizing the same tag on a polymeric support. Thus, it is the solution reagent that has become immobilized on the polymeric support, while the protein is free in solution, at times with pH control, surfactant additives, organic solvents, and so forth. We anticipate that these studies will lead to a general approach to improve and make more selective and specific future protein reaction chemistries. A wide variety of polymeric reagents already exist that could then be applied to novel protein tagging, for several reasons, such as enhanced detector response in HPLC/HPCE modes. An entirely separate issue relates to recovering bioactive (biorecognition), tagged proteins from any of these solution/polymeric reactions, in addition to limiting the sites of tagging and total number of tagged protein products. In part, we address that question as well, demonstrating in initial results, that tagged proteins can be recovered from some of these reactions that still recognize their antibodies in the form of immunoaffinity chromatography purification.

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Experimental

Materials

Styrene-divinylbenzene copolymer (12% cross-linked, 102 Å templated, 16-20 μm) was obtained from Supelco Corporation (Bellefonte, PA, USA). 6-aminoquinoline (6-AQ), triethylamine (TEA), cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), and sodium tetraborate were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). AAs, peptides, proteins, and trypsin-TPCK were all purchased from Sigma Chemical Company (St. Louis, MO, USA). Bovine growth hormone releasing factor (GHRF) was donated by The Upjohn Company (Kalamazoo, MI, USA). Sodium acetate, phosphoric acid, sodium phosphate (monobasic and dibasic), sodium hydroxide, and hydrochloric acid were obtained from Fisher Chemical Company (Fair Lawn, NJ, USA). HPLC grade solvents were generously donated by EM Science, Inc. (Gibbstown, NJ, USA) as their Omnisolv® grade. 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (6-AQC) (Waters AccQ.Fluor™ Reagent) and borate buffer (pH 8.8) were obtained as a kit from Waters Corporation (Milford, MA, USA).

Solutions and HPLC Mobile Phases.

AAs, peptides and proteins were dissolved in water, except insulin, which was dissolved in 50 mM HCl. CTAB, SDS, and Brij 35 were dissolved in water to a concentration of 20%. Mobile phase A consisted of 140 mM sodium acetate and 17 mM TEA, titrated to pH 5.05 using phosphoric acid; mobile phase B consisted of 60% acetonitrile in water (v/v), mobile phase C consisted of 20 mM sodium phosphate (monobasic) titrated to pH 3.0 with phosphoric acid; and mobile phase D consisted of 80% acetonitrile in water (v/v).

Preparation and Characterization of the 6-AQC Polymeric Reagent.

The synthesis of the polymeric benzotriazole intermediate followed a literature procedure [18-20]. The attachment of the analytical label, 6-AQ, to the polymeric intermediate involved the synthesis of quinolyl-6-isocyanate (Scheme I). This was first prepared by adding 1 ml TEA and 0.3 g 6-AQ in 10 ml dried THF dropwise to an ice-cooled flask containing a solution of 0.6 g triphosgene in 10 ml dry THF with stirring. The mixture was stirred for 30 min at 0^0 C, and then rotoevaporated to remove the solvent at 50^0 C. The solid residue showed an absorbance at 2250 cm^{-1} in its IR spectrum (mineral oil), which suggested the existence of an isocyanate group. The IR spectrum was obtained on a Perkin-Elmer Model 1310 infrared spectrophotometer (Perkin-Elmer Corporation, Norwalk, CT). To this solid, 20 ml toluene and 1 g of polymeric benzotriazole intermediate were added. The slurry was stirred and heated until the temperature gradually increased to 65^0 C, and then held there for a further 1.5 h. The reaction slurry was filtered over a sintered glass funnel. The solid polymeric reagent was washed with 10 ml toluene, 2 x 10 ml THF, 7 x 10 ml DCM, and then dried in vacuo at room temperature, overnight. 1.09 g of the polymeric reagent was obtained, Scheme I.

The amount of 6-AQC attached to the polymeric intermediate was determined by a base promoted hydrolysis of the final polymeric reagent. The procedure used for characterization was performed as described elsewhere [6-7]. An authentic sample of 6-AQ was used together with its calibration plot in HPLC-UV to determine the final loading.

Off-line Derivatizations of AAs and Small Peptides with the Polymeric Reagent.

Off-line derivatizations of AAs and peptides were performed as described elsewhere [6-7]. An aliquot of 25 µl of the analyte solution, 25 µl of ACN, 25 µl of saturated

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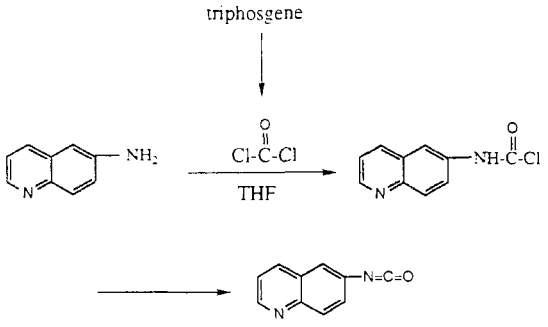
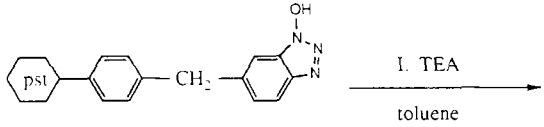
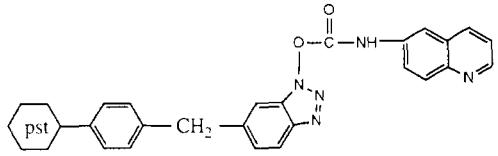




Benzotriazole Intermediate



6-AQC Tagged Solid Phase Reagent

Scheme I. Synthesis of quinolyl-6-isocyanate, and its attachment to the polymeric benzotriazole substrate.

sodium borate solution, and 25 µl of 20 mM CTAB solution were added in a disposable pipet packed with the 6-AQ tagged reagent (10-20 mg). The pipet was kept at 70^0 C for 10 min. After derivatization, the solid reagent was washed with 1.0 ml of 70% ACN-H_2O, and an aliquot of the solution was injected into the HPLC system.

Trypsin Digestion of Cytochrome c.

A solution of 2 mg/ml cytochrome c (cyt c) (horse heart) was prepared in 100 mM, pH 8, ammonium bicarbonate buffer. Trypsin-TPCK was dissolved in the same buffer at a concentration of 0.1 mg/ml. An aliquot of 0.5 ml of the trypsin solution was added to a

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digestion vial which contained 0.5 ml of the cyt c solution. After vortexing, the vial was incubated at 37^0 C for 24 h, and the digestion was terminated by heating the solution at 100^0 C for 5 min [6]. The digested solution was lyophilized and reconstituted with 1.0 ml H_2O, and 50 µl of this solution was derivatized with 20 mg of the polymeric reagent.

Derivatization of Proteins With Solution and Solid Phase Reagents.

A 2 mg/ml GHRF solution was prepared by dissolving 1 mg GHRF in 0.5 ml H_2O. In order to prevent precipitation of GHRF derivatives, a surfactant such as SDS, CTAB, or Brij 35 was used in the derivatization. For solution reagent derivatizations, 60 µl borate buffer (0.2 M, pH 8.8), 20 µl surfactant solution (20%), and 10 µl or 20 µl GHRF solution were first mixed in a 55 x 6 mm glass tube. A portion of different volumes of 6-AQC solutions (3 mg/ml or 0.3 mg/ml) were added and vortexed quickly. The reaction solution was kept at room temperature (r.t.) before being injected into the HPLC system. When necessary, the solutions were incubated in a water bath at 50^0 C for 10 min to hydrolyze the phenolic esters of 6-AQ derived from Tyr. The derivatizing procedure for insulin and glucagon was nearly the same as for GHRF, except surfactants were sometimes omitted.

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For derivatizations with the immobilized reagent, 25 µl borate buffer, 25 µl surfactant solution, 25 µl ACN, and 25 µl protein solution were mixed, then put into a disposable reaction cartridge containing the polymeric reagent. The mixture was vortexed at r.t. After the reaction, the slurry was filtered through a glass wool cartridge and washed with 200 µl ACN/H2O, 70/30, v/v. A portion of the solution was injected into the HPLC system. When desired, the solution was incubated at 50^0 C for 10 min before injection.

Chromatographic Separations.

The HPLC gradient system consisted of a Waters Model 6000 pump, a Waters Model 501 pump, a Waters Model 660 Solvent Programmer, a Waters Model 420 Fluorescence detector (254 nm/395 nm, ex/em) (Waters Corporation, Milford, MA, USA), a Rheodyne Model 7125 injector (Rheodyne Corporation, Cotati, CA, USA), and a Spectra Model 100 UV detector (Thermo Separation Products, Fremont, CA, USA). Data were collected by a Linear recorder (Linear Laboratories, Fremont, CA, USA), a HP 3394A integrator (Hewlett Packed Corporation, Palo Alto, CA, USA), and a Macintosh SE 20 computer (Apple Corporation, Cupertino, CA). For the separation of AA and small peptide derivatives, a YMC ODS column (5µm, 300 Å, 250 x 4.6 mm i.d.) (YMC, Inc., Wilmington, NC, USA) was used. Gradient elution conditions for the

AA derivatives involved: initial mobile phase at 100% A, and B was linearly increased to 30 % over 30 min. For the elution of small peptide derivatives, B was linearly increased to 40% over 30 min. In the separation of protein derivatives, a Delta Pak C4 column (5μm, 300 Å, 150 x 3.9 mm) (Waters Corporation) was used. Gradient elution conditions for tagged proteins involved: initial mobile phase at 30% C, and D was linearly increased to 80% over 40 min. All separations were performed at r.t. Specific detection conditions are indicated in the figure legends.

Preparation and Usage of an Immobilized Antibody (Ab) HPIAC Column for Recovery of Tagged, Bioactive GHRF.

a. Development of GHRF Ab HPIAC Column (Streptavidin-Biotin Linkage)

We have chosen a streptavidin-biotin type linkage of the Ab to support for the HPIAC Ab purification columns. This was prepared by taking 10 mg streptavidin (Prozyme, Inc., El Cerrito, CA, USA) and dissolving this in 2 ml phosphate buffered saline (PBS), pH 9.0 (100 mM sodium phosphate, 150 mM NaCl, pH 9.0). To this solution, 0.5 g POROS epoxide support (PerSeptive Biosystems, Inc., Framingham, MA, USA) was added. The slurry was well mixed, and 2.5 ml of 2 M Na_2SO_4 in PBS (pH 9.0) was then added. The mixture was shaken gently at r.t. for 22 hrs. After this time, the support was washed in a Buchner funnel with 5 x 100 ml PBS (pH 7.4) (10 mM sodium phosphate, 150 mM NaCl, pH 7.4), and resuspended in 2.5 ml PBS (pH 7.4) with 0.1% NaN_3. A portion of this support was packed into an ID cartridge (PEEK, 2.1 mm i.d. x 30 mm) (PerSeptive Biosystems).

For the biotinylation of the purified Ab, 0.5 mg NHS-LC-Biotin II (Pierce Catalog and Handbook, NHS-LC-Biotin II Technical Literature, Pierce Chemical Company, Rockford, IL, USA) was dissolved in 0.5 ml DMF. Of this solution, 10μl was added to a centrifuge tube that contained 150 μg of the purified GHRF monoclonal Ab (mAb) in 300 μl 50 mM $NaHCO_3$ buffer, pH 8.5. The mixture was incubated at ice temperatures for 2 hr. The unreacted biotinylation reagent was removed by centrifuging the product using a Centricon-30 Microcon (Amicon Corporation, Danvers, MA, USA). After centrifugation, the sample was diluted in PBS (pH 7.4) buffer and again centrifuged. After this process was repeated a total of three times, the biotinylated Ab was immobilized onto the packed ID cartridge prepared above.

b. HPIAC of Derivatized GHRF Products.

An HPLC system was used for the HPIAC studies of previously tagged proteins. Derivatized GHRF was injected onto the Ab column, which was first equilibrated with a loading buffer (PBS, pH 7.4). After immobilization, the column was washed with

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loading buffer until a stable baseline was obtained. An eluting buffer was then passed through the HPIAC column (150 mM NaCl, titrated to pH 2.0 with HCl), and the elution buffer was continued until the GHRF derivatives were fully eluted (as evidenced by UV/FL detection). The eluted derivatives were collected and then analyzed separately by reverse phase HPLC. The gradient elution HPLC conditions used a mobile phase A of PBS, pH 7.0, a mobile phase B of ACN/H_2O, 80/20 (v/v), and initial conditions of 30% B. B was linearly increased to 80% over 30 mins for gradient elution, and FL detection utilized 254nm/395nm, excitation/emission wavelengths (ex/em).

Results and Discussion

The solution, 6-AQC reagent is known to be very effective for derivatization of AAs, providing very high FL sensitivity in HPLC [8]. Based on the literature, we have synthesized and evaluated a polymer based reagent with a benzotriazole linkage containing the same 6-AQ tag with a carbamate linkage to the polymer. This has been used for derivatization of amines and AAs (Scheme II) [7]. However, for practical applications, our prior results were not adequate, because of a low loading capacity on that first synthesized reagent. That original material, though identical in structure to that prepared in this work, involved a slightly different synthetic scheme [7]. We have previously discussed, as have others, the various synthetic approaches possible to prepare polymeric, carbamate reagents involving various tags [19-20].

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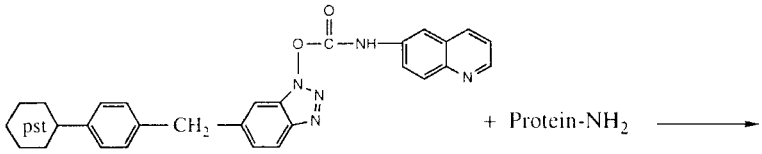
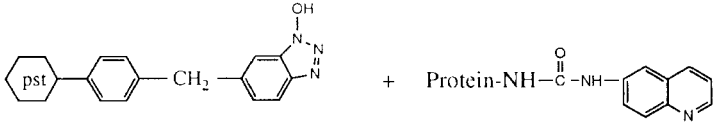
In part, this work was designed to develop a better approach for the synthesis of this same polymeric reagent, Scheme I. By using 6-AQ and triphosgene as the starting materials, this newer procedure for the synthesis of the intermediate quinolyl-6-isocyanate became much simpler than that previously described [7], and fewer side reactions or products were present. A peak at 2225 cm^{-1} in the IR spectrum (Experimental) clearly showed the existence of an isocyanate (-NCO) type structure, Figure 1. Using the actual quinolyl-6-isocyanate reagent, instead of that from rearrangement of the quinolyl acyl azide [7], to react with the solid phase benzotriazole intermediate, a final polymeric reagent was obtained with high loading of tag and few adsorbed impurities, Scheme I.

The method for the determination of loading capacity involved HPLC-UV quantification, as described elsewhere [6-7]. When using such a base promoted hydrolysis method, authentic 6-AQ was used as the external standard. The loading capacity of the 6-AQ tag for this reagent was found to be 0.45 mmol/g, which was sufficient for most polymeric derivatization reactions [5-7, 19-20]. This loading was



6-AQC Tagged Solid Phase Reagent



Derivative of Protein

Scheme II. Derivatization of proteins with the polymeric reagent.

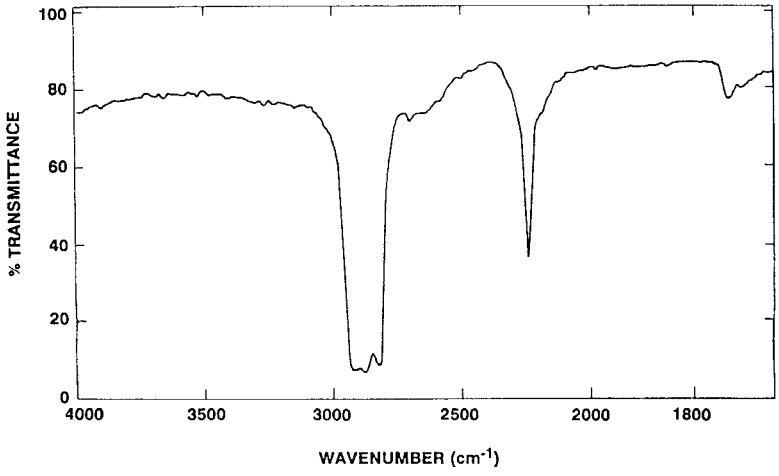


Figure 1. IR spectrum of quinolyl-6-isocyanate.

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also higher than what we previously reported for the first synthetic approach (viz., 0.18 mmol/g) [7].

As has been reported [6-7], this reagent can be stored for a relatively long period of time without affecting its reactivity. The previous work showed that a >1 month stored reagent, without any special protection, had almost the same derivatization yields as a freshly prepared material [7]. However, several decomposition peaks were observed in those chromatograms, when using a stored reagent for off-line derivatizations. As these can interfere with analyte derivative peak(s), it was desirous to both increase the loading of the 6-AQC tag on this newly prepared polymeric reagent, and also to reduce the number and amounts of physically adsorbed impurities that could lead to chromatographic interferences. With the current synthesis protocol, these desirable goals have now been realized.

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Reactivity of AAs and Peptides with the Solid Polymeric 6-AQC Reagent. The off-line derivatizations of AAs and peptides with the 6-AQC tagged polymeric reagent were performed under the same optimized conditions used for the 9-FA tagged polymeric reagent [6]. For quantification of the derivatization efficiency, a calibration curve of the authentic standard derivative of phenylalanine, viz., 6-AQC-L-Phe, was used. The preparation and characterization of this authentic, external standard have been reported elsewhere [21].

The derivatization efficiency was largely affected by the amount of polymeric reagent (mass) used in the derivatization reaction. When 10 mg of reagent was used for the derivatizations, percent derivatizations for Ala, Met, Leu, and Phe were 19.7, 42.9, 54.7, and 74.6%, respectively. When 20 mg of reagent was used, reaction yields of these AAs were: 63.0 (13), 64.8 (9.3), 87.9 (5.5), and 94.3 (5.1), respectively. The data represent the average value and, in parentheses, percent relative standard deviation (%RSD, n=3). These results were obtained using gradient elution HPLC with UV detection, Figure 2. Figure 2 also compares the same derivatized mixture of AAs by both UV and FL modes. If even more of the reagent were used, one might obtain higher derivatization efficiencies, but this was not attempted. The more hydrophobic AAs, such as Phe, not only provided higher derivatization efficiency, but also better reproducibility in their reactions with polymeric reagent, as previously observed [6-7]. Though we have previously reported reactions of typical AAs with the earlier prepared polymeric 6-AQC reagent, the current results represent a substantial improvement in terms of conversion efficiencies and overall chromatographic performance, Figure 2 [7].

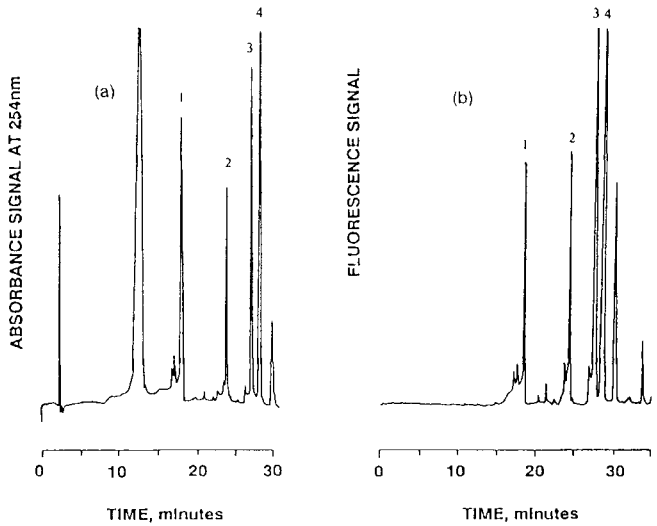

Figure 2. Chromatograms of the polymeric reagent derivatization of an amino acid mixture of Ala, Met, Leu, and Phe (1-4). HPLC conditions as indicated in Experimental. (a) UV detection at 254 nm; (b) FL detection at 254/395 nm, ex/em.

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The derivatization of small peptides containing different numbers of AAs was also performed with this polymeric reagent. The yields of derivatization were all >60%, except for Gly-Ala, a highly hydrophilic dipeptide. Chromatograms for mixtures of these peptides are not described here [21]. Its percent derivatization was only [average (%RSD), n=3] 47.3 (2.3), while others were: Gly-Val: 65.4 (3.6), Ala-Phe: 73.8 (4.0), Phe-Gly: 60.5 (2.4), Leu-Phe: 72.3 (6.6), Phe-Gly-Gly-Phe amide: 71.6 (1.9), Trp-Ala-Trp-Phe: 79.4 (7.5). Again, the more hydrophobic the peptide, the higher the derivatization yield obtained.

Derivatization of Enzymatically Digested Protein.

Trypsin digested cyt C was chosen as an illustrative example of a peptide digest mixture. After derivatization, the derivatives of these peptides were separated on a gradient elution HPLC system (Experimental). There were several major peptide derivatives, Figure 3a-b. Resolution of the 6-AQC tagged peptides was not fully optimized. These initial, qualitative results again demonstrated that peptides from a digested protein can be derivatized with polymeric reagents [6]. We did not previously

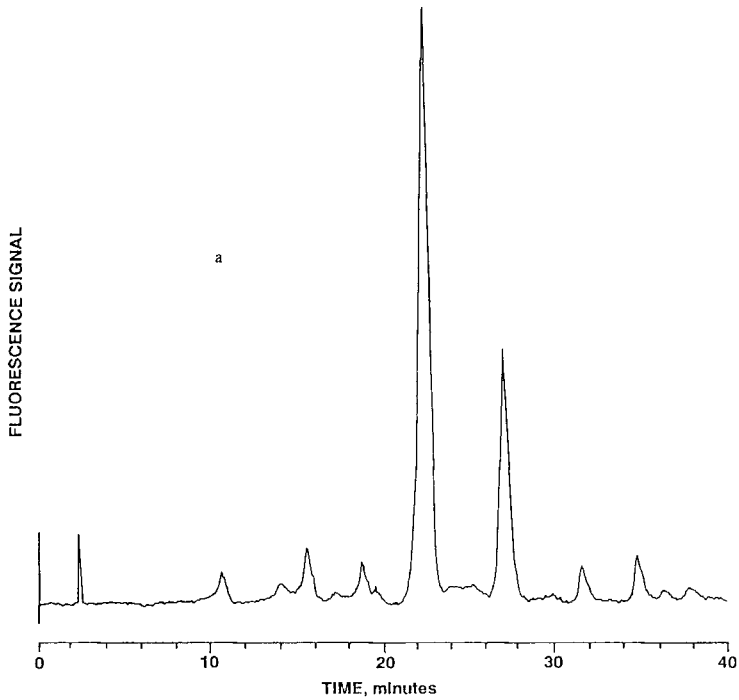

Figure 3. Chromatograms of the polymeric reagent derivatization of a peptide digest derived from cyt C. HPLC conditions as indicated in Experimental. Detection: Fluorescence 254/395 nm, ex/em. (a) blank; (b) enzyme digested cyt c derivatives.

(continued)

demonstrate peptide tagging with any polymeric 6-AQC reagent [7]. Each peptide from the current digest may not have been 100% derivatized. Based on the above results with standard, known peptides, it appears these peptide digests may show different percent conversions. These results may be of a more qualitative use for peptide mapping, as opposed to the quantitative conversion of AAs and peptides when using a solution 6-AQC reagent [8, 22-23]. This is a general disadvantage when using a hydrophobic, immobilized reagent for tagging peptides [1].

Derivatization of Proteins with Solution and Solid Polymeric Reagents. Generally, derivatization with an electrophilic reagent may result in the attachment of

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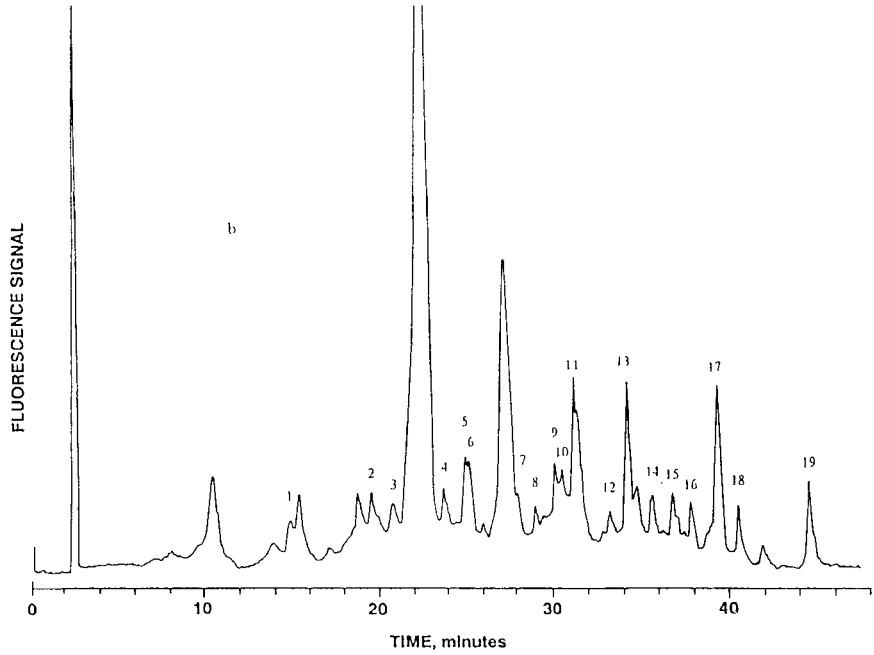

Figure 3 (continued)

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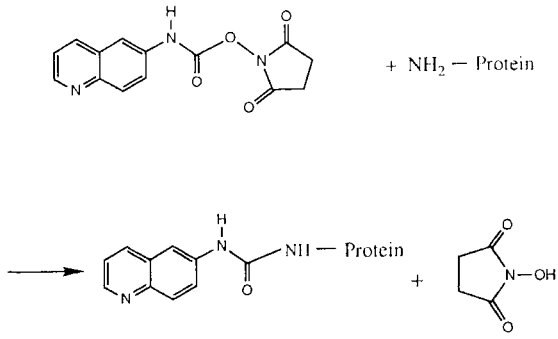
the tagging groups to all amino, thiol, and hydroxyl groups on proteins, such as amino terminus and Lys side chains, phenol on Tyr, thiol on Cys and hydroxyl on Ser and Thr [8-10]. However, the chemical reactivities and final stabilities of these functional groups differ. Most reagents neither discriminate between these groups to get selective tagging, nor completely derivatize each and every possible site/group. There are some SH selective reagents, as well as some that will be selective towards Ser and Thr [9-10].

In addition, the derivatives formed from certain functional groups are unstable; they may hydrolyze simultaneously with formation or slowly thereafter [8]. Usually, the derivatization of proteins yields multiple products, which may lead to questionable identification of the original protein(s) and increased (elevated) DLs. Such results detract from the application of precolumn, solution derivatization of proteins for the purpose of enhancing detection, in any separation method (HPLC, HPCE).



Scheme III. Derivatization of proteins with the 6-AQC solution reagent.

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Solution 6-AQC can react with several nucleophilic centers, such as amino and phenolic groups in proteins, Scheme III. The urea products derived from amine derivatizations are quite stable, which makes the derivatization of amino groups highly efficient and reproducible [8-10]. However, the derivatization of phenolic group on Tyr is often incomplete, and the carbamate structure derived from phenol derivatization is usually unstable [8-10]. This structure slowly hydrolyzes at r.t. As shown in Figure 4, multiple products were observed at r.t from the derivatization of insulin, glucagon, and bovine GHRF. This was the case even when these reactions were performed under conditions of high molar ratio of reagent to protein. The products formed could be simplified if the derivatization was performed at a higher temperature, or if the r.t. reaction products were subsequently heated to the same higher temperature (50^0 C), since hydrolysis of the phenolic carbamates present was then accelerated.

A single tagged product (one HPLC peak) was obtained for each of these standard proteins by incubating the reaction mixture at 50^0 C for 10 min, Figure 5. Further studies (see below), using different stoichiometric ratios of reagent to protein, have shown that this single peak (product) probably corresponded to a derivative in which all of the free amino groups (terminal and Lys side chains) were completely derivatized but no phenolic group remained tagged. We do not currently believe that reactions at the higher temperature inhibit the Tyr reaction, but rather that any adduct once formed will rapidly hydrolyze under these conditions ($t_{1/2}$ Tyr <6 min. at 50^0 C) [24].

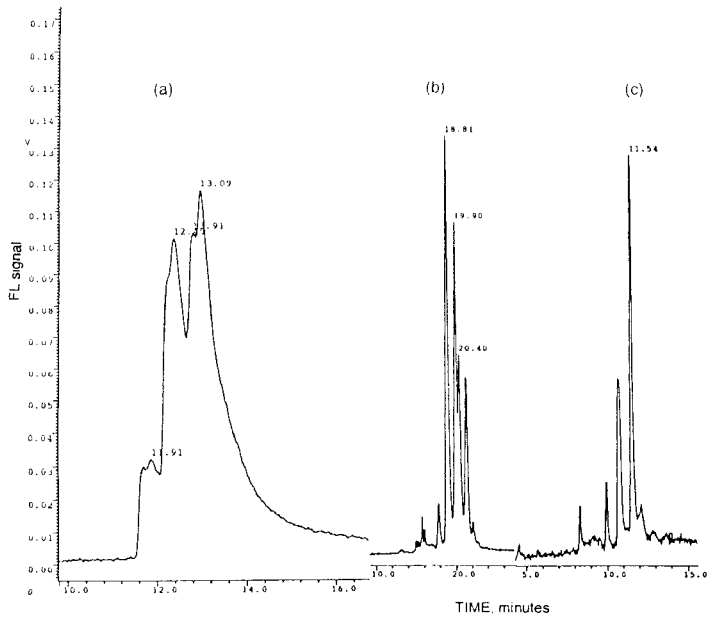

Figure 4. Chromatograms of protein derivatives. Reaction conditions: 10 µl of protein (1 mg/ml), 70 µl borate buffer (0.2 M, pH 8.8), and 20 µl 6-AQC solution reagent (3 mg/ml), mixed at room temperature and incubated for 10 min. For GHRF, another 20 µl CTAB (20% in water) was added. HPLC conditions are indicated in Experimental. Detection: FL 254/395 nm, ex/em.(a) insulin, (b) glucagon, (c) bovine growth hormone releasing factor (GHRF).

Mass spectrometric (MS) analysis of these protein products (Figures 4-5) has not, as yet, been attempted, in order to confirm the AA sites of tagging. Under the above reaction conditions, detection of proteins could be enhanced in HPLC and HPCE by precolumn derivatization with the solution 6-AQC reagent, Figure 5. This has been further demonstrated by the elevated temperature derivatization of insulin and GHRF. Picomole amounts of these proteins could be detected with FL, Figure 6. These particular HPLC-FL conditions were not fully optimized in terms of pH [8]. Ideal FL responses occur at neutral or slightly basic pH values, whereas the optimal HPLC conditions we have used were acidic, pH 5.0. It is likely that by operating at a more basic pH, perhaps by postcolumn pH adjustment, these minimum DLs could be further lowered [8].

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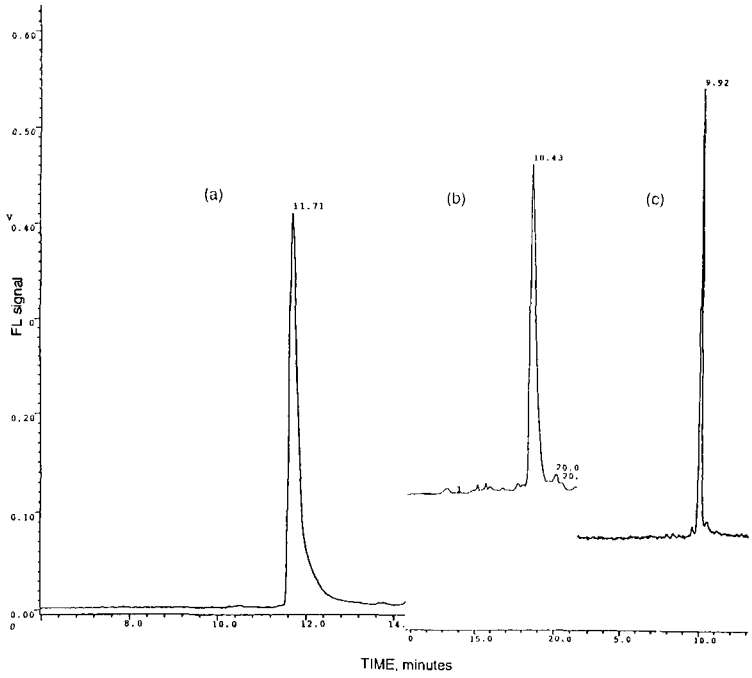

Figure 5. Chromatograms of protein derivatizations at elevated temperature. Reaction conditions are the same as in Figure 4, except the reaction solutions were incubated at 50^0 C over 10 min. (a) insulin, (b) glucagon, (c) GHRF. HPLC and detection conditions as in Figure 4.

Further experiments have shown that phenolic groups on Tyr can only be derivatized when the solution reagent is present in high excess. If the amount of the reagent was controlled at low levels, e.g., less than a 1/1 equivalence of free amino groups in the proteins, then the phenolic groups were not readily derivatized. Free amino groups are better nucleophiles than phenols, and thus should react more rapidly with the same, perhaps limited reagent. If there is a large excess of reagent, e.g., Figure 4, then most likely all amines and perhaps phenols are derivatized by the end of 10 mins. At a low molar ratio of reagent to protein, reactions at r.t and at 50^0 C yielded the same chromatograms (same number and types of products). This again suggested that no phenolic groups were being derivatized, and also that the derivatization of phenolic groups is much slower than amino, compatible with the literature [9-10].

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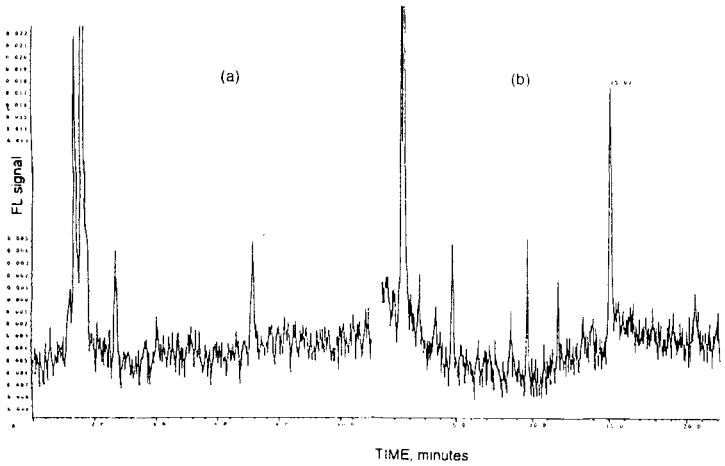

Figure 6. Determination of the detection limits of protein 6-AQC derivatives. (a) 10 ng (10µl x 1 ng/µl), about 1 picomole of insulin, (b) 20 ng (20 µl x 1 ng/µl), about 3 picomoles of bovine growth hormone releasing factor (GHRF). HPLC conditions: Experimental and mobile phase A consisted of 20 mM sodium acetate titrated to pH 5.0 with phosphoric acid, mobile phase B consisted of ACN/H_2O, 80/20, v/v. Gradient elution: B was linearly increased from 30% to 80% over 30 min. Detection: FL 254/395 nm, ex/em.

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The possible product differences became even clearer when comparing derivatizations between the solution 6-AQC (excess reagent/protein) and the 6-AQC tagged polymeric reagent. The reaction with the 6-AQC polymeric reagent often yielded fewer tags on the protein (compare Figures 4 vs. 7). These products were very similar to those obtained from the solution reagent derivatization when using a molar ratio of 1/1 (reagent to protein), Figures 7-9. These Figures 7-9 make direct comparisons of both the solution and polymeric reagent reaction products for three different proteins (insulin, GHRF, glucagon). These chromatograms represent both UV and FL responses to the tagged proteins present, and almost identical chromatograms were obtained by simultaneous UV/FL detection, other than for the presence (at times) of unreacted starting protein by UV (214 nm), as below. Untagged, starting proteins (except glucagon) showed no FL responses under these same HPLC-FL conditions. We chose 214 nm detection in the UV to ensure detection of all proteins and peptides, as well as any unreacted species.

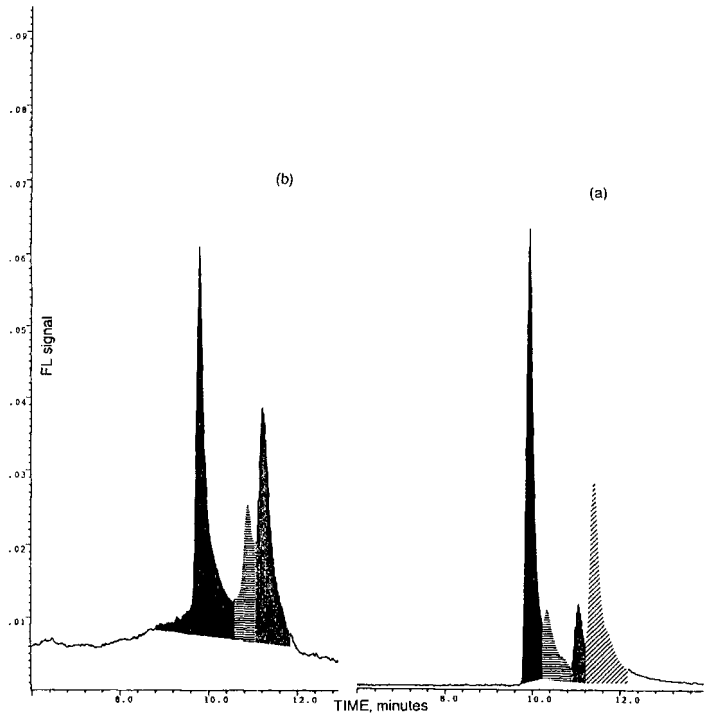

Figure 7A. Chromatograms of polymeric reagent vs. solution reagent derivatized insulin with FL detection. Reactions were performed at room temperature: (a) derivatized with 6-AQC solution reagent at a molar ratio of reagent to insulin of 1:1. (b) derivatized with polymeric reagent. HPLC conditions as in Figure 4. Peak shadings do not attempt to identify individual proteins.

In the case of insulin, Figures 7A-B, the shaded peaks represent those products derived just from tagging of the protein, after subtraction of the blank chromatograms (reagent blanks). Figure 7A (FL) shows no residual starting insulin, which does appear at a retention time of about 9.2 mins in Figure 7B (UV). Otherwise, the peak profiles are identical, which means that we are indeed seeing all of the tagged proteins formed by HPLC-UV/FL. These results should be directly compared with Figure 4, which represents products formed when the solution 6-AQC reagent was in large excess (Experimental). When the molar ratio of solution reagent/protein was

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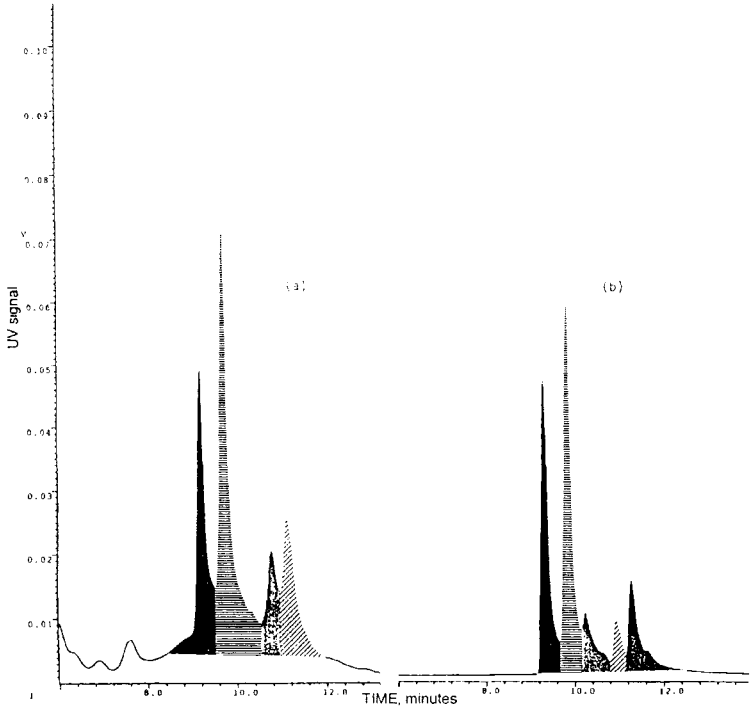

Figure 7B. Chromatograms of polymeric vs. solution reagent derivatized insulin with UV detection at 214 nm. Reaction conditions are the same as in Figure 7A. (a) derivatized with the 6-AQC solution reagent at a molar ratio of reagent to insulin of 1:1; (b) derivatized with polymeric reagent. HPLC conditions as in Figure 4. Peak shadings do not attempt to identify individual proteins.

closer to 1/1, the number of products observed was reduced in every case, Figures 7-9, to varying extents. Even though the polymeric reagent contained a large excess of available tags to protein present, far fewer products were formed than from the analogous, solution situation, Figure 4. This again suggested that not all of the potentially taggable sites on the proteins can reach the immobilized tags to be reacted, for example, phenolic groups on Tyr. Perhaps only those more active, exposed amino groups (N-terminal and Lys) were reacted by the polymeric, immobilized reagent. When the solution reagent was present in a limited amount (1/1),

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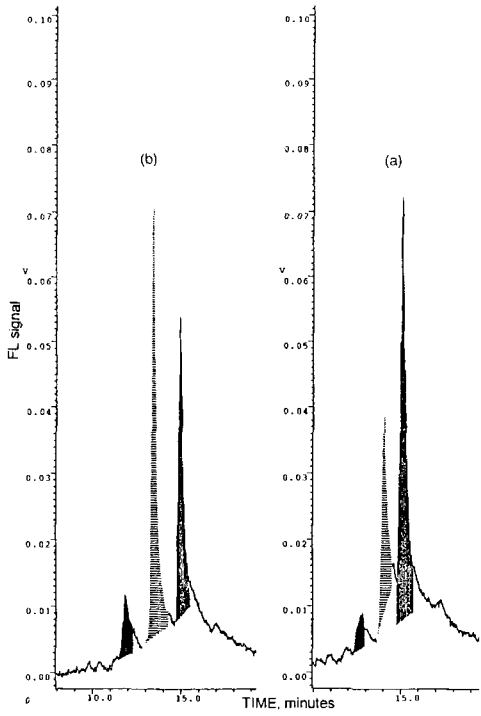

Figure 8A. Chromatograms of polymeric vs. solution reagent derivatized GHRF with FL detection. Reactions were performed at room temperature. (a) derivatized with polymeric reagent; (b) derivatized with the 6-AQC solution reagent at a molar ratio of reagent to GHRF of 1:1. HPLC conditions are the same as in Figure 4. Peak shadings do not attempt to identify individual proteins.

fewer products were formed (Figures 7-9) than in Figure 4, and those were most likely derived from N-terminal and Lys groups, for the above reasons.

In comparing, Figures 7A-B, solution vs. polymeric reagent for insulin tagging, though the products appeared to be the same, they were not formed in the exact same relative ratios (peak area comparisons). There were subtle, but seemingly real differences in the relative (not absolute) ratios of tagged products formed in going from solution to polymeric reagent. This may have been due to steric hindrance in the case of the polymeric reagent, obviously absent for the solution case.

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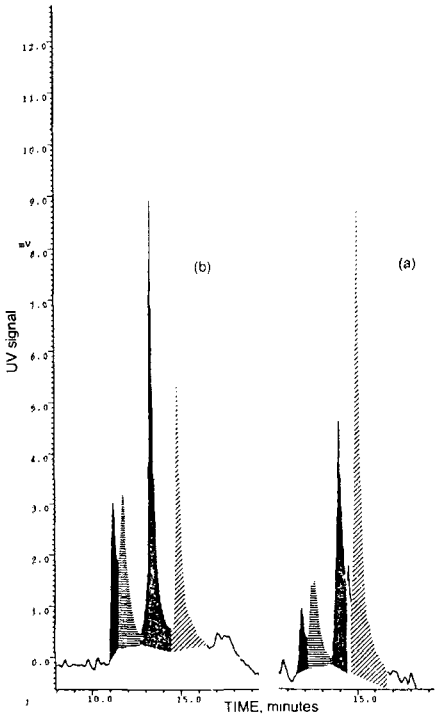

Figure 8B Chromatograms of polymeric vs. solution reagent derivatized GHRF with UV detection at 214 nm. Reaction conditions are the same as in Figure 8A. (a) derivatized with polymeric reagent, (b) derivatized with the 6-AQC solution reagent at a molar ratio of reagent to GHRF of 1:1. HPLC conditions are the same as in Figure 4. Peak shadings do not attempt to identify individual proteins.

There is a reversal of the relative ratios of formation of the two products coming from GHRF, Figures 8A-B. Again, Figure 8A (FL) does not show the presence of unreacted GHRF, but Figure 8B (UV) clearly shows this additional peak at a retention time of about 10.4 mins, just before the first tagged GHRF peak, also present (reduced) in Figure 8A (FL). As expected, all of these hydrophobically tagged peptide/protein peaks elute later than underivatized starting proteins.

In the case of glucagon, Figures 9A-B, there is again a difference in the relative ratios of products. This still suggests some degree of site selectivity, which could/should vary

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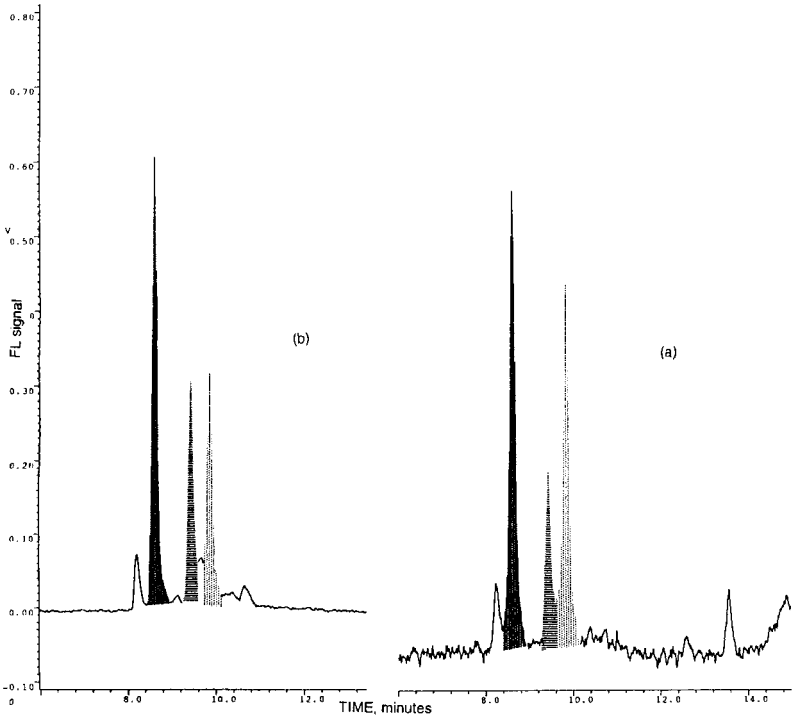

Figure 9A. Chromatograms of polymeric vs. solution reagent derivatized glucagon with FL detection. Reactions were performed at room temperature. (a) derivatized with polymeric reagent; (b) derivatized with the 6-AQC solution reagent at a molar ratio of reagent to GHRF of 1:1. HPLC conditions are the same as in Figure 4. Peak shadings do not attempt to identify individual proteins.

from protein to protein, and may also be reaction conditions dependent. Figure 9A (FL) illustrates the presence of three tagged products, with unreacted glucagon eluting at about 8.0 mins in both Figures 9A and 9B. Separate injections of unreacted, starting protein were injected for all proteins under these very HPLC-UV/FL conditions to ensure identification of the untagged protein peak. There is a small FL response for what appears to be the untagged glucagon peak in Figure 9A, which is considerably larger relative to the tagged products by UV in Figure 9B. This may be due to the presence of Trp in the individual, respective proteins.

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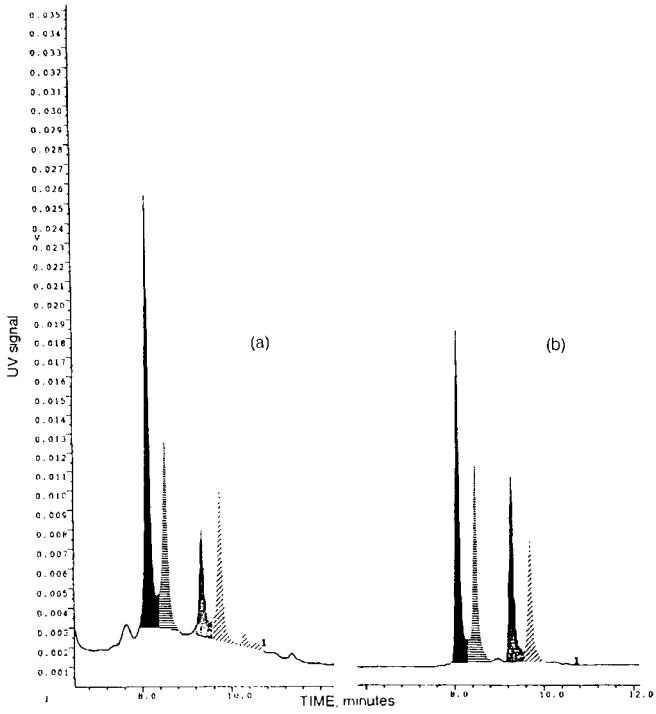

Figure 9B Chromatograms of polymeric vs. solution reagent derivatized glucagon with UV detection at 214 nm. Reaction conditions are the same as in Figure 9A. (a) derivatized with polymeric reagent, (b) derivatized with the 6-AQC solution reagent at a molar ratio of reagent to GHRF of 1:1. HPLC conditions are the same as in Figure 4. Peak shadings do not attempt to identify individual proteins.

Polymeric Reagents With Outer Tags First Removed; Possible Selective Tagging of Specific Sites on Proteins.

These particular polymeric reagents had tags on their outer surface and within the more restricted pores [20, 25]. Because the polymer support used for preparation of these reagents was made from a controlled pore silica substrate using a template polymerization method, the final reagents contained a narrow, fixed pore size, which was reaction solvent dependent [25-26]. We have now shown that the size of these pores changes with the reaction solvent, and that a size selectivity occurs with

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different sized amine type analytes [26]. It is entirely possible that if the outer surface tags were first removed with a large, bulky amine, leaving a polymeric reagent with tags only on the inner pore surfaces, reaction selectivity towards proteins will be more pronounced and obvious. These studies are now in progress [26].

The incomplete reaction of amino groups, with the current polymeric reagent, may be due to steric hindrance restricting access to functional groups within the protein's interior. This may only allow the functional groups on the outer surface of the proteins to be tagged. This could be useful for keeping the protein derivatives bioactive. Such studies are now in progress, as below.

The 6-AQC tagged polymeric reagent seems to be less chemically reactive than the solution reagent, since the same products were obtained from reactions first performed at room temperature or then filtered and heated to 50^0 C. We may conclude that the phenolic groups in these proteins cannot be readily derivatized with the immobilized reagent. This result is reasonable given the reagent's structure and perhaps accessibility of Tyr sites in the proteins. The bond (carbamate) holding the 6-AQ tag in the polymeric reagent is perhaps less reactive than that in the analogous solution reagent, because the activating groups [benzotriazole (polymeric) vs. N-hydroxysuccinimide (solution)] are different. There are basic differences in the overall, solution reactivities of these functional groups, combined with the fact that one reaction is in a homogeneous solution and the other on a heterogeneous support. In general, solution reactions using the same labile linkage are kinetically faster than the analogous, immobilized reaction, just because of the number of effective collisions per unit time with the same analyte [15]. Also, carbamate linkages in solution reagents tend to be less labile or reactive than activated esters (NHS) [19-20].

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It is therefore more difficult for this tagging group on the polymeric reagent to react with weaker nucleophilic centers, such as phenolic groups. There is also the further issue of accessibility of the reactive sites on the polymeric vs. solution reagents to perhaps buried phenolic sites in the protein.

Effects of Surfactants on Derivatizations of Proteins, Bioactive vs. Bioinactive, Tagged Protein Products.

We have also undertaken preliminary studies with surfactants to assess their effect on product distribution and reactivity with both solution and polymeric reagents. Surfactants have some effect on protein derivatizations with the polymeric reagent. This effect was compared by using GHRF as a standard protein example. The neutral

surfactant Brij 35, cationic surfactant CTAB, and anionic surfactant SDS, were tested with the polymeric reagent. The reaction with Brij 35 had the lowest yield of products, SDS was better than Brij 35, and the reaction with CTAB had the highest yield of tagged products. These results agreed with those obtained for typical AAs using an analogous polymeric 9-FA reagent [6]. Since the reaction was performed in a basic buffer, where most proteins were negatively charged, cationic surfactants could form ion pairs with proteins, and enable them to penetrate the hydrophobic surface of the polymeric reagent for subsequent reaction. SDS could not form ion pairs with proteins under the same conditions. Brij 35 is a hydrophilic surfactant, and it would protect proteins from approach to the surface of the polymeric reagent to some extent.

The presence of such surfactants with solution reactions has also been initially investigated. At a high molar ratio of reagent to protein, no significant differences were observed with or without surfactants present. All the amino groups in the protein must have been fully derivatized, though in the one case studied, the final products were not recognized by their antibody (Ab) (GHRF) (not bioactive). At a lower ratio of solution reagent to protein, the products were still not bioactive if SDS and CTAB were present in the reaction solution. When the derivatization of GHRF was done at a molar ratio of about 1/1 with 0.5% Brij present, the final tagged products were now bioactive. They could now be recognized and captured by their N- or C-terminal Ab high performance immunoaffinity column (HPIAC)(Experimental). Figure 10 illustrates the RPC chromatograms of those solution tagged proteins first captured (b, c) on an immobilized Ab column, as indicated. Figure 10a illustrates, again by RPC, the total mixture of tagged products, only some of which are recognized on the two different immunoaffinity columns (N- and C-terminal Abs to GHRF). Apparently, there are but three tagged GHRF products formed under these particular, solution reagent, Brij conditions, Figure 10a. Only one of these is being recognized by the C-terminal Ab column, Figure 10b. All three tagged proteins; however, are recognized to differing degrees by the N-terminal Ab column, Figure 10c, when compared with Figure 10a.

There remains a significant problem in tagging of proteins by solution reactions, perhaps exemplified by the recent works of Schultz and Kennedy, and Banks and Paquette [11-12]. In such studies, using conventional solution reagents for preparing tagged, bioactive proteins, multiple products are formed, as a function of the reaction conditions (concentration, stoichiometric ratios, temperature, time, etc.). These products can have several tags per protein molecule, making such species problematic as part of an immunoassay or HPLC/HPCE-detection schemes. Still

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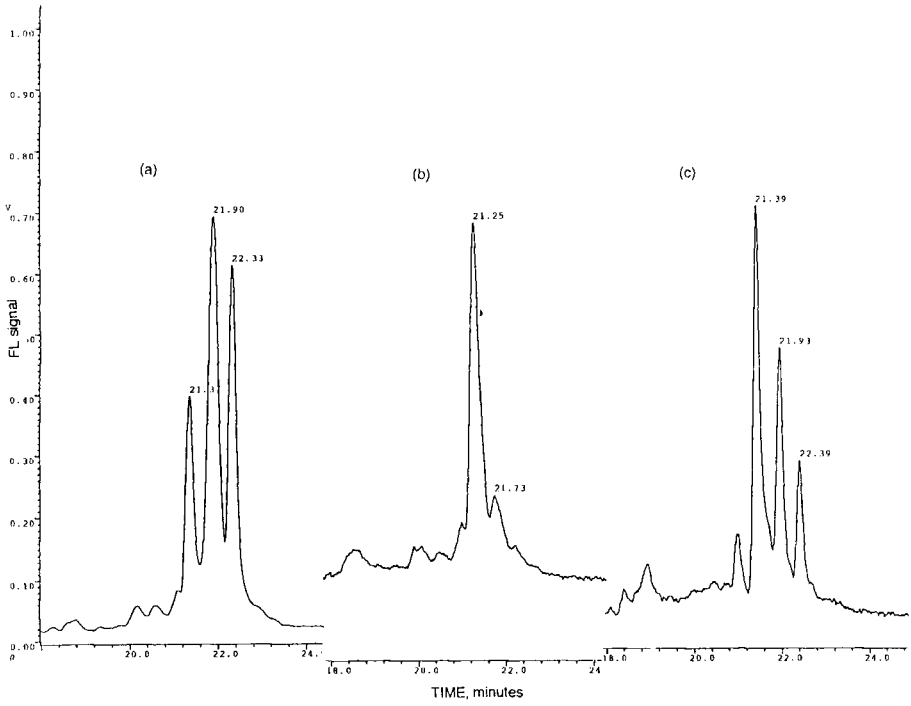

Figure 10. RPC chromatograms of solution tagged 6-AQC derivatives. Reaction conditions used a small amount of Brij present, leading to bioactive, tagged GHRF products. a) total reaction products without initial affinity isolation; b) products first captured by C-terminal Ab column to GHRF, then assayed by RPC; c) products first captured by N-terminal Ab column to GHRF, then assayed by RPC. HPLC conditions: mobile phase A was PBS 7, mobile phase B consisted of ACN/H2O,80/20, v/v. Initial at 30% B, and B was linearly increased to 80% over 30 min in gradient elution. Fluorescence detection: 254 nm/ 395 nm, ex/em.

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today, there are no simple approaches for the preparation of a single, controlled tagged, bioactive protein, except in certain cases where one deals with small peptides or polypeptides. The above approaches address these issues and hopefully will permit us to eventually control reaction conditions leading to a single, tagged, and bioactive protein suitable for analytical applications and determinations.

Identification of 6-AQ Tagged Proteins by HPLC-UV/FL.

A need exists to discuss the differences in retention (elution) times for the variously tagged proteins, for example GHRF, Figures 4-5, 8, 10. In not knowing specific conformations for these tagged species, nor the exact number of sites of tagging in the products, it is impossible to correlate HPLC retention or elution times. It is not clear that we are indeed looking at the exact same tagged species in comparing, for example, Figures 4-5 and 8-10. These may very well be quite different species, which would explain their somewhat different elution times in HPLC. It is possible that we are also dealing with different conformations for these tagged species, concomitant with differences in tagging levels and/or sites. Hence, to correlate their chromatographic properties, at the moment, without knowing more about specific conformations and levels/sites of tagging is problematic. It is clear that further understanding of the exact nature of these tagged species will require extensive MS analyses, work contemplated for the future.

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Conclusions

An improved method for the synthesis of a styrene-divinylbenzene based polymeric 6-AQC reagent has been developed. The obtained polymeric reagent was successfully used for the derivatization of AAs and peptides. The efficiency of derivatization and improved HPLC detectability (UV/FL) of these derivatives makes this reagent attractive for the qualitative analysis of AAs and small peptides. Such newer derivatization methods may be applied for quantitative analysis, in view of their high reproducibility.

The derivatization of proteins with the polymeric reagent usually yielded fewer or altered ratios of tagged products in comparison with the analogous solution reagent. Some bioaffinity sites may be blocked in this way, affecting biorecognition. In general, forming a single, tagged product with bioactivity is useful for enhancing detection of proteins in affinity chromatography (HPIAC). The derivatization of proteins at elevated temperatures yielded a single product when using the 6-AQC solution reagent, but such products were usually bioinactive towards their antibodies (data not shown). These reaction approaches can now be used for precolumn derivatization in reverse

phase HPLC or HPCE, in order to improve the general detection of proteins. Under optimized conditions, picomoles of proteins could be detected with FL, depending on the final number of 6-AQ tags present per protein molecule.

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