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Review

Labeling reactions applicable to chromatography and electrophoresis of minute amounts of proteins

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Abstract

Chromatography and electrophoresis have become extremely valuable and important methods for the separation, purification, detection and analysis of biopolymers and HPLC/HPCE may become the premier, preferable approaches for both qualitative and quantitative analyses of most proteins, especially from recombinant materials. This includes smaller peptides, polypeptides, proteins, antibodies and all types of protein or antibody-conjugates (antibody–enzyme, protein–fluorescent probe, antibody–drug and so forth). This entire Topical Issue of Journal of Chromatography emphasizes the application of chromatography and electrophoresis to protein analysis. This particular review deals with approaches to the selective tagging or labeling of proteins at trace (minute) levels, again using either chromatography or electrophoresis, with the emphasis on modern HPLC/HPCE methods and approaches. We discuss here both pre- and post-column labeling methods and reagents, techniques for realizing selective labeling of proteins or antibodies, applicable approaches to protein preconcentration in both HPLC and HPCE areas and in general, methods for improving (lowering) detection limits for proteins utilizing chemical or physical derivatization and/or preconcentration techniques. There are really two major goals or emphases in that which follows: (1) methods for selective labeling of proteins prior to or after HPLC/HPCE and (2) labeling of proteins at trace levels for improved separation–detection and lowered detection limits. We discuss here a large number of specific references related to both pre- and post-column/capillary derivatizations for proteins, as well as methods for improved detectability in both HPLC and HPCE by, for example, analyte preconcentration on a solid-phase extractor or membrane support, capillary isotachophoresis and other methods. Selective reactions or derivatizations on proteins refers to the ability to tag the protein at specific (e.g. reactive amino sites) in a controlled manner, with the products having the same number of tags all at the very same site or sites. The products are all the same species, having the same number of tags at the same locations on the protein. Selective reactions can also refer to the idea of tagging all of the protein sample at only a single, same site or at all available sites, homogeneously.

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1. Introduction and background to importance of labeling reactions for minute amounts of proteins

In recent years, we and others have described in various review papers, original research articles and book chapters numerous approaches for the pre- and post-column (capillary) derivatization of peptides and proteins [1–28]. With a wide variety of separation modes developed and optimized, the final qualitative and quantitative analysis for proteins has become commonplace and widely applied [29–53]. The separation issue appears to have been adequately addressed by this point in time, especially given the significant separation advances made in various HPCE modes, including capillary zone (CZE) or free solution CE (FSCE), micellar electrokinetic chromatography (MECC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE) and others. With these available modes, together with all of the modern HPLC and HPCE separation modes fully optimized for proteins of all types and varieties, separations abound, but trace detection is scarce.

This Topical Issue of Journal of Chromatography (B) deals with chromatographic and electrophoretic methods in protein analysis, which includes separation, detection, identification, quantitation and method validation for proteins from all types of complex sample matrices. This particular chapter/review deals with novel derivatization approaches for proteins, applicable to both HPLC and HPCE, pre- and post-separation, for eventual trace analysis/detection and absolute quantitation or quantification. Thus, we shall emphasize mainly solution and solid-phase derivatizations that have been described and utilized in HPLC and/or HPCE, as well as unique derivatization approaches for post-column or post-capillary (HPCE) tagging, such as on-line photolysis, microwave digestion, immunodetection (ID) and so forth. We will, at various times, use the same words to denote a chemical modification of the original protein, which could be tagging, derivatization, modification or others. These are all meant to indicate the addition of one or more reagents to the original protein backbone, yielding one or more derivatives. Analogously, non-chemical approaches to protein derivatization, such as photochemical, radiolytic, thermal, X-ray and others, without the use of actual chemical reagents, usually also yield one or more derivatives.

There are basically two different mechanisms or pathways for protein modifications: (1) the addition of another reagent somewhere to the protein chain (or at the carbohydrate sites of antibodies) or (2) a molecular rearrangement of atoms somewhere within the protein (induced by thermal, photochemical, radiolytic or other methods). By and large, solution tagging approaches have been those most widely applied to proteins and peptides and much less has been explored by other routes in HPLC and HPCE for improved separation and/or detection [1–17,54,19,20]. That is, the majority of publications attempting to improve either resolution/separation and/or detection of proteins in chromatography or

electrophoresis have emphasized simple solution tagging approaches, homogeneous reactions performed just prior to injection-analysis, with little effort (with some notable exceptions, as below) expended to optimize selectivity and therefore sensitivity.

Why has this been the case and why is it that very little of practical value and utility has evolved that allows for routine precolumn/capillary tagging of proteins at trace levels for improved resolution and/or detection in either HPLC or HPCE? There are really two issues or points that need to be addressed here: (1) selective tagging of proteins, in general, leading to a single derivative with a single or multiple tags, at high yields with 100% conversion of the starting material/protein and (2) true trace derivatization of proteins at low levels, together with selective tagging with high conversion efficiencies. These could be self-contradictory goals, that is the need to convert proteins at very trace levels with 100% efficiency into a homogeneous (single) derivative with a uniform number and location (!) of tags. This last sentence perhaps says it all and is the general thrust of this review and where much research appears headed in the future. What is needed today in bioanalysis and analytical biotechnology, is a simple method to convert any and all proteins at trace levels, often in complex biofluids, into a single, homogeneous product having a fixed number of tags at specific locations (or all possible locations) and with high efficiency of such conversion and speed. Now we have introduced the concept of speed, since any derivatization that will take forever is of little practical utility. A problem arises in derivatizing proteins at very low levels and that is the speed or kinetics of these reactions become slower and slower, to the point of impracticality. Hence, one must always keep in mind the practical essentials of analysis, one of which is time.

Perhaps now we should discuss why protein tagging, pre-injection, is so more complicated than small molecule tagging reactions, which have been fully optimized and applied for several decades [5–7]. Derivatizations in chromatography and electrophoresis are often applied to improve (i.e., lower) detection limits and sensitivity, whether that be by UV, FL, EC or even MS. Small molecules usually have a single site of reaction and attachment and a single product is often formed in high yield. Thus, amino acids, amines, amino alcohols and other organic groups/compounds, are easily derivatized to a single, homogeneous product with one or more tags, in high yield and often purity. It is also easy to convert multiple amino groups, as in polyamines, into a single derivative with a constant number of tags per parent molecule, so that a single chromatographic or HPCE derivative peak occurs. That is, by and large, an ideal situation, which is perhaps why so many current methods in widespread usage in HPLC/HPCE utilize pre-injection derivatization for small molecules, especially low molecular mass drugs. Indeed, these techniques and approaches have become fully automated within the carousel or injection device in both HPLC and HPCE. Why then is the situation so different for peptides, proteins and antibodies and why is it that very few analysts bother to utilize solution tagging for such biopolymers still today?

The basic problem comes down to the chemical fact that proteins often have several sites of reactivity, especially amino groups, but also for thiols, phenols, aromatics, disulfides and so forth. These nitrogen groups can be N-terminal, heterocyclic, lysine derived, arginine derived, peptidic and so forth. In general, with most electrophilic reagents, it is the N-terminal and lysine free amino groups that compete for the reagent. These do not have the same reactivity and often other nucleophiles (thiols, phenols) can also react along with the free amino groups. For a simple protein like insulin that can have several different amino groups, this will lead to several different tagged products, singly tagged at different amino acid sites, ditagged, tritagged and so forth [21–24]. These are usually formed at different rates of reaction, with different efficiencies of conversion and in different yields. Thus, mixtures of products will arise, for almost all and any protein, size dependent, with the use of solution reagents. Though there have been some notable efforts to control the selectivity of these solution (homogeneous) reactions, by and large these have not been very successful. Thus, solid-phase synthesis can yield a single protein derivative, but this is totally impractical for real trace analysis of recombinant proteins in biofluids for pharmacokinetic or metabolic studies. Similarly, careful control of pH and the

ratio of solution reagent to protein analyte may, at times, also lead to fewer products having a uniform number of tags per protein backbone. These approaches are research efforts in and of themselves and are not truly rugged, robust, or routine for most applications. And, this is true for proteins present in samples at high concentrations or levels.

Now, the formation of multiple products is not in and of itself necessarily a bad result, if these products can be made to coelute in HPLC or HPCE. In that case, a single peak will result, hopefully resolved from other sample components and identification and quantitation can result [21]. One can therefore force the chromatography or electrophoresis to produce a single peak for what is really a mixture of protein derivatives. Alternatively, one can denature all of the protein derivatives prior to injection and also realize a single HPLC peak for what is really still a mixture of protein derivatives, only now eluting as a single peak [21]. This too will yield the desired result of a single HPLC/HPCE peak for what was a mixture of derivatives, which simplifies the final identification and quantitation of the original protein analyte.

However, in general, the formation of multiple products prior to HPLC and HPCE is highly undesirable, in that one usually obtains several peaks for the original protein. These numerous peaks then make it more difficult to identify the original protein on the basis of its retention times or detection characteristics. Also, quantitation of the protein suffers through the formation of numerous derivatives, since the original mass of protein is now divided amongst its tagged products, reducing the peak area/height of any one of these alone. There is yet a tremendous need to derive more selective tagging approaches for proteins and antibodies, so that the final separation–detection methods will indeed yield a single, well characterized and defined peak. Ideally, one would like to control where the reaction occurs, the efficiency and time and the conversion yield of the starting protein. This is what we mean by selective tagging of proteins or peptides— the formation of one homogeneous derivative having a constant number of tags per protein molecule, in a high conversion yield, in a short period of time and with efficient consumption of the reagent. It is also important to remember that if we are successful in placing a single derivative tag onto a single amino acid site on that protein, then we will not realize maximal detectability of the starting protein. Ideally, one would like to realize complete tagging of all possible sites (e.g, amino groups), to a single, homogeneous product, which will provide maximal detector response. The successful conversion of a starting protein to a single, fully tagged, homogeneous product is much simpler than it sounds on paper, because of the vast differences in reactivities of different amino (nucleophilic, taggable) sites on the protein. The perhaps simplistic approach of forcing reaction conditions to drive all of the starting protein to a single, fully tagged product, does not always work. This can be due to reduced reactivities, steric hindrance to reactions, protection by neighboring groups on the protein backbone and so forth.

One of the goals of this review is to describe how one can derivatize or tag a protein in a complex biofluid type matrix, for example, when that protein is present at meaningful, practical, trace levels. This is, of course, entirely different than tagging a standard, pure protein, at a high concentration in a neat buffer matrix. Unfortunately, most of the current literature is replete with demonstrations of our ability to tag proteins at high levels when they are pure in neat solutions and then dilute these derivatives several fold in order to demonstrate detection limits of the derivatives, but alas not for the original proteins at trace levels. In other words, it is one simple thing to dilute a derivative once formed from a pure protein at high levels and demonstrate its calibration plot and linearity. It is an entirely different matter to efficiently and rapidly tag a protein at trace levels, with chemical selectivity and uniformity, when that protein is in a complex biofluid type matrix. That is, the rates of reaction for proteins change and are drastically reduced, when their concentration is lowered and these rates can become so limiting that the entire approach becomes impractical and ineffectual. The bimolecular reaction for very low levels of analyte protein becomes essentially first order in the analyte and going to higher and higher concentrations of reagent does not increase the reaction rate of protein tagging.

Never mind that we cannot make the tagging reaction selective and uniform, we cannot now even make the reaction work efficiently on a timescale

that is practical and usable. As the level of protein falls, the number of effective collisions with reagent also falls, to the point where the overall rate of the reaction is now useless or seriously impedes the overall progress of the analysis. We have reached the point of diminishing returns, where the conversion efficiency is very low and we are not able to form a single, homogeneous product. This is, unfortunately, the current state-of-the-art in pre-injection derivatizations for both HPLC and HPCE.

This is unfortunate for HPLC, but it is a disaster for HPCE, whose true strength resides in protein separations. Unfortunately, native proteins are poor UV absorbers or fluorescers and thus their detection limits in the absence of precolumn tagging are very high (poor). This is a fact of life in all HPCE and in the absence of preconcentration techniques, we are at the low ppm detection levels. These are not trace concentration levels for real samples. Perhaps the entire reason for the introduction of laser induced fluorescence (LIF) detection in HPCE has been because of the poor detection limits possible for native protein species? But this has then required a successful precapillary derivatization method for these very same proteins, at any concentration level, something that is yet unrealized. We are in the dubious position of having an excellent resolution technique, namely HPCE in all of its various modes, without being able to derivatize proteins in high efficiency and selectivity and at trace levels when needed. By and large, virtually all solution tagging approaches for proteins, pre-injection, especially for larger and larger proteins, lead to multiple products separable by the HPCE methods employed [21–24]. This is not the way the world should work. This is contrary to what is really and truly needed with real biofluid type samples having proteins present at trace (ppt) levels. But this is why we are now writing this very review article to address these very issues, the lingering problems and some potential methods of overcoming our current disabilities via future research efforts.

In a recent review chapter, Lee and Yeung have eloquently summarized the current state of affairs with regard to detection of proteins in all of HPCE [54]. They say 'To the extent that innovative separation strategies and new applications continue to materialize, inadequate detection sensitivity remains the major obstacle to the acceptance of CE by the practical analyst'. However, the present detection sensitivity of commercial absorbance detectors is a far cry from what is required for practical analyses on numerous occasions. As a result, the successful permeation of CE into the real world of analysis would rely on the development of reasonably sensitive, inexpensive and general detectors. Further improvements in conductivity, absorbance, RI and indirect fluorometric detectors are thus necessary. Even though financially well-endowed laboratories may now procure LIF detectors commercially, the implementation of CE for the purpose of trace to ultratrace analysis is not yet straightforward. Concurrent developments of selective and highly fluorescent labeling reagents and reliable derivatization protocols are needed to realize the full potential of LIF in CE.

2. Preconcentration of proteins from biofluids for direct detection and/or derivatization. Analyte preconcentration on a solid support

2.1. HPLC applications of protein/peptide preconcentration on a solid adsorbent support

Because it will be virtually impossible to derivatize proteins at trace levels using solution approaches, i.e., homogeneous conditions, one is thus forced to consider some type of sample preconcentration in both HPLC and HPCE before (!) the derivatization occurs. Several workers have now described such approaches. At times, they have utilized a solid-phase extractor (SPE) and preconcentrator in HPLC (precolumn) or within the capillary in HPCE, followed by a solution reaction. Or, they have used a solid-phase reagent (SPR) that can function as both the extractor and preconcentrator, with the reagent already in place for subsequent reactions on the preconcentrated analyte. However, by and large, these SPE and SPR approaches have not been described for proteins, with some notable exceptions (as below).

In the area of preconcentration of peptides or proteins prior to HPLC, followed by either solution or solid-phase reactions, Krull et al. have described the application of various SPRs for preconcentration and derivatization of smaller peptides, but not yet for

larger proteins [21–24,55–59]. In such an approach, the peptides can be isolated from a biofluid matrix in high yield, preconcentrated on the hydrophobic solid support, washed free of other matrix components that might also derivatize and then by simply elevating the temperature for a specific period of time, the solid-phase reaction occurs on the peptides. Such reactions are not selective, there is no reason a priori why they should be, just because the peptide is preconcentrated on the solid support. But it is clearly possible to use this SPE or a combined SPE–SPR approach to preconcentrate proteins from a trace level biofluid sample and then perform some other type of solution derivatization, for example. This too would not be selective for specific regions or sites on the protein, no more than for a totally homogeneous derivatization reaction. In this solid-phase approach, the hydrophobic adsorbent acts to extract and preconcentrate the peptide or protein and can then function as a support for a subsequent solution or solid-phase reaction.

Rosenfeld in Canada has described this approach for smaller molecules, prior to derivatization–GC analysis, especially for steroids from biofluids [60,61]. Several other examples exist of using preconcentration followed by solution derivatization, but always for smaller molecules, almost never for proteins or peptides, with very few exceptions (see below). Though a good deal of work has evolved in using SPEs for preconcentration of proteins and peptides in HPLC, very little work has been described wherein the already preconcentrated analyte is then derivatized [30,44,50,62–71]. This is really what is needed, it would appear, in order to realize successful tagging of trace level proteins in complex biofluids.

2.2. HPCE applications of protein/peptide preconcentration on a solid adsorbent support or membrane

In HPCE, sample preconcentration has assumed an even more important role, due to the rather poor detection limits (too high) for most analytes, especially peptides and proteins. There have been some quite notable approaches described for sample/analyte preconcentration in HPCE, especially the work of Guzman, Phillips, Swartz, Zare, Tomlinson, Landers and others. Most of this work has been applied to smaller molecules, with much less for proteins or peptides, which again is where it is really needed in HPCE-detection areas [1,2,6,72].

As already mentioned, perhaps too often, the major disadvantage to HPCE is its low concentration sensitivity. Because the entire system volume is only a few microliters, detector flow cells require narrow, short path lengths that ultimately limit UV absorbance or FL sensitivities. The frequently reported mass detection limits in the pmol/fmol range for sample injections of low nanoliter volumes actually translate to only parts per million (ppm) or high parts per billion (ppb) concentration detection limits, easily obtainable in HPLC. Thus, there is a great desire to improve sensitivities for analyte detection in HPCE.

An example of the difference between reported mass and concentration detection limits is the work of Nickerson and Jorgenson [73]. By using LIF for improved detection of naphthalene dicarboxaldehyde (NDA) amino acid derivatives in CZE, they report detection of 2.5×10^{-18} mol of arginine. These mass detection limits are impressive by any yardstick. However, because of only nanoliter injection volumes, the concentration detection limits are on the order of 10^{-8} *M*. Many other reports of detection limits that offer low mass detection limits often have concentration limits of only 10^{-6} *M*. Thus, the resolving power and efficiency of HPCE, coupled with trace analyte sensitivities in small sample volumes, is the goal of many analytical laboratories. Progress in the area of HPCE derivatizations using LIF detection has lowered mass detection limits to the zmol–ymol range, with concentration detection limits on the order of 10^{-12} *M*. For instance, Cheng and Dovichi [74] have used LIF detection for fluorescein isothiocyanate (FITC) derivatized amino acids for detectabilities of fewer than 6000 molecules, or 10^{-21} mol. These mass detection limits translate into 10^{-12} *M* concentration detection limits.

Several recent review articles have addressed the separation mechanisms and methods of detection [12,34,40,75–78] available in HPCE and on the discussion of fundamental attempts to lower concentration detectabilities [79]. Some of the following sections will address the issue of improved detection of trace amounts of analytes in HPCE through

derivatization for improved determination of proteins in a biological matrix. However, it should also be noted that there are alternative methods for improving concentration sensitivities. These include alternative detector designs for improved sensitivity and sample concentrating techniques, such as sample stacking or C_{18} or isotachophoretic (ITP) preconcentration. Detectability of an analyte will ultimately hinge on the careful choice of preconcentration conditions, detector design and derivatization reagent.

2.3. Alternate methods for improved detection in HPCE. Sample stacking and isotachophoresis in HPCE

A major disadvantage of HPCE is, of course, the limited amount of sample that can be loaded onto the capillary. By concentrating a large amount of a dilute sample at the beginning of the capillary, prior to separation, loadability is increased and detectabilities can be lowered several orders of magnitude.

The most popular method of preconcentration in HPCE is sample stacking. Sample stacking has recently been reviewed [79] and several researchers have been investigating the fundamentals and applications [80,81]. Sample stacking depends on a matrix difference (pH, ionic strength) between the sample buffer and capillary buffer, so that the electric field across the sample zone is greater (more intense) than in the capillary region. In sample stacking, a large volume of sample in a low concentration buffer is introduced for preconcentration at the head of the capillary. The capillary is filled with a buffer of the same composition, but a higher concentration. When the sample ions reach the capillary buffer and the lower electric field, they stack into a concentrated zone. Sample stacking has increased detectabilities 1–3 orders of magnitude.

Another method of preconcentration is to apply isotachophoresis (ITP) prior to the free zone HPCE separation of analytes [32–35,37,41,42,82–84]. ITP is an electrophoretic technique which allows microliter volumes of sample to be loaded onto the capillary, in contrast to the low nanoliter injection volumes typically associated with HPCE. The technique relies on inserting the sample between two buffers (leading and trailing electrolytes) of higher and lower mobility, respectively, than the analyte. The technique is inherently a concentration technique, where the analytes concentrate into pure zones migrating with the same speed. The technique is less popular than stacking methods because of the need for several choices of leading and trailing electrolytes and the ability to separate only cationic or anionic species during a separation. Nevertheless, impressive increases in detectability have been demonstrated. Stegehuis et al. [82] used ITP coupled with capillary zone electrophoresis (CZE) for the determination of *o*-phthaldehyde (OPA) and FITC amino acid derivatives. ITP was performed prior to CZE separation in a capillary with a wider diameter than the CZE capillary. This allowed a 5000-fold increase in the loadability of the sample onto the CZE capillary, although the decrease in capillary diameter required a 1:100 split of the running buffer. Fig. 1 shows the remarkable effect of ITP preconcentration on the separation of FITC-derivatized amino acids. This

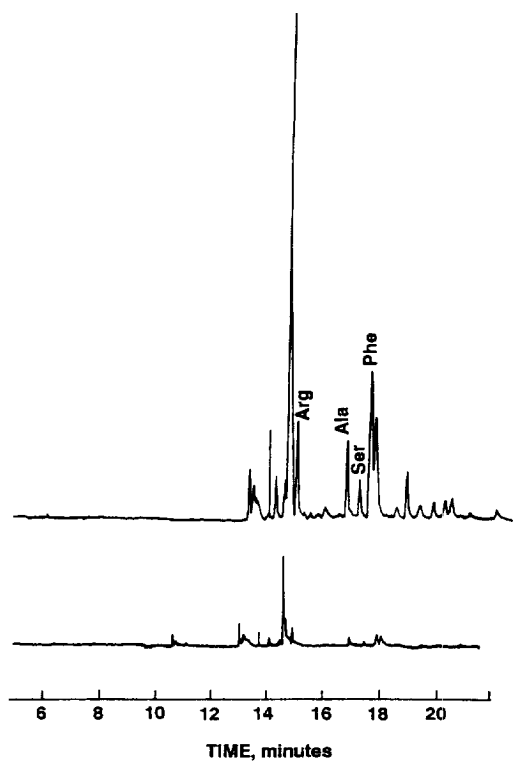

Fig. 1. The remarkable effect of ITP preconcentration on the separation of FITC-derivatized amino acids [82].

technique is also suitable for initial preconcentration of proteins, at times just prior to a second HPCE separation mode, such as ITP–CZE [85–87]. However, thus far no derivatization of such initially preconcentrated proteins has been described in HPCE modes.

2.4. Alternate methods for improved detection in HPCE. Sample preconcentration and reactions on solid supports or membranes in HPCE

Samples can also be concentrated at the head of the capillary using techniques typically associated with chromatography, notably concentration of hydrophobic analytes using a bonded C_{18} stationary phase (SPE), or specific isolation and concentration of an analyte using an affinity ligand or a membrane [88–99]. For these applications, the capillary can be considered to contain an on-line SPE cartridge. The capillary is conditioned with buffer, sample is loaded onto the concentrator and eluted with a short plug of ACN or elution buffer. Waters Corporation has actually commercialized their C_{18} packed capillary, which can then be used for multiple assays of crude samples for nonselective recognition, extraction, adsorption and effective preconcentration of hydrophobic analytes [92].

Tomlinson and colleagues have described, in a number of publications, an ability to improve on-line preconcentration in HPCE using an immobilized membrane to minimize the bed volume of the adsorptive phase [93–99]. This same group has also demonstrated the enhanced performance possible via a combination of membrane preconcentration together with conventional HPCE analyte stacking or transient ITP after the analytes have been eluted from the membrane [93]. These are perhaps the best approaches yet described to sample preconcentration in HPCE and several research groups appear to be utilizing these methods [98,99]. However, it does not yet appear that this type of protein preconcentration by membrane extraction has yet been followed by solution tagging for enhanced detectability, in general.

Guzman et al. have described a series of immobilized reactors (enzyme based) as well as preconcentrators containing immobilized antibodies (Ab, Abs) to selectively extract and preconcentrate specific antigenic (protein) analytes from biofluids, Fig. 2 [88–91,100]. They have described the fundamentals of placing different glass bead or silica based adsorbents within the entrance of the capillary, fixed in place by porous glass or polymeric frits, which can then be used repeatedly and reproducibly through dozens of individual assays of biofluids. The Abs were bound to controlled-pore aminopropyl glass beads and packed into a 100 μm I.D. capillary. The Ab sites were saturated with sample, then an elution buffer was applied to separate the retained analytes. Although recovery yield varied from 20–65%, because of irreproducibility of the preparation of the bound Ab concentrator, the concentrator is reusable and up to 350 ng of analyte can be loaded onto the capillary.

This idea of using immobilized Abs for selective preconcentration is significant, because it really represents a form of affinity recognition or affinity electrophoresis within the capillary. It is perhaps the most selective and specific method of preconcen-

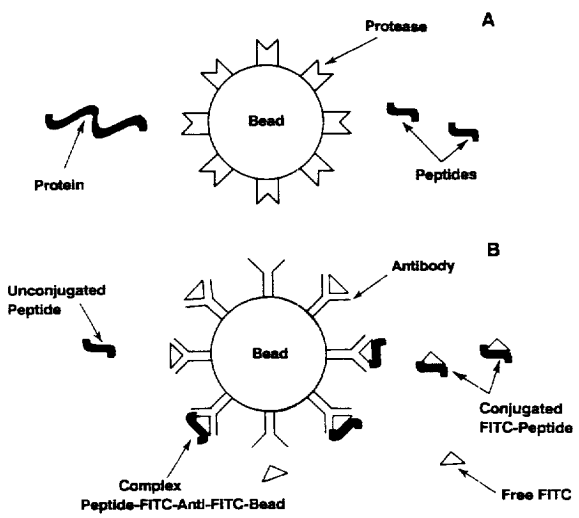

Fig. 2. Schematic representation of the proteins conjugated to controlled-porous glass beads. Four proteins were independently conjugated to microbeads: cytochrome *c*, bovine serum albumin, *S. aureus* V8 protease, and anti-FITC monoclonal antibody. (A) cartridge containing an immobilized protease and interacting on prolyl hydroxylase alpha-subunit to generate peptides (B) cartridge containing immobilized anti-FITC monoclonal antibody, the corresponding ligand FITC and the reaction of FITC with peptides to generate derivatized peptides (from Ref. [100], reproduced with permission).

tration possible. After preconcentration of the unique analyte, the sample matrix is washed free of the support and with another change of buffer make-up, the Ab–antigen (Ag) bonds are broken and the antigenic analyte species is eluted for HPCE resolution and final detection. This is clearly equivalent to how immunoaffinity chromatography or immunodetection has operated in HPLC areas, but now transferred to the HPCE format [101–110].

In yet another potential approach, Guzman has suggested how the once trapped antigenic analyte species can also be solution derivatized on the immobilized Ab within the capillary preconcentration region. He has termed this an analyte concentrator-reaction chamber (AC-RC) system. By the introduction of a solution reagent into the buffer medium, the trapped analyte could be derivatized within the capillary. After a suitable period of time, perhaps with an elevated temperature, the excess reagent is eluted with the HPCE operating buffer and a change in elution buffer releases (as above) the now-tagged analyte species for final resolution–detection steps. The idea of using an immobilized Ab or any biorecognition element within the preconcentration bed/zone is clearly novel and together with in situ derivatizations, should lead to very selective tagging and perhaps even uniformly tagged final derivatives, which remains to be demonstrated [91]. This is potentially a solution to the generic problem of trace derivatization of proteins in biofluids at trace levels. In this basic approach, injection of the biofluid would be followed by selective, Ab based extraction and preconcentration of the analyte protein of interest. Elution of unwanted matrix components would then be followed by a solution reagent, establishment of the necessary reaction conditions in the capillary and then elution–separation–detection of the now-tagged protein derivative. Of course, selective tagging would also be desirable on the SPE within the capillary, but that is a separate issue, further discussed below.

In another clever approach, Guzman et al. have described a two microreactor system within a capillary in HPCE, now used for consecutive protein digestion and peptide derivatization, employing an on-line analyte concentrator to map proteins using HPCE [100]. In this arrangement, the protein was first extracted and preconcentrated on an Ab-based solid-phase support placed near the entrance to the capillary. After the capture of the desired protein from a complex biofluid type sample, the protein was eluted by a buffer change, when it then entered a microreactor containing another immobilized Ab support. This final solid-phase reactor now carried the derivatization reagent, namely fluorescein isothiocyanate (FITC). That is, the second microreactor had FITC non-covalently held to its Ab, which was covalently bound to the solid support of the microreactor, all within the capillary entrance. The peptides that eluted from the first microreactor thus reacted with Ab-bound FITC and these derivatives were then eluted and identified. It is not clear that each peptide from the protein digest formed but a single derivative, nor where the tag was located. This is an alternative approach to peptide/protein tagging, where now the reagent was Ab immobilized and the protein/peptide in solution, rather than having an Ab immobilized protein with a solution reagent. Both approaches appear tenable and possibly valuable for trace protein preconcentration and derivatization within the capillary in HPCE. This appears to be the first time that a solution type derivatization reagent/reaction was utilized on a solid-phase support within the capillary in HPCE and where that analyte preconcentrator also contained a biorecognition element, here Ab based.

Beattie et al. have recently described the use of solid-phase concentrators for on-line preconcentration of metallothionein (MT) prior to isoform separation by CZE methods [111]. In this study, the authors investigated the use of an inexpensive and easy to assemble C_{18} concentrator that was fitted to the capillary inlet to extend the detection limits of MT isoform analysis. Samples were pressure loaded onto the concentrator for up to 5 min, eluted with 33% ACN and then subjected to CZE in borate or phosphate buffers. Sensitivity enhancement was greater than 700-fold and the relationship between loading time and isoform absorbance peak area was linear when only a small proportion of the concentrator capacity was used. Analysis of sheep liver extracts demonstrated that both sensitivity and specificity were enhanced. A discussion of other possible solid-phase concentrators is also presented [111]. It is thus clear that generic or Ab based solid-phase or membrane concentrators have been

widely used in numerous HPCE applications. What is less common is the combination of in situ protein preconcentration together with (!) selective tagging methods that lead to a single derivative from a trace level protein analyte in a complex biofluid type sample.

The idea of using an immobilized Ab microreactor which also contains a solution reagent, as in Guzman's work, has been extended by Zare, Krull and co-workers to the use of immobilized reagents, preinjection and in situ (within the capillary) in HPCE [1,12,23,24,55,56,112,113]. In Krull's initial efforts, a SPR was placed in the sample vial in a carousel of the HPCE instrument [1,12,113]. Derivatizations of small molecules occurred by raising the temperature of the carousel for a fixed period of time, after which an aliquot of the derivatized sample was automatically injected into the HPCE capillary. This approach permitted automated derivatizations of the analytes, at times in complex biofluids, with good reproducibility and high conversion efficiencies, in a totally automated and repeatable manner. It was an on-line approach, in that the derivatization and sample injection of the derivatized product were both fully automated and instrument/computer controlled. In subsequent work, Szulc and Krull developed an in situ, within the capillary approach to automated derivatizations in HPCE [Szulc, M.E., Krull, I.S., unpublished results (1994–95)]. In this situation, the SPR was placed within the capillary entrance, totally analogous to a solid-phase preconcentrator or an immobilized Ab column or a microreactor having immobilized enzymes, as described above. When the sample was introduced, passage through the hydrophobic SPR extracted the hydrophobic analyte amine, preconcentrated this analyte and then by raising the temperature of the capillary bed for a fixed period of time, the desired derivatization reaction would ensue. With at least small amines, this approach was reasonably reproducible, but not for long periods of time or for large numbers of repeat injections. That is, the method suffered from poor reproducibility over long periods of time and the SPR capillary had to be regenerated or replaced almost every other day. It was never really possible to demonstrate good repeatability within one day or reproducibility and ruggedness/robustness from day-to-day or from SPR capillary-to-capillary.

In analogous studies, Zare's group has also investigated the possible implementation of SPRs within the entrance to the capillary and they have obtained a US patent for this application to sample tagging in HPCE [112]. They have described in this patent, but never in a separate, reviewed publication, the possible application of SPRs for general sample tagging by automated introduction of samples into the capillary. However, they have never actually shown repeatable or reproducible results, qualitative or quantitative, or the ability to utilize this technique from day-to-day or from laboratory-to-laboratory. Repeatability, reproducibility, sample quantitation, method validation, ruggedness and/or robustness have also never been demonstrated by either Krull's or Zare's group in the application of SPRs within the capillary in HPCE. There may well be some very good reasons.

3. Post-separation methods of protein labeling in HPLC/HPCE reagents and approaches for post-column detection of proteins and peptides in HPLC/HPCE

As mentioned throughout, detection of minute amounts of proteins and peptides after HPLC and particularly HPCE is critical to fully realize the analytical benefits of these techniques, especially when the sample size is limited. However, pre-column labeling of analytes can pose challenging problems and push the separation system to its limits. Indeed, it is with the rapid improvements in separation technology leading to much higher resolving power that some of the weaknesses of pre-column labeling become apparent.

Post-column detection can circumvent many of these problems and at the same time permit high-sensitivity detection. The advantages of the post-column arrangement primarily reside in the fact that it is the native species that are separated and the selectivity of the reaction, in terms of the number and position of the attached labels, is not so critical provided the reaction is reproducible. The limitations of post-column detection are that the derivatization reagent must be carefully selected in terms of its spectral properties, reaction kinetics must be fast enough to maintain the integrity of the separation,

reaction volumes must be sufficiently large to allow effective mixing of solutions but small enough to minimize the effects of band dispersion and reaction solvents must be miscible and compatible with the mobile phase or operating buffer.

3.1. Specific reagents and approaches for post-column detection of proteins and peptides in HPLC

Here we will focus on post-column detection for proteins and peptides and we will not cover all the various post-column reactor configurations. For a complete review of such, with applications to a wide range of analytical problems, the reader is referred to a review by Brinkman [114]. By far, the most common post-column detection arrangement involves the introduction of a FL group to the analytes as they exit from the separation capillary, prior to detection. Two reagents have been commonly used for FL labeling of proteins and peptides following separation: Fluorescamine [115] and *o*-phthalaldehyde (OPA) [116]. Both reagents are non-fluorescent and react with available primary amines very rapidly (seconds) in aqueous solution to form fluorescent species. These and other reagents targeting amines and other reactive sites have been reviewed recently [117,118].

It is interesting to note that the same problems and concerns outlined in this review and other similar reviews, have persisted for many years. For example, Frei commented on the lack of sensitivity associated with UV–Vis absorption detection for HPLC of biological samples some twenty years ago [119,120]. To address such concerns, Frei et al. [120,121] designed a post-column FL detector for HPLC utilizing fluorescamine to quantitate the nonapeptides oxytocin, lysine-8-vasopressin and ornipressin. With optimized reaction conditions, such as solvent composition, pH, mixing arrangements (T-piece, with reagent flow opposing eluent flow and a spiral mixing device), they were able to achieve detection limits of less than 9.0 ng peptide per injection [120] with the spiral mixer. In a subsequent paper [122] they fine-tuned the spiral mixer (length and flow-rate) adjusted reaction temperatures to tolerate reaction times up to several minutes and coupled the post-column reactor (PCR) with a step-gradient system, to effect preconcentration of the peptides at the head of the HPLC column prior. Other examples of oxytocin analysis include Little et al. [123] who designed a packed-bed PCR for fluorescamine or OPA. Brown [124] utilized a similar fluorescamine PCR detector with on-line preconcentration at the head of a reversed-phase HPLC (RPLC) column to quantitate oxytocin from large volumes of parenteral intravenous solution mixtures. Limits of quantitation of 15 ng were reported.

Mabuchi and Nakahashi [125,126] employed post-column derivatization with fluorescamine to profile middle molecular mass (300–5000 Da) peptides excreted in serum [125], hemodialyzates [125] and urine [125,126] from normal and uremic individuals. Size-exclusion [125] and reversed-phase ion-pair chromatography [126] were used to separate the peptides isolated at the picomole level from body fluids and generate profiles that differed between healthy and uremic individuals. Newcomb [127] reported post-column fluorescamine detection as part of a strategy to isolate and characterize peptides in the sinus gland of a crab species, *C. carnifex*.

To screen peptides for the presence of proline or N-terminal blocking groups, Schlabach [128] used UV–Vis absorbance detection on-line with post-column FL detection. The absorbance channel provided a universal profile of the isolated peptides while one of two types of post-column reactions were used to detect an N-terminal blocking group or the presence of proline residues in a peptide. To determine blocked N-terminal peptides, the fluorescamine post-column derivatization was run below pH 8.4 to direct reaction to the N-terminal. Thus any peaks present in the absorbance channel but not the FL channel were attributed to the blocked N-terminus. To find proline-containing peptides, a two-step hypochlorite/OPA reaction [129] was used post-column to activate proline residues for OPA derivatization. In this case, two separate chromatographic runs were required to screen for total OPA reactivity and OPA reactivity in the absence of the proline activator hypochlorite (lysine derivatization). The emphasis here was thus on characterization using a suite of different detection schemes rather than high-sensitivity analysis. In another approach to characterization, a stream-sampler was used to sample a portion of the chromatographic effluent for post-column fluorescamine derivatization [130]. The stream-sampler acquired

5-ml aliquots from the eluent every 10 s for mixing with fluorescamine and transfer to a fluorometer, the rest of the effluent was available for further fraction collection and assays. Tryptic and cyanogen bromide peptide maps from as little as 10 nmol of myelin, ovalbumin and BSA and 75 nmol of lysozyme were performed using a stream-sampling post-column fluorescamine detection system [130]. These levels and amounts of peptides can now be routinely detected in HPLC–PDA without any prior derivatizations.

The use of post-column fluorescamine detection for complete micro-characterization of proteins has been discussed [131] including (1) post-column quantitation of amino acids from protein and peptide hydrolysates (2) HPLC-based peptide mapping (3) two-dimensional chromatography/electrophoresis and (4) SDS-PAGE electrophoresis with post-separation fluorescamine staining. Stein and co-workers [132,133] developed a fluorescamine-based post-column detection method for monitoring the activity of elastase on elastin. Specifically, the dipeptide Val–Pro resulting from the enzymatic action of elastase on elastin was targeted for quantitation by post-column FL, with Gly–Leu as an internal standard [132]. The method was utilized to measure elastin in swine aortic tissue [133] as a potential diagnostic tool for a variety of pulmonary and cardiac disease processes. More recently, Svensson [134] determined the nonapeptide felypressin in pharmaceuticals down to the level of 0.6 ng using column-switching as a clean-up step, followed by post-column fluorescamine detection. Lee [135] demonstrated quantitation of 10 ng of salmon calcitonin from biodegradable microspheres and injection and nasal spray formulations using gradient elution RPLC with a fluorescamine PCR. Boppana [136] reported a comparison of pre- and post-column derivatization of peptides with fluorescamine. Fluorescamine mixed, in an autoinjector, with the peptide solution just prior to injection onto the HPLC column was reported to be more sensitive than post-column fluorescamine detection of the same species. Limits of detection for the pre-column assay were found to be ~75 pg for angiotensin.

The other commonly used reagent for post-column fluorescence detection is *o*-phthalaldehyde (OPA) [116], which reacts rapidly with amine compounds to form highly fluorescent isoindole species. Boppana [137] used OPA post-column FL for the determination of a synthetic hematoregulatory peptide from dog and rat plasma. Using anion-exchange precleanup of the plasma with RPLC separation of the peptides, detection limits of 1 ng were achieved with limits of quantitation (LOQ) of 5 ng in a 0.25 ml plasma sample. Stegehuis [138,139] demonstrated actual detection limits of 5 ng of enkephalin-γ-endorphin [138] from plasma and 1 ng of two related β-endorphin metabolites from human plasma [139]. To quantitate enkephalin-γ-endorphin in plasma, on-line continuous flow dialysis was used to eliminate possible interferences from biopolymers, followed by analyte concentration on an Amberlite XAD-2 precolumn and analytical separation by gradient-elution RPLC [138]. More information and improved sensitivity could be achieved on two endorphin-related peptides using gel permeation chromatography (GPC), instead of the on-line dialysis to remove interfering biopolymers [139]. The molecular mass range of interest from the GPC was reconcentrated on an Amberlite cartridge, followed by separation by gradient RPLC. A heart-cut was taken from the RPLC for ion-pair separation, with preconcentration at the head of the HPLC column. With post-column OPA FL detection using the UV mode of an argon ion laser source, they were able to achieve detection limits of 1 ng for each peptide. Further investigations compared laser-induced fluorescence (LIF) detection of pre-column FITC-labeled endorphin-related peptides (argon ion, 488 nm) with post-column OPA LIF detection (argon ion, multiline 351.1 and 363.8 nm) [140]. Post-column OPA detection (limit of detection was ~80 pg) was preferred due to the large number of artifact peaks from the pre-column FITC reaction (limit of detection was ~18 ng peptide injected).

The tripeptide glutathione plays an important role in cellular defense against oxidants or free radicals, as such there is substantial interest in measuring glutathione levels. Post-column OPA FL is attractive for glutathione determinations since addition of the nucleophile (e.g., mercaptoethanol) for the OPA reaction is not required as the reduced form of glutathione contains both a thiol and a primary amine. Leroy [141,142] utilized post-column OPA detection to measure as little as 0.2 ng of glutathione in human plasma [141] and cultured cells [142].

Total glutathione [141] was measured by first reducing with DTT then precipitating proteins and analyzing by RPLC. Unbound glutathione was determined by DTT reduction and chromatographic analysis after protein precipitation [141]. This method compared favorably to the post-column FL detection with pyrenemaleimide [143] which was shown to be less selective than the OPA method. Furthermore, the pyrenemaleimide post-column reactor required an additional solvent delivery system, needed to make the mobile phase suitably alkaline for reactions in organic solvent. Intracellular glutathione was measured (3 min analysis time) with method detection limits of 0.5 ng from cultured cells using a 5-cm long RPLC column using post-column OPA fluorescence detection [142]. Fujita et al. [144] used post-column FL detection following RPLC separation to quantitate glutathione conjugates with various aliphatic aldehydes in the mid-pmol range (12–111 pmol) injected. The conjugates were found in rat red blood cells following incubation with several alkenals and hydroxyalkenals [144].

Engelhardt et al. have developed a knitted open tubular, post-column reactor that showed good mixing with minimal peak dispersion [145] and recently reviewed post-column reaction detection and drew comparisons with flow-injection analysis [146]. A unique application described by this group involves microwave-assisted hydrolysis of separated proteins prior to on-line post-column OPA derivatization [147]. In this scheme detection sensitivity for proteins was substantially enhanced by microwave-enhanced hydrolysis of the proteins just prior to the OPA PCR. Hydrolysis of the protein provides the primary amine of each resulting amino acid for derivatization with OPA, thus increasing the fluorescence signal. The microwave-enhanced hydrolysis was carried out in 45 s at a microwave power of 750 W in NaOH, since the subsequent OPA reaction favors alkaline conditions. An enhancement of 25–50 fold in signal was observed for the hydrolysis reaction followed by OPA labeling over OPA labeling without microwave-assisted hydrolysis. Detection limits of 0.8 ng injected for bovine serum albumin were reported [147].

Chow [148] compared pre- and post-column OPA detection with UV absorbance detection for amino acid oligomers (Gly–Gly^6), where these were expected to form on pre-biotic mineral surfaces. They concluded, for this particular sample set, that pre-column OPA derivatization and UV absorbance detection was the preferred mode in terms of analysis time and sensitivity.

Calmodulin was determined from bovine brain and pea plant extracts at the level of 50 ng using a high-performance affinity chromatography (HPAC) system with OPA post-column detection [149]. The affinity column was prepared by coupling melittin to glycidyloxypropyl-silica. Calmodulin binds melittin in the presence of Ca^{2+} and is released from the column by Ca^{2+}-free buffer. Selectivity for Calmodulin in the presence of other calcium-binding proteins was demonstrated.

An alternative to RIA for the determination of the activity of human growth hormone releasing factor (GRF) based on post-column OPA fluorescence detection was proposed by Nakazawa [150]. Rat growth hormone secreted in rat pituitary cell culture in response to treatment with GRF was determined by the developed method as an indirect probe of GRF activity. The method correlated well with the RIA control data and showed superior reproducibility for rat growth hormone over RIA. Detection limits of 3 ng injected on a C_4 RPLC column were demonstrated.

Schlabach utilized a dual-detector system in which a UV absorbance and a post-column OPA fluorescence or a native fluorescence detector were operated in series [128,151]. Absorbance detection provided a universal indicator for the presence of a peptide, while native FL was indicative of the aromatic amino acids and fluorescence-to-absorbance ratios were used to distinguish tyrosine-containing peptides from those containing tryptophan [151]. Post-column derivatization and FL detection with OPA was more sensitive to lysine residues [151]. The dual-detector approach, using either native FL or the post-column OPA FL detection was shown to be useful for characterizing the tryptic peptide maps from human growth hormone [151].

Glutamine synthetase activity was quantitated using ion-exchange chromatography with post-column OPA fluorescence detection to measure the γ-glutamylhydroxamate product [152]. Detection limits for γ-glutamylhydroxamate of 50 pmol injected allowed the assay of glutamine synthetase

activity from extracts of fish retina (33 nmol/min/mg), rat clonal C6 glioma cells (1 nmol/min/mg), mouse clonal NIE 115 (0.33 nmol/min/mg) and N18TG2 neuroblastoma cells (0.33 nmol/min/mg).

While fluorescamine and OPA are the most prevalent post-column derivatization reagents, other reagents have been intermittently investigated and used for protein and peptide analysis. The proteins human serum albumin, β-lactoglobulin and myoglobin were monitored by post-column reaction with 4-fluoro-7-nitro-2-oxa-1,3-diazole (NBD-F) following size exclusion separation [153]. Low molecular mass amines were optimally detected with high acetonitrile content and pH 8.5, while the optimum protein conditions were lower pH (7.9) with a high salt content. Under optimal conditions detection limits for the protein species were between 100 and 300 ng. Tissue collagen and protein synthesis rates were measured by HPLC with post-column FL detection of 7-chloro-4-nitrobenzofuran (NBD-Cl) derivatives [154].

The opioid peptides, many of which contain an N-terminal tyrosine, play an important role in pain sensation. Ohkura and co-workers [118,155–160] have described post-column derivatization of these compounds with hydroxylamine, Co(II) and borate at pH 8–9 to form FL species that feature an excitation maximum at 330 nm and emission maximum at 440 nm. Seven biogenic opioid peptides were determined by RPLC separation with sensitivity ranging from 0.5–1.5 pmol injected [158]. The method was utilized to determine enkephalinases A and B [157] and for assaying enkephalin-generating enzyme activity [159]. Five peptides from the tryptic digestion of opioid peptide precursors in rat brain tissue were quantified with this technique with demonstrated detection limits of 0.7–2.8 pmol injected [160]. Naphthalene-2,3-dicarboxaldehyde (NDA) has also been used in a post-column arrangement to form fluorescent products with some opioid peptides [161]. Mercaptoethanol served as the reaction nucleophile because its kinetics are more favorable for post-column reactions than cyanide. Detection limits of 36 fmol of leucine-enkephalin, using the 457.9 nm line of the argon ion laser, were reported.

Ohkura quantitated glycated albumin using post-column FL detection with derivatization by the carbohydrate-specific [118] reagents 4-methoxybenzamidine (*p*-MBA) and meso-1,2-bis(4-methoxyphenyl)ethylenediamine (*p*-MOED), with limits of detection of 100 pmol and 150 pmol, respectively [162]. Tryptophan 5-monooxygenase activity in rat brain tissue homogenate was assayed by quantitation of the formed 5-hydroxytryptophan by post-column FL detection of benzylamine [163]. Another approach to post-column FL detection for HPLC [164] or FIA [165] involves two reaction coils. The first coil serves to mix hypochlorite in pH 7.5 phosphate buffer with the eluting proteins to chlorinate the peptide bonds. In the second reactor, the chlorinated species are labeled with thiamine hydrochloride and sodium nitrite in pH 7.5 phosphate buffer. A flow-through FL detector monitored the emission at 440 nm upon excitation at 370 nm. The method was applied to extracts of *E. coli* cell debris and jack bean meal, with detection limits comparable to absorbance detection a 210 nm, 10 ng BSA injected.

Fatty acid binding protein (FABP) from rat liver was measured by monitoring the FL enhancement when it binds dansyl-undecanoic acid [166]. The eluate from a GPC column was mixed in a post-column coil with dansyl-undecanoic acid in phosphate buffer and the FL was monitored at an excitation wavelength of 350 nm and emission at 500 nm.

Using an approach that involves both pre-column labeling and post-column reaction detection, Okabayashi [167] determined small amines and biological thiol compounds, including glutathione (detection limit, 13.2 fmol) and coenzyme A (detection limit, 18.8 fmol). The compounds of interest were first conjugated, pre-column, with non-fluorescent Eu^{3+} chelates and separated by RPLC. The post-column reactor involved the replacement reaction of Eu^{3+} with 3-naphthoyltrifluoroacetone, which emits at 617 nm upon excitation at 344 nm.

Ninhydrin reacts selectively and rapidly with the guanidino moiety of compounds under alkaline conditions to form FL products (λ_{ex}=390 nm, λ_{em}= 470 nm). The application of ninhydrin to post-column derivatization of small molecules containing a guanidino group, such as creatinine and methyl guanidine, suggests the possibility of detecting arginine-containing peptides at high sensitivity. The sweetener aspartame, a dipeptide methyl ester, was determined by post-column ninhydrin FL detection

[168] following ion-exchange chromatography. The method was applied to the determination of aspartame in beverages such as fruit juices, colas, coffee and tea [168]. Agater [169] used post-column ninhydrin FL detection to characterize the peptides that resulted from enzymatic digestion of soya and meat protein products. Rhodes and Boppana developed a rapid, specific and highly sensitive HPLC method for the post-column ninhydrin detection of arginine-containing peptides in biological matrices [170,171]. Optimization of post-column reaction conditions and the use of microbore HPLC columns (2.1 mm) resulted in on-column detection limits as low as 50 fmol for naturally occurring peptides, such as bradykinin and bombesin. Application to the determination of a synthetic octapeptide vasopressin antagonist in human plasma were shown to result in a quantitative response, which is linear over approximately three orders of magnitude with limits of detection, in plasma, of 45 ng [170,171]. Post-column derivatization with ninhydrin has also been applied for the determination of other biologically important samples.

Benzoin also reacts with the guanidino moiety to form FL products (λ_{ex}=325 nm, λ_{em}=435 nm) at a sufficient rate to be utilized for post-column derivatization [118]. Ohkura et al. utilized a post-column benzoin FL derivatization for the analysis of pmol quantities of arginine-containing peptides by high-performance liquid chromatography [172,173]. Several physiologically important peptides that feature arginine residues, such as kallidin, bradykinin, angiotensins and melanocyte stimulating hormone, were demonstrated with this system. Identification of arginine-containing fragments in the tryptic digest of β-melanocyte stimulating hormone were made by tryptic mapping, with selective benzoin detection and comparison with tryptic maps obtained using the more general detection by UV absorption detection at 215 nm [172]. Benzoin post-column detection was also used to determine the neuropeptide substance P in the hypothalamus tissue of rat brain [173]. The method was sensitive enough for quantitation at peptide levels as low as 580 fmol/mg of protein in the brain homogenate [173].

In a departure from the traditional techniques that utilize post-column mixing of chemical reagents to introduce FL moieties to the solutes of interest, Krull [174] developed a post-column system in which photons impinging on a knitted open-tubular reactor act as the reagent to activate the eluting species for sensitive electrochemical detection. They demonstrated a signal enhancement for both electroactive and non-electroactive amino acids [175] when the reactor was irradiated with UV light. The post-column photolysis system was applied to several small peptides and proteins, with limits of detection at the low nanogram level. The technique has been expanded to include proteins separated by SEC, RPLC, ion-exchange and hydrophobic interaction chromatography [176] and peptides [177,178]. Related to the approach of using post-column photochemical reactions in HPLC, Lurie et al. have applied this to a variety of drugs of abuse with HPLC–PDA/MS interfacing [179]. The on-line photolysis step converts the original drugs to derivatives having different MS and UV properties, which aids in identifying the original drug when compared with an authentic standard. This approach could also prevail with peptides and proteins in HPLC–PDA/MS analysis.

Another approach to electrochemical detection involves the electrogeneration of bromine in a separate flow channel, followed by mixing with the column effluent [180,181]. Detection is accomplished by monitoring the bromine concentration using reductive amperometry. When a compound that consumes Br_2 elutes (e.g. thiol or disulfide containing species), the bromine current is correspondingly reduced. Glutathione, at the 0.5 pmol level [180,181] and the proteins lysozyme and cytochrome *c*, at 1.9 and 0.6 pmol levels [180], were reported.

Peptides can be determined by dual electrode amperometry following post-column mixing with Cu(II). In the Biuret reaction, initially used in a post-column reactor arrangement for UV–Vis detection of proteins by Schlabach [182], Cu(II)-peptide complexes that can be oxidized to Cu(III) complexes are formed with peptides larger than dipeptides. Warner and Weber [183] utilized the selective dual electrode arrangement, in which an upstream anode oxidizes the Cu(II) to Cu(III), then the current at a downstream cathode resulting from the reduction of Cu(III) back to Cu(II) is measured. In a subsequent study the influence of tyrosine (Tyr) on the signal was investigated [184]. In the dual

electrode configuration, the anodic sensitivity was enhanced by the presence of Tyr, while the cathodic response was depressed. Tsai and Weber [185] examined conditions (pH and electrode potentials) for the Biuret post-column reaction of N-blocked and free N-terminal dipeptides at the pg level. Recently, Chen et al. [186] characterized detection properties of the Biuret reaction using rotating ring-disk voltammetry of model bioactive peptides. Detection limits ranging from 0.016 to 0.4 pmol injected for several classes of peptides was reported, along with an example of a tryptic digest from of cytochrome *c*.

Chemiluminescence (CL) represents another approach to post-column reaction detection [187,188]. The analytes may be labeled pre-column and mixed with chemiluminescence reagents in a post-column format for sensitive detection, without an incident light source. Alternatively, post-column mixing with the appropriate reagents for generation of chemiluminescence with native species eliminates the need for a pre-column labeling scheme. In keeping with the theme of this review, this section will center only on chemiluminescence detection formats as applied to protein and peptides. Hara and co-workers [189–191] utilized a Cu(II)/luminol/peroxide CL system following gel permeation chromatography to determine as little as 50 ng, injected, of ovalbumin [189]. They also developed a Cu(II)/1,10-phenanthroline/peroxide CL post-column reactor following metal chelate affinity chromatography [190] and ion-exchange chromatography [191]. With this CL system, detection limits of 5 ng (bovine serum γ-globulin) were reported. Chemiluminescence detection of 'native' proteins, peptides and amino acids was demonstrated using electrogenerated tris(2,2′-bipyridine)ruthenium(III) in a FIA mode [192] and amino acids from a peptide hydrolysate by HPLC [193].

Fujinari et al. [194,195] developed a chemiluminescent HPLC detector to specifically monitor nitrogen containing compounds without pre- or post-column derivatization. The eluent was split between a UV absorbance detector and the CL detection cell. The eluent in the CL detection channel was pyrolyzed (at least 1000°C) which converts all chemically bound nitrogen into nitric oxide. The dried gases are mixed with ozone to produce excited state NO_2 which emits a photon upon relaxation to the ground state. A preliminary study [194] demonstrated the system for the analysis of amino acids and wheat gliaden proteins. Subsequent work [195] centered on the characterization of two peptides from a casein hydrolysate.

3.2. Specific reagents and approaches for post-capillary detection in HPCE

As an analytical technique capillary electrophoresis (CE) is now beginning to move into a more mature state as an accepted, routine, quantitative tool. As a result, CE is being adapted to more real-world applications, in which the concentration of sample components are frequently very low and solutes do not usually present themselves in the large volumes and at the high concentrations generally required for clean, rapid chemical modification and easy manipulations. Furthermore, when working with limited samples volumes, such as biofluids or particularly at the level of individual cells, pre-column manipulation of reagents, sample losses and dilution become more restrictive. Post-column detection for CE thus becomes attractive, since some sampling handling steps and their associated problems are eliminated.

As with HPLC, the spectral and chemical characteristics of fluorescamine and OPA make them the reagents of choice for post-capillary CE detection. Naphthalene dicarboxaldehyde (NDA), with mercaptoethanol, has also been successfully implemented [196]. An early reactor design by Tsuda [197] incorporated three pumps to deliver post-column reaction buffer, fluorescamine and equalize pressure at the inlet and outlet ends of the capillary. Rose and Jorgenson [198] described a co-axial design in which the separation capillary was sleeved inside a larger capillary to mix with the OPA reagent. The OPA was delivered by hydrodynamic pressure to a stainless steel T-piece around the separation capillary into a reaction reactor capillary. Detection limits of 6.3 fg glycine and 380 fg myoglobin were reported. Later reported improvements to this design [199] included the use of a He–Cd (325 nm) laser source for excitation of the OPA derivatives, larger diameter capillaries and helium pressure to deliver the OPA solutions to the reactor capillary. Detection limits of 780 fg myoglobin injected were reported more than

double the previous design [198], however peak efficiency (6.0×10^5 plates) was 10-fold higher than the previous design. The higher mass detection limits were attributed to the use of larger diameter capillaries (for easier and more durable construction), concentration detection limits were almost identical.

In a recent report, Zhang and Yeung [200] described a co-axial geometry post-capillary reaction detector. The reactor was constructed using smaller capillaries (15 μm separation capillary and 30 μm reaction capillary), with independent electroosmotic control of the OPA reagent delivery. As shown in Fig. 3, the reaction capillary is sealed in the bottom of a micro-centrifuge vial which serves as the OPA reagent reservoir. The junction between the separation capillary (outer diameter etched to fit inside the reactor capillary) and the reaction capillary resides in the OPA reservoir, which is grounded. Reagents and migrating solutes are mixed in the reactor capillary and electrically driven to the LIF detector (325 nm line of the He–Cd laser). Calculated mass limits of detection for the proteins human hemoglobin A and carbonic anhydrase I were roughly 150 fg and 110 fg, respectively. Detection limits for small peptides were poor compared to the proteins, ~2–5 pg, except glutathione (~95 fg). The system was applied to examine the amine contents of single human erythrocytes from normal adult, diabetic adult and newborn blood samples.

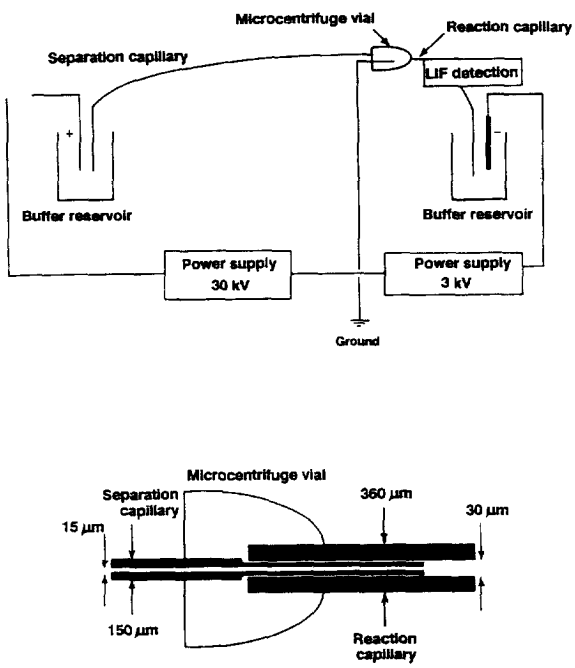

Fig. 3. Schematic representation of the co-axial post-capillary reactor and CE system for OPA derivatization of the contents of single human red blood cells [200]. (top) CE system with post-capillary reactor (bottom) enlargement of the post-capillary reactor.

On-column derivatization of amino acids with OPA was accomplished using a connector in the form of a cross [201]. The cross was formed by laser drilling a hole through the fused-silica separation capillary and sealing two pieces of fused silica concentric with the drilled hole. LIF detection was accomplished downstream of the cross with a He–Cd laser operating at 325 nm. Linearity extending over three orders of magnitude was shown with detection limits of approximately 2 pg histidine injected. Rose [202] constructed a free-solution OPA post-column reactor, in which solutes migrate into a large volume OPA reservoir, mix with the reagents by diffusion and convection and are detected a short distance from the capillary outlet using fiber-optic illumination from a Hg arc lamp. Once built, the reactor somewhat eased the plumbing and positioning requirements for post-capillary detection, however FL background was high and band-broadening significant.

Fluid gap reactors have been used with some success for the post-capillary derivatization of biological compounds [203,204]. Albin [203] used a four-way T to align the gap junction and provided for OPA or fluorescamine reagent flow to the gap region. The separation capillary was 50 μm I.D. and positioned approximately 10–50 mm from the end of the reactor capillary (75 μm I.D.). Migrating solute crossed the gap due to the electric field, while the larger inner diameter of the reaction capillary ensured that derivatization reagents were drawn into the reactor simultaneously. Mixing of the reagents and solutes was by diffusion and convection on the path downstream (8 cm) to the FL detector (Xenon lamp). Post-capillary fluorescamine derivatization of the tryptic peptides from β-lactoglobulin were shown. Mass detection limits for OPA-gly were reported to be 247 fg, which compared favorably with those obtained by pre-capillary OPA labeling (210 fg). Gilman [204,205] designed a gap junction

in which the separation capillary (10 μm, I.D.) and the reaction capillary were separated by fracturing the capillary. Thus, the analytical capillary and the reactor capillary were the same inner diameter. For ease of handling and stability the fractured capillary was epoxied to a glass slide under a laboratory-made reagent reservoir. Introduction of the post-capillary derivatization reagents, OPA [204] or NDA/2-mercaptoethanol [205], was primarily due to reagent diffusion into the gap. Reactor performance in terms of peak efficiency, signal-to-noise and detection limits were thoroughly examined. Column performance was not compromised by the gap reactor, plate numbers for amino acids were as high as 200 000 and detection limits for glycine and iron-free transferrin were 9.8 fg (130 amol) and ~130 fg (5.2 amol). The same design was used with NDA/2-mercaptoethanol as the post-capillary derivatization reagent [205]. The NDA system was applied to the separation of components from homogenates of the right and left pedal ganglia of Planorbis corneus and the contents of a single human erythrocyte [205]. Experiments with amino acid, peptide and protein standards gave detection limits roughly two-fold greater than those obtained with the OPA post-capillary gap reactor.

Zhu and Kok [206] utilized a version of the gap reactor for OPA derivatization of amino acids. Two capillaries were fixed a short distance apart inside a porous PTFE tube mounted in a reagent vial (1 ml) and through which OPA reagent solutions were driven by air pressure. Pressure was applied to the inlet end of the capillary via the same pressure system to prevent backflow into the separation capillary. Detection was accomplished downstream from the reactor using a xenon lamp fluorometer and a Hewlett–Packard Bubble Cell capillary. An electropherogram from an untreated urine sample was shown in comparison with amino acid standards known to occur in urine. The best detection limits reported ranged from 0.05–0.1 pmol injected amino acid (the particular amino acids used were not specified).

Emmer and Roeraade [207,208] utilized both coaxial [207] and gap junction [208] designs to construct a post-capillary enzyme reactor to monitor enzymatic activity of separated sample components. Substrate (NADP) was introduced to a T-piece, by a syringe pump, around the separation capillary and into the reactor capillary for mixing with migrating zones approximately 3 mm from the detection window. Two UV detectors were utilized one before and one after the reactor capillary for universal (210 nm) and specific detection of the produced NADH (340 nm), respectively. This arrangement was capable of detecting as little as 0.5 fmol glucose-6-phosphate dehydrogenase. A subsequent modification to the reactor design incorporated a gap junction between two capillaries of the same inner diameter. Substrate delivery was controlled by a separate high voltage power supply. The gap junction mixing device and entire CE system are shown in Fig. 4. Reported detection for glucose-6-phosphate dehydrogenase with this arrangement were 90 amol injected. The possibilities of improving the sensitivity using stopped-flow electrophoresis to allow build-up of product in the reactor was discussed; however in this work the difficulty in timing and diffusion counteracted any significant sensitivity enhancement.

In the continuing efforts towards miniaturization, Jacobson et al. [209] used post-column OPA FL detection for CE on a glass microchip. They described a flow-gated system for analyte injection and electrical delivery of reagents to a T-channel micromachined on the chip. Separation lengths of 7 mm and post-column reactor length 10.8 mm were used to demonstrate the potential of microchip-based CE system utilizing post-capillary detection.

Deacon et al. [210] employed an on-capillary version of the Biuret reaction [182–186] for electrochemical detection of small peptides. They investigated in-capillary Cu(II) complexation by simply incorporating $CuSO_4$ into the operating buffer, but found an unacceptable increase in detector noise with time. However, pre-rinsing the capillary with Cu(II) to saturate the walls prior to separation in 2.5 m*M* NaOH containing no Cu(II) gave the best performance. The best detection limits reported for this system was for the tripeptide, Gly–Gly–Gly at 7×10^{-7} *M*.

Chemiluminescence detection can be attractive for capillary electrophoresis, since detection can be accomplished with low background and without an incident radiation source. The literature on CL detection capillary electrophoresis is relatively large [211] and growing. As such, we will attempt to

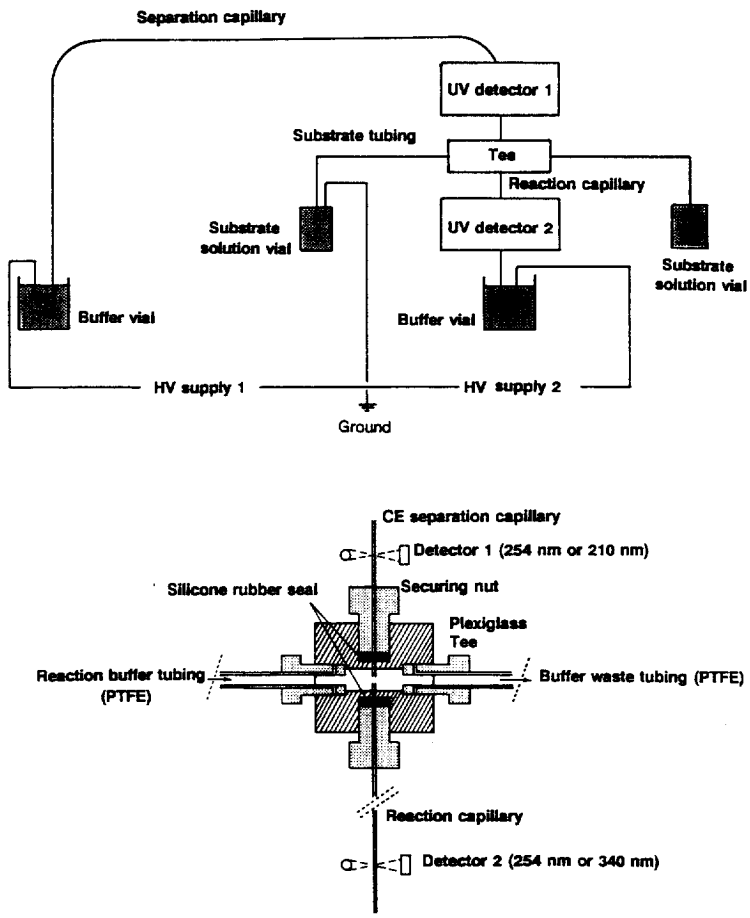

Fig. 4. Schematic representation of the gap junction post-capillary reactor and two-detector CE system for performing enzymatic activity assays of solutes migrating form the separation capillary. (top) the entire CE system (bottom) expanded view of the gap-reactor T-piece. Reprinted with permission from Ref. [244].

summarize the relevant literature for protein and peptide analysis by CE with CL detection. The transient nature of the CL mostly dictates a post-capillary reaction coil for mixing migrating analyte and CL reagents [212]. Hara and co-workers [213–215] utilized the formation of supramolecular complexes of Eosin Y [213] and Rose Bengal [214,215] with various proteins for the basis of chemiluminescence detection by bis(2,4,6-trichlorophenyl) oxalate (TCPO)- peroxide [213–215]. Mixing of the CL reagents with migrating proteins was accomplished post-capillary in a four-way chamber in which CL reagent and CL reaction buffer flow were from opposing directions, mutually perpendicular to the outlet of the CE capillary and subsequently improved with a co-axial arrangement of capillary and CL reagent introduction [215]. Absolute detection limits of 4 fmol (BSA) were reported using the Rose Bengal system.

Covalently bound fluorescent labels can be detected by CL using the TCPO-peroxide system [216–218]. Hara utilized rhodamine isothiocyanate (RITC) [216] bound to BSA and demonstrated detection limits of 1 fmol by CL detection following CE separation. Replacing RITC with TRITC and employing sample stacking injections the minimal detectable amount of BSA was lowered to 220 amol [217]. Utilization of pre-capillary fluorescamine labeling allowed for more rapid chemical modification and elimination of the reagent peak from the

electropherogram [218]. Although with this system reported detection for BSA were 0.7 fmol and the separation efficiency was somewhat lower compared to previous reports with other fluorophores. Ruberto and Grayeski [219] formed acridinium esters with peptides for chemiluminescence detection following post-capillary mixing with peroxide in basic solution. Interestingly, the attachment of multiple acridinium labels increased the CL sensitivity. However, no mention was made as to the possibilities of multiple CE peaks due to the multiple labeling that might be expected when the acridinium moiety is attached to the peptides pre-capillary.

Gilman reported electrogenerated CL detection of small amines, including a tripeptide, labeled with N-(4-aminobutyl)-N-ethylisoluminol via N,N-disuccinimidyl carbonate (ABEI-DSC) at subfemtomole levels [220]. A carbon fiber electrode was shown to have a more stable response than the platinum wire electrode, although detection limits were higher (worse) with the carbon fiber. Liao performed electrophoretic separations of native amino acids and a peptide in a buffer containing luminol and peroxide [221,222] for indirect CL detection. Post-capillary mixing with Cu(II) generated chemiluminescence at the detector window. Complexation of the Cu(II) with an amino acid or peptide reduced the background CL. Preliminary results were demonstrated with a few amino acids and a peptide [221], with a later paper more fully investigating the details of the system [222] using amino acids as the model solutes.

4. Pre-separation methods of protein labeling in HPLC/HPCE

4.1. Specific reagents applicable for selective labeling reactions in HPCE

The major disadvantage to HPCE is its low concentration sensitivity due to the limited (capillary) system volume, only a few μl and short path length for the detector flow cell. In order to improve sensitivities for analyte detection in HPCE, preconcentration and/or derivatization of the analyte and the use of a sensitive detector can be considered. The methods of preconcentration and sample stacking were already precisely described in a previous section. In this section, we will describe several labeling reactions for proteins and detection methods possible for derivatized proteins. We will also describe some applications using the labeled proteins. Because Krull et al. have already reviewed derivatization reactions in HPLC/HPCE [12,13], this review will mainly deal with recent papers published from 1994 to the present.

Krull et al. [12] and Arriaga et al. [223] have compared the two basic derivatization approaches, pre- and post-column for HPCE. In order to obtain maximum sensitivity, precolumn derivatization is preferred [224]. One prime requirement for using the precolumn derivatization method is the stability of the derivatives before and during the separation. Precolumn derivatization also needs high concentrations of the reagent and analyte, in order to obtain a high (fast) reaction yield. It is simply not possible to continuously decrease the analyte concentration and increase the reagent level so that the final reaction rate remains practical and realistic. During precolumn derivatization, a hydrolysis of the FL reagent often produces FL byproducts. Unreacted reagent and FL hydrolysis products often share the detection properties of the derivative, so that it is necessary to remove the excess reagent and/or its hydrolysis product(s) before separation, or to (chromatographically) resolve these species from the desired derivative peak.

Banks and Paquette have investigated hydrolysis of various FL reagents and separation of the mixture containing reagent, reaction product(s) and hydrolysis product(s) using HPCE (225). They compared three amine reactive FL probes, each containing different reactive moieties, fluorescein isothiocyanate (FITC), carboxyfluorescein succinimidyl ester (CFSE) and 5-([4,6-dichlorotriazine-2-yl]amino) fluorescein (DTAF), in terms of the degree of conjugation to myoglobin, the rate of reaction, freedom from hydrolysis and stability of a conjugate with lysine. They reported that each of the reactive probes demonstrated the ability to achieve a similar degree of conjugation. They also described that for the relative rate of conjugation between probes and stability of the resulting conjugate, CFSE demonstrated superior performance, followed by DTAF and then FITC, for both the protein myoglobin and amino acid L-lysine. However, they indicated that the

FITC conjugation reaction was much easier to control, which may be significant for applications that require a precise degree of such control [225].

Fluorescamine (4-phenylspiro[furan-2(3H), 1-phthalan]-3,3-dione) is a commonly used FL reagent for amino acids, peptides and proteins [226–228]. This reagent reacts readily and rapidly at an alkaline pH with primary amines to form intensely FL substances. The reaction is complete within a two minute incubation after mixing the analyte and reagent [226]. Guzman et al. have indicated that free fluorescamine was quickly hydrolyzed in aqueous solution, but the derivatives formed with primary amines were not. Thus, they concluded that the comparative rates of the hydrolysis and derivatization reactions are crucial parameters for any precolumn derivatization reaction with fluorescamine [226].

Gump and Monning have performed a precolumn derivatization of proteins, such as myoglobin, α-chymotrypsinogen A, ovalbumin and conalbumin, with fluorescamine, naphthalene-2,3-dicarboxyaldehyde (NDA) and *o*-phthaldialdehyde (OPA) [228]. The derivatized proteins were detected with UV and FL detection, after separation by SDS, using non-gel sieving HPCE. They described that under favorable conditions, UV absorption detection limits with the labeled proteins at 280 nm were approximately equivalent to the detection limits of underivatized proteins at 200 nm. They could obtain attomol detection limits using FL detection with the derivatized proteins. In their studies, although precolumn labeling decreased the efficiency of the separation, it did not give rise to multiple peaks because of heterogeneous labeling. The migration velocity of the labeled proteins was slightly different from the unlabeled molecules, but this did not significantly impair molecular mass determinations. Using precolumn derivatization with FL detection, they characterized the proteins in a fertilization membrane isolated from a single amphibian embryo [228].

Wang and Beale [229] labeled proteins covering a wide molecular mass range (6000–97 000 Da) with FITC for separation in a linear polyacrylamide gel column in which a step-gradient in acrylamide concentration was established. Detection was accomplished by LIF with an argon ion (488 nm) laser source in an epi-illumination format [230] in which solute zones can be monitored along the entire length of the capillary or effective column length can be adjusted to optimize resolution and analysis time. The gel gradient permitted separation of the zones whose molecular mass differed by at least 3–4 kDa within 7 min. As the solutes migrated into the more dense gels, high resolution between closely sized solutes (within 1000 Da) was achieved within 15 min.

Fadden and Haystead have developed a quantitative and selective method for FL labeling of phosphoserine residues on peptides and proteins [231]. Phosphoserine was derivatized with 1,2-ethanedithiol and then the thiol-serine residues were coupled to a FL probe by an iodoacetate reaction (Fig. 5). They indicated that this FL probe tagging of phosphoamino acids on proteins and peptides offers direct quantitative evaluation of cellular phosphorylation states at the attomol level in tissue samples derived from plants, animals and humans, without the use of radioisotopes, antibodies, or mass spectrometry [231].

Lim et al. have constructed a low-cost and highly sensitive LIF detector for precolumn derivatization systems [232]. Using their constructed detector, they quantitated TRITC (tetramethylrhodamine isothiocyanate) or Lissamine 30 labeled proteins. The limit of detection (LOD) at a signal-to-noise ratio of 2 for Lissamine 20 was about 1.4×10^{-21} mol at 10 nl sample injection and the relative standard deviation (R.S.D.) for 1.5×10^{-11} *M* of Lissamine 20 solution

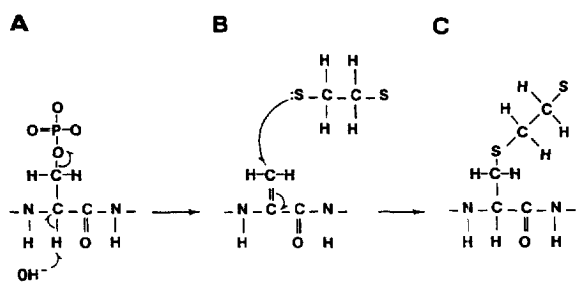

Fig. 5. Specific chemical reactions for FL labeling of phosphoserine residues on peptides and proteins. Phosphoserine was derivatized with 1,2-ethanedithiol, and then the thiol-serine residues were coupled to a FL probe by an iodoacetate reaction (Reprinted with permission from Ref. [231].

was about 15%. They applied their LIF detection for the study of tryptic digests of insulin and for urinary protein profiling [232].

Pinto et al. [233] have suggested that the disadvantages of precolumn technology are that it requires both a high concentration of labeling reagent (10^{-2}–10^{-4} *M*), resulting in a high detector background and a high concentration of peptide (10^{-3}–10^{-6} *M*), which is a major limitation in the case of real samples. Thus, for the sample preconcentration step before tagging, they have performed solid-phase FL labeling reactions with picomol amounts of insulin in very dilute solutions and then analysis by HPCE. Using oxidized insulin chain B as a test peptide, they demonstrated the use of an Immobilon CD membrane to capture and preconcentrate peptides. Insulin B chain was labeled with a FL reagent, 3-(2-furoyl)quinoline-2-carboxaldehyde, while it was still attached to the membrane. Unwanted FL by-products could be washed away with methanol without significant removal of the labeled insulin chain B, which then was extracted with a low pH buffer. However, this did not lead to a uniformly tagged, homogeneous species. They were able to detect 2.4×10^{-21} mol of labeled insulin B chain using electrokinetic capillary chromatography with LIF detection [233].

HPCE can also prove useful for binding studies of proteins. Reif and Freitag investigated the complexes formed between proteases, substrates and the protease inhibitor α2-macroglobulin, using HPCE with LIF detection [234]. They investigated the formation of a complex between FITC-labeled proteases, such as trypsin, plasmin, or α-chymotrypsin and the (unlabeled) protease inhibitor α2-macroglobulin, under optimized analysis conditions. They indicated that complex formation showed a strong dependence on the incubation conditions (pH, salt concentration, temperature and incubation time), but once formed, the complexes were stable under CZE conditions (e.g., pH of the electrophoresis buffer of 10.5). Treatment with SDS (5 min at 90°C or 30 min at 75°C) did not destroy the complexes, whereas treatment with mercaptoethanol (reduction of disulfide bonds) eliminated the peak from the electropherogram [234]. These findings suggested the formation of a covalent bond between the protease and the inhibitor during complex formation. They also concluded that further optimization needs to be carried out with respect to the quantitation problems of binding assays in HPCE.

Shimura and Kasai [235] have used angiotensin derivatives labeled at their N-terminal amino group with 5-carboxytetramethylrhodamine succinimidyl ester as p*I* markers for CIEF with FL detection. They indicated that proteins have many possible reactive sites for an amino-reactive FL dye and this might result in the formation of highly heterogeneously labeled products with respect to p*I*, since the labeling reactions may change the charge of proteins. On the contrary, peptides have only a limited number of reactive amino groups and many of them still focus well at their p*I* values. Using CIEF with LIF detection (He–Ne laser, 1 mW, 543.5 nm), they detected around 3×10^{-12} *M* (0.8 amol) of FL-labeled peptides.

There have been many attempts to obtain a single or controlled FL labeled product by several research groups [236–238]. Zhao et al. [236] have described the incomplete labeling of ε-amino groups on lysine residues. This was caused by bulky FL labels, resulting in the production of $2n-1$ reaction products, where n is the number of α and ε amino groups in the peptide. Thus, they have tried to obtain a single FL labeled peptide by first taking the peptide through one cycle of the Edman degradation reaction. After this first reaction, all ε amino groups were converted to the phenyl thiocarbamyl and the cleavage step exposed one α amino group at the N-terminus of the peptide. The fluorescent label was attached to that N-terminus [236].

Cobb and Novotny have investigated the use of two different amino-selective fluorogenic reagents for the selective derivatization of arginine- and tyrosine-containing peptides [237]. One such scheme utilized a selective reaction of benzoin with the guanidine moiety to derivatize arginine residues occurring in a peptide. The second scheme involved the formylation of tyrosine, followed by reaction with 4-methoxy-1,2-phenylenediamine. Using HPCE with FL detection, they could detect 270 amol of model arginine-containing peptides and 390 amol of model tyrosine-containing peptides [237]. They indicated that both derivatizations were useful for high

sensitivity peptide mapping applications, in which only the peptides containing the derivatized amino acids were detected.

Banks and Paquette have investigated the conjugation reaction between FITC and myoglobin by CZE with UV detection, eventually to get a singly labeled protein [238]. From the kinetic analysis of their data, the reaction was first order with respect to myoglobin and 1.3 orders with respect to FITC. They indicated that the separation of peaks attributable to the incorporation of $n=1, 2,...$ 7 fluorochrome labels into the protein had some significance for the generation of singly labeled protein probes, such as antibodies.

HPCE is one of the more useful techniques for performing rapid, automated immunoassays. By using FL probes with LIF detection, it is possible to detect trace levels of compounds separated in HPCE. Schultz and Kennedy have described two types of CZE-based immunoassays, competitive and non-competitive, using FITC-insulin and antiinsulin Fab [239]. Shimura and Karger have also described a highly sensitive microscale analytical procedure called affinity probe capillary electrophoresis (APCE) [240]. As an example of this approach, they labeled an Fab fragment of a mouse monoclonal antibody (anti-human growth hormone) with tetramethylrhodamine-iodoacetamide at a hinge region, thiol group. Samples were mixed with the purified labeled antibody fragment and the associated complex was separated by CIEF with detection by LIF. They could determine around 5×10^{-12} M of methionyl recombinant human growth hormone using their method [240].

HPCE can be also applied for studying enzyme activities [241,242]. Some advantages to using HPCE over conventional enzymatic assays are that the formation of products and loss of substrate can be simultaneously monitored, small volumes can be easily handled, the analysis is short and quantitation is easy. Craig et al. have assayed galactosidase by monitoring the generation of the FL products, when the FL substrate fluorescein-di-β-D-galactopyranoside was used [241]. By using 40 pl of the enzymatic mixture, they detected 6.5×10^{-14} M galactosidase or 1.6 molecules based on the detection of galactopyranoside. In another paper by these same workers, they assayed alkaline phosphatase by monitoring the conversion of the FL substrate AttoPhos into the highly FL product AttoFluor [242]. They used HPCE with LIF detection to monitor this reaction. From their results, the concentration limit of detection (3σ) of alkaline phosphatase was 1.5×10^{-17} M (2.1 fg/ml), which corresponds to a mass limit of detection of nine molecules (1.5×10^{-23} mol) contained within a 1-μl sample volume [242].

Hietpas and Ewing have reviewed derivatization methods for the analysis of single cell components by HPCE with LIF detection [243]. Recent advances have led to the use of this technique for the identification of amino acids and proteins in many different cell lines, ranging in size from 8 to 140 μm. They have compared pre-column, on-column and post-column methods of derivatization in order to determine which results in the least amount of sample handling and dilution, thereby leading to better detection limits for the analytes of interest. They concluded that future advances in these analyses will likely focus on new reaction chemistry for rapid and quantitative derivatization, as well as instrumental advances to immobilize cells in microvials and to minimize dilution during derivatization while maintaining the integrity of the separation [243].

Recently, several detection methods based on the LIF detector have been developed [230,244]. Beale and Sudmeier [230] developed an LIF detector for HPCE, in which the entire capillary can be scanned during the course of the electrophoretic run. The detection geometry was based on an epi-illumination format with confocal optical detection geometry. They demonstrated the capabilities of the scanning detector with CIEF and minimization of analysis times, using FITC labeled proteins. They have further presented a method for directly determining the diffusion coefficient of proteins. Once the diffusion coefficient was obtained, then expected peak widths could be calculated and compared with experimental ones [230]. Wu et al. [244] have demonstrated an LIF imaging detection system for CIEF, again using FITC labeled proteins. Because the imaging system provides information along the whole capillary column, the separation could be completed as soon as the desired resolution was achieved. The overall system could be useful for studying separation mechanisms, interactions be-

tween analytes and between analytes and capillary wall [244].

4.2. Specific reagents applicable for selective labeling reactions in HPLC. Pre-column protein labeling for HPLC

The general inability to selectively label protein species to form well defined derivatives has resulted in relatively few citations on the subject over the last several years. Nearly all of the approaches for improving the detection limits of protein derived species using HPLC with pre-column derivatization have focused on the identification and separation of the resulting amino acid or peptide derivatives after degradation of a protein analyte, such as with acid or base hydrolysis, Edman sequencing and enzymatic digestion. Such protocols have entailed the use of *o*-phthalaldehyde (OPA), phenylisothiocyanate (PITC) and other well known amino acid derivatization reagents and have been thoroughly investigated and reported in the literature. Our focus here is to highlight those reports that have utilized protocols targeting whole, intact proteins or biologically relevant peptides using traditional or more site specific reagents and schemes.

Most approaches for selective derivatization have focused on a particular, unique functional group present in a protein or peptide in order to avoid the formation of multiple derivative species. Not surprisingly, the relatively low frequency of thiols (as on the cysteine side chain) compared to other heteroatoms makes for an attractive target for site selective derivatization strategies. Several papers have appeared focusing on the derivatization of glutathione (GSH) [245–248], which is a tripeptide (γ-Glu–Cys–Gly) that is present in tissues and plasma throughout the human body and plays a significant role in many biological processes, such as protection from free radical oxidants, mediation of the sulfhydryl states of proteins, etc.

Derivatization to produce FL active derivatives of GSH have been successfully made with monobromobimane [246,248] and *o*-phthalaldehyde [247] and separated with reversed-phase HPLC, Fig. 6 [248]. These reports addressed the fact that GSH is present in biological matrices in several forms corresponding mainly to reduced glutathione (GSH), oxidized glutathione (GSSG) and protein bound glutathione (GSSPro). Detection limits for these assays ranged from 10–250 n*M*.

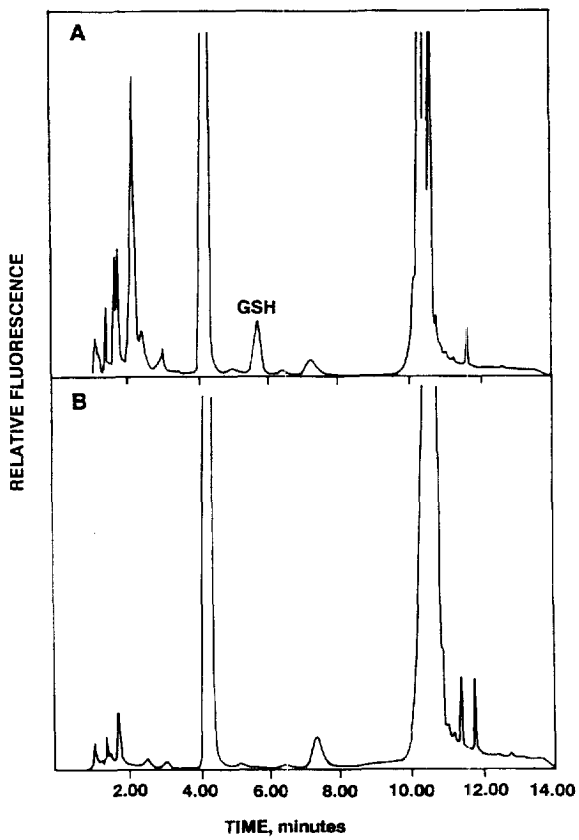

Fig. 6. Typical chromatogram of (A) Br-derivatized plasma sample and (B) plasma samples treated with N-ethyl maleimide. Econosphere C_{18} cartridge column (150×4.6 mm I.D., particle size 5 μm). Mobile phase A consisted of 30 m*M* tetrabutylammonium hydroxide (TBA) in 25% methanol, while mobile phase B consisted of 30 m*M* TBA in 100% methanol. The elution profile was 0–10 min isocratic with mobile phase A, followed by a column wash with 90% mobile phase A for 5 min. The flow-rate was 1.5 ml/min (Reproduced with permission from Ref. [248]).

Toyo'oka and Imai studied the derivatization of the thiol groups within egg albumin using 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [249]. Their results demonstrate one of the challenges associated with the derivatization of proteins; the tertiary and quaternary structure of a protein can strongly influence the degree of derivatization. Under non-denaturing conditions, only one of the four thiol groups of egg albumin was derivatized. The use of

detergents and heat was needed for the internalized thiols to become accessible to ABD-F for successful derivatization.

Although the derivatization schemes just described achieved lower limits of detection over direct UV detection, by imparting FL properties to the derivatives, glutathione and similar thiols were here derivatized with N-(4-anilinophenyl) maleimide (APM) and electrochemically detected with a glassy carbon electrode (+1.0 V versus Ag/AgCl) following separation with reversed-phases HPLC, Fig. 7 [245]. The detection limit in this case was reported to be 300 p*M*.

Other cases have been reported where a uniqueness in structure greatly assisted in selective derivatizations. A N,O-acyl transfer reaction between

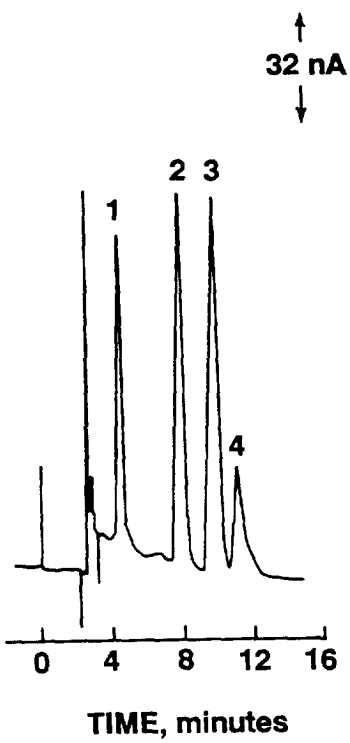

Fig. 7. Separation of a mixture of thiol compounds derivatized with APM: (1) glutathione; (2) L-cysteine; (3) N-acetyl-L-cysteine; (4) D-penicillamine (each 5 ng). Applied potential: +1.0 V. A μBondapak C_{18} column (30.5×0.63 cm I.D.) was used under ambient conditions. The mobile phase was acetonitrile–aqueous 0.5% (w/v) ammonium dihydrogenphosphate (4:7, v/v, pH 3.0, adjusted with H_3PO_4). The flow-rate was 1.0 ml/min (Reproduced with permission from Ref. [245]).

cyclosporin A and isocyclosporin A facilitated by methanesulfonic acid produced a free secondary amino group that was successfully derivatized with dansyl chloride [250]. Unfortunately, the method was hampered by sample losses and a poor derivative yield and did not lead to an improvement in the detection limit versus direct UV detection.

Ohkura and colleagues have studied the use of benzoin in the presence of mercaptoethanol as a pre-column derivatization reagent for peptides containing the guanidino moiety of arginine [251,252]. This reagent was successfully used to quantitate angiotensins I, II and III, as well as leupeptin-related peptides after NaBH4 reduction. The limit of detection of the angiotensin standards ranged from 27 to 130 fmol (0.28–1.2 ppb), while the limits of detection of leupeptin were determined to be 150 fmol (0.64 ppb) as a standard and 250 pmol/ml (106 ppb) in mouse serum and 500 pmol/g in mouse muscle. Also, this group examined the use of 1,2-diamino-4,5-dimethoxybenzene (DDB) as a reagent specific for the tyrosine residues of opioid peptides [253]. The detection limit of leucine-enkephalin in tissues was determined to be 5.6 pmol/g (500 fmol). The use of benzoin to derivatize arginine containing peptides was later extended to opioid and tachykinin peptides with reversed-phase separations [254] with fmol detection limits.

Opioid peptides were also derivatized pre-column with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide [255]. NDA targeted the α-amino group of the peptides of interest. Here the authors used a multi-dimensional HPLC system to selectively isolate the derivatives of interest from other matrix components and then to perform the desired separation and quantitation. The results reported were consistent with those reported by Ohkura's group, but this protocol realized a slightly lower limit of detection (100 fmol).

In another case, the 26-mer peptide melittin was enzymatically modified using a transglutaminase and a dansyl FL probe to produce a singly tagged dansyl derivative [256]. Although complete conversion was not achieved, derivatized and native melittin were separated by SEC and RPLC, Fig. 8. The limit of detection of the derivative was not reported.

Reports for the derivatization and HPLC separation of whole proteins have been limited. Fried and

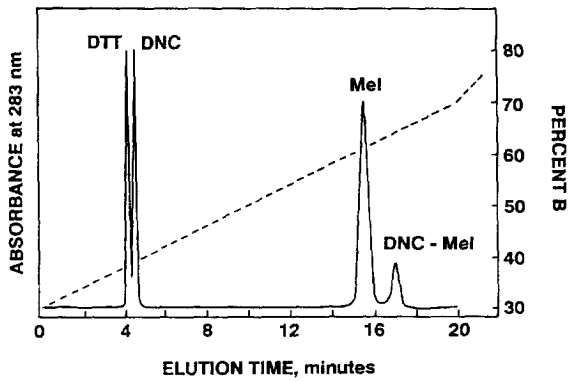

Fig. 8. Reversed-phase separation of melittin and DNC-melittin under optimized gradient conditions. Separation was accomplished with a μBondapak C_{18} column (30×0.78 cm I.D.) used with a 20-min linear gradient from 0.1% TFA–acetonitrile (70:30, v/v) to 30:70 (v/v). The flow-rate was 1.0 ml/min. (Reproduced with permission from Ref. [256]).

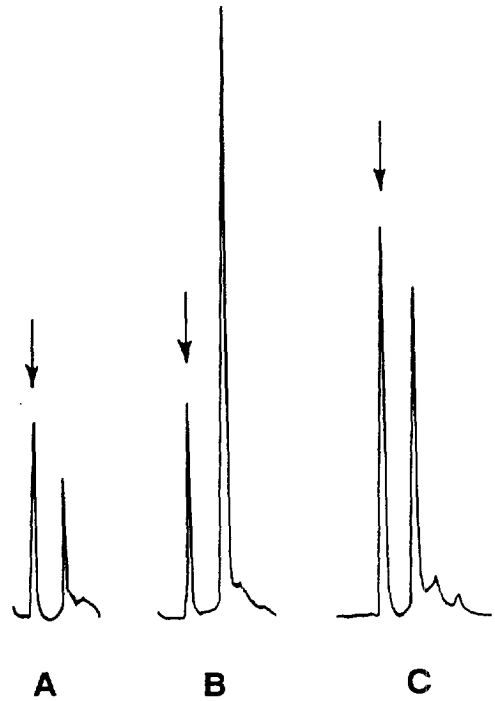

Fig. 9. Large proteins and small peptides coelute in the excluded volume of the TSK column when equilibrated in 0.1% SDS. (A) Elution pattern of the 22-amino acid residue peptide produced from myoglobin by cyanogen bromide cleavage (2.5 K peptide) after derivatization with OPA. Five microliters was used in the standard assay. (B) Elution pattern of *E. coli*-galactosidase protein (subunit M_r=116 K) after derivatization with OPA. Five microliters were used in the assay. (C) Elution pattern of the 2.5 K peptide and 116 K β-galactosidase protein derivatized as a mixture of 5 ml each in the standard assay. Separation was accomplished with a TSK guard column (4.5×75 mm) used with a 0.1% SDS mobile phase. The flow-rate was 1.0 ml/min operated at ambient temperature (Reproduced with permission from Ref. [257]).

colleagues developed a protein quantitation assay using OPA derivatization [257]. Proteins derivatized with OPA were separated from excess OPA and other fluorescent products with SEC. As presented, the method was specific for total protein content since all proteins and peptides coeluted in the void volume, Fig. 9, with a limit of detection for BSA approximately 3 ng (150 ppb).

Gel permeation chromatography with FL detection was successfully used to separate and quantitate serum proteins that were bound pre-column with indocyanine green (ICG) [258]. ICG is a FL dye that was found to bind to proteins. A semiconductor laser was used as the excitation source and was able to generate a detection limit of 1.3 pmol of BSA.

Similarly, ferrocenic derivatization reagents were evaluated for protein derivatization using HPLC with electrochemical detection [259]. The most successful reagents were 3-ferrocenylpropionic acid and ferrocenylmethyl-succinimidyl-glycine-hydrochloride. These reagents exhibited detection limits in the low n*M* region for BSA.

In the above discussions, we have emphasized entirely solution approaches for the derivatization of proteins and peptides, precolumn in HPLC. Certainly, these have been the most popular approaches, but it has also been possible to utilize immobilized or solid-phase reagents for similar tagging of proteins, though described far less. As above described, a solution reaction between a protein and the derivatizing reagent often forms multiple derivatives and gives broad peak with reduced detectability for the original protein [260]. Also, the formation of multiple products is explained by having different protein conformations present, since each conformation can react at different reaction sites and rates, depending on reaction conditions. These reactions are easy to perform, rapid and usually inexpensive. Two com-

mon reagents used in the solution analysis of peptides and proteins are: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (6-AQC) [261–265,271] and 9-fluorenyl methylchloroformate (9-FMOC) [266]. Analysis of hydrolyzed proteins with 6-AQC shows high compositional accuracy and subpicomole detection limits. A shift in the FL emission maximum between amino acid derivatives of 6-AQC and the reagent itself allows the direct injection of the derivatization mixture without interference from excess reagent and/or its hydrolysis product(s).

It is also possible to perform reactions of proteins with immobilized (polymeric or solid-phase) reagents. However, this can prove difficult due to unsuccessful contacts between reactive sites on the protein and the immobilized reagent. In an immobilized reagent, the underlying, solid support (silica, alumina, polystyrene, polystyrene–divinylbenzene) contains an attached reagent with an activated linkage at the end that releases its tag in the presence of a protein [267,268]. A derivative of 6-AQC has been used as a polymeric reagent for the derivatization of proteins [269,270]. A comparison of the chromatograms for the polymeric vs. solution reagents with derivatized insulin is shown in Fig. 10. The Waters AccQ-Fluor reagent (Waters trade name for the 6-aminoquinol-N-hydroxiysuccinimidyl carbamate solution reagent) was used as the solution analog. Approximately the same number of derivatives were formed by both solution and solid-phase approaches, but specific reaction sites and the extent of reactions are unknown. The specific nature of these insulin derivatives with regard to the number of tags per molecule and their sites of attachments was not determined.

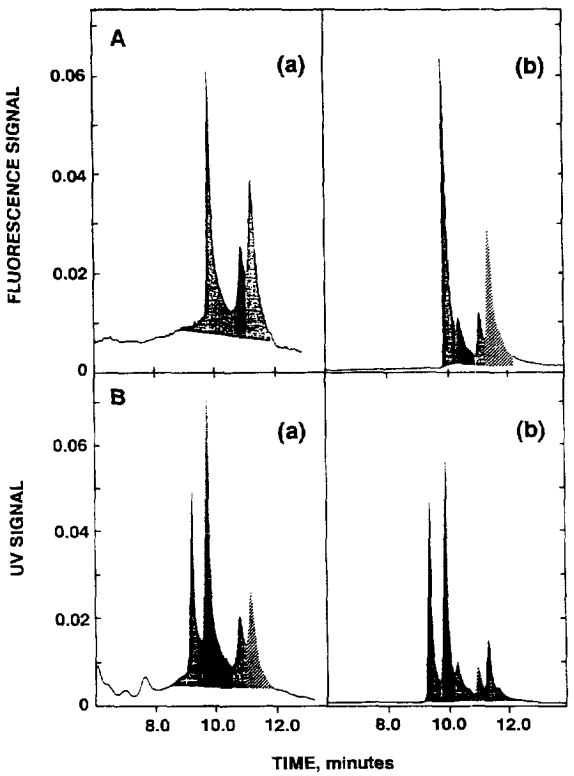

Fig. 10. Chromatograms of polymeric reagent vs. solution reagent derivatized insulin with FL (A) and UV detection at 214 nm (B). HPLC conditions are described elsewhere. Peak shadings do not attempt to identify individual proteins. (a) Insulin derivatized with 6-AQC solution reagent at a molar ratio of reagent to insulin of 1:1; (b) insulin derivatized with polymeric reagent (Reproduced with permission from Ref. [269]).

In another immobilized reagent approach, a specific type of macroporous polymer support (controlled pore size, particle diameter, percent crosslinkage, etc.), with 9-fluorenylacetyl (9-FA) as the tagging reagent, was used in a so-called size selective immobilized reagent derivatization of proteins [24,23]. The key in this particular approach has been to remove the outer surface layer of reagents on the polymer with an excess of protein or amine, so that only that reagent inside the pores is now available for reactions with a protein. Large molecules, such as proteins, show discrimination in their reactive sites compared to analogous reactions in solution. A removal of the outer tags has a large effect on the products formed as a result of this unique derivatization approach. Experiments with insulin showed a single derivative formed, with a single tag (N-terminus?) using a size selective reagent, Fig. 11. A much smaller number of tagged species [1,2] formed from alkaline phosphatase using the same immobilized reagent [24,23]. It is apparent that size selective derivatizations may provide the most selectivity among all tagging approaches for proteins. Lingering problems of percent conversion, rates of reactions, efficiency of derivatization and the usual kinetics of heterogeneous reactions on large biopolymers remain to be solved [24].

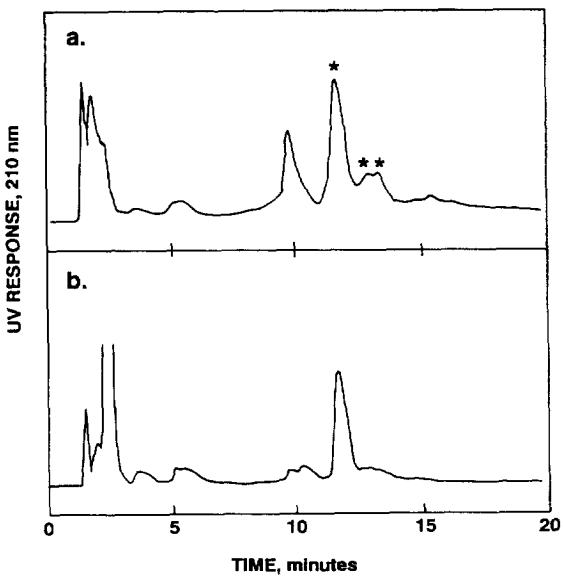

Fig. 11. Chromatograms for insulin derivatized with the usual immobilized reagent (a) and with the size selective reagent (b). The peak without an asterisk (first eluting peak) is untagged, residual insulin and peaks with the asterisk were formed, insulin derivatives. HPLC conditions are described elsewhere [24,23]. (Reproduced with permission from Refs. [24,23]).

5. Problems remaining for selective tagging of proteins in HPLC/HPCE, pre-separation. Resolving lingering problems.

Truly selective labeling of large biopolymers, in particular proteins and to a large extent peptides, that contain a variable number of different reactive groups (i.e., amines, thiols, etc.) remains an elusive target. The task becomes even more formidable if one elects to impose further criteria of minimal effects on the physical properties of the analyte and possibly the retention of biological activity subsequent to labeling. It is apparent that simple control of solution parameters will not, in most cases, afford the requisite selectivity and complete conversion to the desired product. That is, careful control of the ratio of reagent to protein or pH or temperature or solvents alone, does not appear able to control the degree or extent of tagging nor the placement of such solution tags.

Currently successful alternatives seem to be limited, such as permanent blocking of side chains with subsequent cleavage of the N-terminal residue [236] for attachment of the label. A brute force method, in which the desired products are purified by preparative techniques prior to utilization, can be successful in those applications where minute quantities of purified material are to be used for many subsequent analyses, i.e., as an affinity probe [240]. Such targeted analyses represent an important class of emerging analytical techniques capable of extremely sensitive assays in exchange for exhaustive, labor-intensive preparation of the selectively labeled probe (e.g., immunoaffinity capillary electrophoresis). However, for the case of a general analytical probe for proteins and peptides, independent of biological activity, there is not yet a satisfactory solution. Some promise can be found in the post-capillary reaction techniques being developed for CE; however, the limited chemistries available for this approach is restrictive. Perhaps in the short term, while on-going research begins to tackle the problems associated with a general strategy for selective protein labeling, further consideration should be given to characterization of small quantities of pre-isolated protein. This is a critical area routinely encountered by our colleagues in the biological and biomedical fields. In this vein, one typically thinks of peptide mapping, epitope mapping, pinpointing substrate binding or sub-unit contact sites and mapping glycosylation or other modification sites. Once the protein of interest has been micro-isolated (in perhaps a small quantity but at reasonable concentration), protective chemistry or targeted modification strategies can be brought to bear to locate sites critical for binding or activity. In some cases, the peptide fragments from micro-scale digestion techniques (solution or solid-phase) followed by solution or solid-phase labeling of the resulting fragments may be more amenable to controlled derivatzation. For example, following tryptic digestion, control of the label position and number of labels incorporated should be possible based on solution pH. But this may only work for smaller peptides with a limited number of reactive sites. Where cleavage occurs at lysine or arginine residues, then no peptide will have more than an N-terminal amine and at most a single amine side chain (from cleavage at lysine). This would still permit more than a single derivative to be formed by conventional, solution reactions, though forcing conditions might lead to a homogeneous, fully tagged product.

Are there other viable approaches that we have not considered as yet? The notion of using an immobilized protein on a solid support, as described by others, such as an Immobilon membrane or a PS–DVB hydrophobic support does not appear, protein dependent, able to lead to selective derivatizations. Is it possible that by first denaturing the protein one could then expose all reactive sites, thereby enabling these to all react with the same basic kinetics (other than for N-terminal sites), leading to a more homogeneous, perhaps fully tagged, single product? Careful studies on initial denaturation of proteins followed by forcing, solution reactions may be another route to form single, homogeneously tagged products? Yet another possible approach might be to immobilize the protein onto a solid support via its Ab or a receptor binding protein that has first been attached to the solid support. Formation of the standard, immunoaffinity complex of Ab–protein might cover certain otherwise reactive sites on the protein, perhaps restricting the number of products that then form with conventional solution reagents. Though such an approach might also derivatize the immobilized antibody as well, if it does not touch the recognition sites on the Ab or antigen, the same immobilized support might be used over and over again with different samples of the same antigen before recognition is fully lost. The formation of a sandwich complex between the immobilized Ab, antigenic protein and a secondary antibody, might lead to even more potentially reactive sites on the protein covered or bound. This again might lead to fewer reactive sites available for the solution reagent to locate, thus leading to fewer, final tagged products, it is imagined. Such an approach could, of course, employ monoclonal Abs or polyclonal or mixed sandwiches. None of these potentially interesting, perhaps viable, approaches have yet appeared in the analytical literature. It is quite likely that reactions on proteins in the presence of their antibodies or substrates have been attempted, but mainly for structural studies.

This idea of first immobilizing the protein prior to reactions with a solution reagent is the analogous approach to using an immobilized, perhaps size selective reagent with the protein in solution. In this already described approach, the protein has limited access to the reactive sites within the restricted access reagents. When the protein first forms an immobilized complex with its Abs prior to reaction, then the solution reagent has a reduced or limited access to the protein's reactive sites. In this manner, the end results of limited tagging, not necessarily just at the N-terminus as with a size selective, immobilized reagent, may lead to limited tagging elsewhere in the protein backbone. These approaches remain to be explored.

6. Summary and conclusions. Prospects for the future

The HPLC/HPCE determination of proteins relies on methods to improve detectability, which is crucial for such analytes at minute or trace levels. This becomes an even more complex problem when trace levels are present in complex biofluids, such as serum or urine. In the area of post-column derivatizations for proteins in HPLC or HPCE, this is somewhat routine by this time and numerous reagents and instrumentation is available in HPLC areas. However, in HPCE there is no commercial post-column reaction detector yet available and thus if derivatizations after the separation are desired or necessary, then a laboratory-made system must be constructed and optimized. To some extent, this has slowed the development of post-column protein tagging methodologies in HPCE applications.

In the areas of precolumn derivatizations, for either HPLC or HPCE, these are yet problematic areas because most solution reagents are just not able to selectively derivatize most proteins and surely not at trace levels in a homogeneous approach. Thus, most applications for improved protein detection in HPLC/HPCE do not rely on precolumn tagging, just because such approaches cannot yet be made routinely selective and with high efficiency of conversions. At trace levels of protein analyte, some type of sample preconcentration is needed, in both HPLC and HPCE, prior to direct analysis or derivatization with solution reagents. Virtually nothing has yet been described for successful, on-line, precolumn tagging of proteins at trace levels in either HPLC or HPCE leading to selective derivatizations. Though several attempts have been described to effect selective tagging of proteins at trace levels in both HPLC and

HPCE, by and large these approaches have not been very successful. A good deal of careful chemistry needs to be applied to proteins at trace levels for selective, precolumn derivatization, with any solution reagent. Even then, the success rate in forming a single, tagged derivative appears quite low, at least for most proteins at trace levels. It is almost always the case that such efforts result in either untagged starting material or other tagged products present along with the desired, single or homogeneously tagged protein derivative. Even today there do not appear to be many successful descriptions of precolumn, selective tagging of proteins at trace levels in biofluids. The idea of using an immobilized antibody to preconcentrate the protein from a biofluid, then followed by solution tagging appears promising. Though this has, to some extent, been applied in HPCE by Guzman et al., but by using an immobilized reagent with the protein free in solution, it does not appear to have been attempted in immunoaffinity or affinity HPLC areas. Also, as a general approach to the selective tagging of antibodies or proteins using their immobilized counterpart, very little has yet been described for any separation–detection scheme.

The use of immobilized reagents for selectively preconcentrating and derivatizing proteins from complex sample matrices remains a promising area for further development. In such an approach, the solid-phase support functions to extract and preconcentrate the protein, especially if one were to use an affinity support. This can then be followed by solid-phase tagging of the now-concentrated protein, followed by desorption with a suitable buffer or organic solvent to elute the derivatized protein(s). Thus, it is conceivable that one could combine the selective preconcentration effects of an affinity or immunoaffinity support together with a solid-phase reagent bed in a single cartridge. This could then conceivably recognize the analyte protein of interest, cleanup that extract from matrix components and then perform a solid-phase derivatization on the immobilized protein, perhaps. Alternatively, one might utilize a solution reagent to tag the preconcentrated protein on the solid-phase extraction (affinity) support, followed by removal of excess solution reagent and release of the now (perhaps) selectively tagged protein derivative ready for HPLC or HPCE separation–detection steps. These are just some areas that remain for future research and development in the areas of labeling reactions applicable to chromatography and electrophoresis of minute amounts of proteins.

7. List of abbreviations

ABD-F	4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole
ACN	Acetonitrile
Ab	Antibody
Ag	Antigen
6-AQC	6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate
APCE	Affinity probe capillary electrophoresis
APM	N-(4-Anilinophenyl) maleimide
CGE	Capillary gel electrophoresis
CZE	Capillary zone electrophoresis
CEC	Capillary electrochromatography
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CFSE	Carboxyfluorescein succinimidyl ester
DDB	1,2-Diamino-4,5-dimethoxybenzene
DTAF	5-([4,6-Dichlorotriazine-2-yl]amino)-fluorescein
EC	Electrochemical detection
FSCE	Free solution capillary electrophoresis
FL	Fluorescence
FITC	Fluorescein isothiocyanate
9-FA	9-Fluorenylacetyl
9-FMOC	9-Fluorenyl methylchloroformate
GSSG	Oxidized glutathione
GSSPro	Protein bound glutathione
GSH	Reduced glutathione
GPC	Gel permeation chromatography
HPLC	High-performance liquid chromatography
HPCE	High-performance capillary electrophoresis
HPAC	High-performance affinity chromatography
ID	Immunodetection
ICG	Indocyanine green
Immobilon	Trade name for one particular type of polyvinyldifluoride polymer
LIF	Laser induced fluorescence

LOD	Limit of detection
MECC	Micellar electrokinetic capillary chromatography
MS	Mass spectrometry
MT	Metallothionein
NDA	Naphthalene dicarboxaldehyde
OPA	*o*-Phthaldialdehyde
PITC	Phenylisothiocyanate
PDA	Photodiode array detector
ppm	Parts-per-million
RIA	Radioimmunoassay
R.S.D.	Relative standard deviation
RPLC	Reversed-phase liquid chromatography
SPR	Solid-phase reagent or reactor
SPE	Solid-phase extractor
TRITC	Tetramethylrhodamine isothiocyanate
UV	Ultraviolet

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