analytical chemistry

ARTICLE

pubs.acs.org/ac

Development of Soluble Ester-Linked Aldehyde Polymers for Proteomics

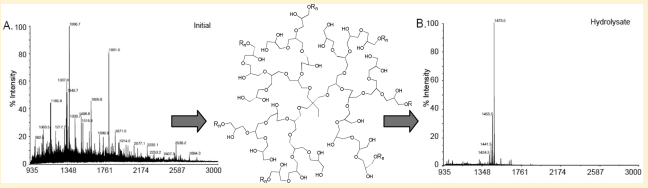
Patrick Beaudette,[†,‡] Nicholas A. A. Rossi,[†,§] Pitter F. Huesgen,[†,‡] Xifei Yu,[†,§] Rajesh A. Shenoi,[†,§] Alain Doucet,[†,¶] Christopher M. Overall,[*,†,‡,∥] and Jayachandran N. Kizhakkedathu[*,†,§,⊥]

[†]Centre for Blood Research, [‡]Department of Biochemistry and Molecular Biology, [§]Department of Pathology and Laboratory Medicine, [∥]Department of Oral Biological and Medical Sciences, and [⊥]Department of Chemistry, The University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

[¶]Institute of Systems Biology, The University of Ottawa, Ottawa, Ontario, Canada K1N 6N5

Supporting Information

ABSTRACT: High molecular weight hyperbranched polyglycerol (HPG) was selected for development as a soluble polymer support for the targeted selection and release of primary-amine containing peptides from a complex mixture. HPG has been functionalized with ester-linked aldehyde groups that can bind primary-amine containing peptides via a reductive alkylation reaction. Once bound, the high molecular weight of the polymer facilitates separation from a complex peptide mixture by employing either a 30 kDa molecular weight cutoff membrane or precipitation in acetonitrile. Following the removal of unbound peptides and reagents, subsequent hydrolysis of the ester linker releases the bound peptide into solution for analysis by mass spectrometry. Released peptides retain the linker moiety and are therefore characteristically mass-shifted. Four water-soluble cleavable aldehyde polymers (CAP1, CAP2, CAP3, and CAP4) ranging in types of linker groups, length of the linker groups, have been prepared and characterized, each demonstrating the ability to selectively enrich and sequence primary-amine peptides from a complex human proteome containing blocked (dimethylated amine) and unblocked (primary amine) peptides. The polymers have very low nonspecific peptide-binding properties while possessing significantly more reactive groups per milligram of the support than commercially available resins. The polymers exhibit a range of reactivities and binding capacities that depend on the type of linker group between the aldehyde group and the polymer. Using various linker structures, we also probed the mechanism of the observed dehydration of hydrolyzed peptides during matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis.



Tandem mass spectrometry (MS/MS) has become the primary methodology for the rapid identification and measurement of proteins in biological samples.[1,2] In shotgun proteomics approaches, intact proteins are typically first cleaved to peptides by digestion with a protease of canonical specificity such as trypsin.[3] Compared to proteins, tryptic peptide masses can be determined with higher accuracy and precision[4] and are also more amenable to analysis by MS due to improved ionization and solubility properties. However, with each protein yielding many tryptic peptides, proteolytic digestion of a whole proteome significantly increases the sample complexity, so reducing the possibility that every protein present will be identified.[5]

To overcome what has been termed "undersampling",[6] peptide fractionation can be performed before MS analysis to reduce sample complexity. Fewer peptides simultaneously entering the MS ion source minimizes deleterious ion suppression effects and results in higher-quality MS/MS spectra and an increased number of confident peptide identifications. Several methods including the separation at the peptide level by chromatographic methods or at the protein level[7] have been attempted. However, although effective at reducing sample complexity, each additional dimension of sample fractionation exponentially increases the total number of samples to analyze, significantly increasing the amount of MS time and bioinformatics analysis required.

Alternatively, in certain proteomics studies, such as those involving post-translational modifications[8] or relative quantification between samples,[9] complex peptide mixtures are simplified by enriching for a subset of target peptides. For example, His-containing peptides are purified by nickel cation affinity chromatography, and phosphorylated peptides are captured using immobilized metal oxides (titanium, zirconium)[10,11] or antibodies directed toward phosphate groups.[12] Primary amines[13] and cysteine sulfhydryl groups[9,14] can be specifically labeled with biotin reagents, followed by enrichment of biotinylated peptides with streptavidin. In such methods, the affinity ligands are physically supported on an insoluble matrix, often resins or beads composed of highly cross-linked polymers. These supports can be packed into columns or given magnetic properties to facilitate

Received: February 23, 2011
Accepted: July 20, 2011
Published: July 20, 2011

purification by physically separating them from unbound components in the sample mixture. However, the interaction between the solution-phase peptides and the solid-phase ligand is suboptimal and suffers from nonlinear kinetics due to unequal access between reactive groups.[15,16] A low degree of derivatization of the support also affects yields often necessitating larger amounts of sample, which can be limiting. Additionally, the heterogeneous nature of the reaction can result in nonspecific sample loss at the solid−liquid phase interface, particularly for low-abundance species.[17]

As an alternative, replacing insoluble supports with a soluble polymer as the support allows for the restoration of more favorable homogeneous reaction conditions and improved kinetics/efficiency of coupling.[18,19] Soluble dendrimers such as polyamidoamine (PAMAM)[20] have been modified to incorporate both affinity ligands for noncovalent capture of phosphopeptides[21] and chemically reactive groups for covalent capture of phosphopeptides[22] and Cys-containing peptides.[15] The covalent capture of target peptides has the added benefit of permitting a more stringent washing and removal of nonspecific contaminants.[17]

In the study of proteolysis as a post-translational modification, the primary-amine group generated by hydrolysis of an amide bond can serve as a handle to direct enrichment efforts.[23] The primary amine at the N-terminus of the peptide cleavage products can be modified to alter the peptide hydrophobicity and change its chromatographic behavior as performed in combined fractional diagonal chromatography (COFRADIC),[24] or affinity captured by resin-immobilized streptavidin following biotinylation of peptides using small-molecule reagents or enzymes.[13] Very recently our group has demonstrated the utility of a soluble aldehyde-functionalized polyglycerol capable of irreversibly binding internal tryptic peptides to enrich for amine-blocked N-terminal peptides in solution.[25−27]

Here we present a group of novel, water-soluble functional polymer supports for proteomics based on hyperbranched polyglycerol (HPG). These soluble supports are functionalized with amine-reactive aldehyde groups attached through a cleavable ester linkage. Primary-amine containing peptides are selectively and covalently attached by reductive alkylation and later released back into solution for MS analysis by base hydrolysis—a catch-and-release mechanism. The peptide mass is shifted characteristically because the cleaved polymer linker remnant remains attached to the N-terminus allowing for convenient independent identification of bound peptides. We investigated the reactivity of aldehyde groups attached to polymer through various linker groups. These new polymer supports are shown to have virtually no nonspecific binding and very high binding capacity. We also demonstrate the application of the new support for the enrichment and analysis of selected peptides from a highly complex mixture of peptides from cell lysate.

EXPERIMENTAL SECTION

Details of the synthesis of different aldehyde-functionalized polymers, their characterization, and peptide binding and identification analysis are given in the Supporting Information.

RESULTS

Synthesis of Polymer Supports. In order to realize the workflow illustrated in Figure 1, soluble polymer supports with variations in the linker between the aldehyde group and

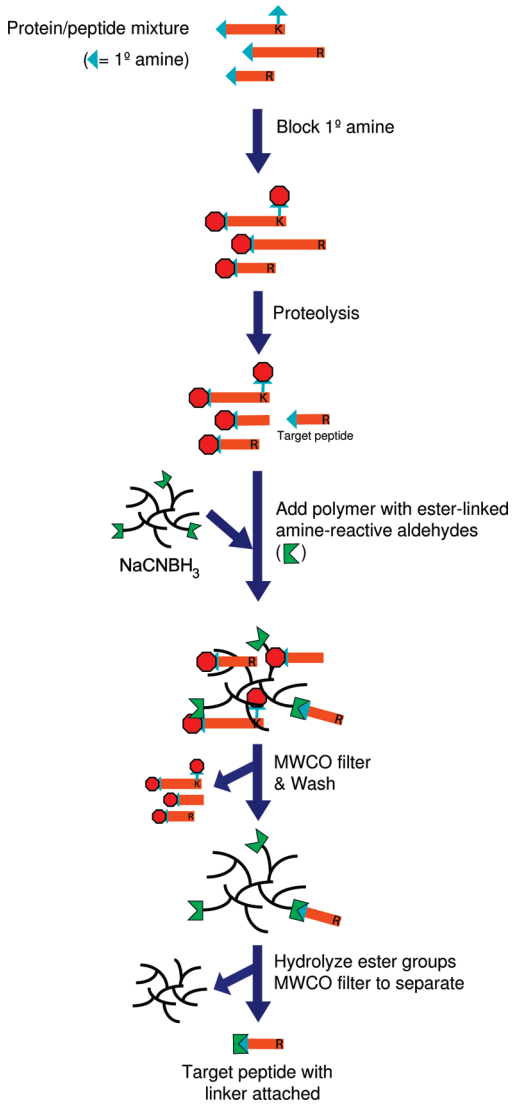


Figure 1. Schematic illustration of a soluble-polymer-based procedure to isolate primary-amine containing peptides from a complex mixture.

the ester group were synthesized (Figure 2) to optimize the peptide binding and release. Four different synthetic pathways were used for the generation of new aldehyde-functionalized HPG (CAP1, CAP2, CAP3, and CAP4) which were compared in terms of the peptide reactivity to the aldehyde groups on the polymer and their cleavage from the different supports. CAP1 and CAP2 (Figure 2) differ in terms of spacer length between aldehyde group and amide group (one carbon and two carbons, respectively). In the case of CAP3, the amide group of the CAP2 linker was replaced by an ester group. Thus, comparisons were made between CAP1 and CAP2, and CAP2 and CAP3. Succinic acid based linker was used in CAP1, CAP2, and CAP3 for linking the polymer backbone and the reactive groups. CAP4 was synthesized for probing the mechanism of dehydration. We followed a previously reported procedure for the synthesis of HPG-ALD,[25] and the chemical structure HPG-ALD is given in Figure 2.

Synthesis of CAP1. CAP1 was synthesized by the quantitative conversion of hydroxyl groups to ester-linked 1,2-diols (Scheme 1S, Supporting Information) in two steps. Initially HPG was

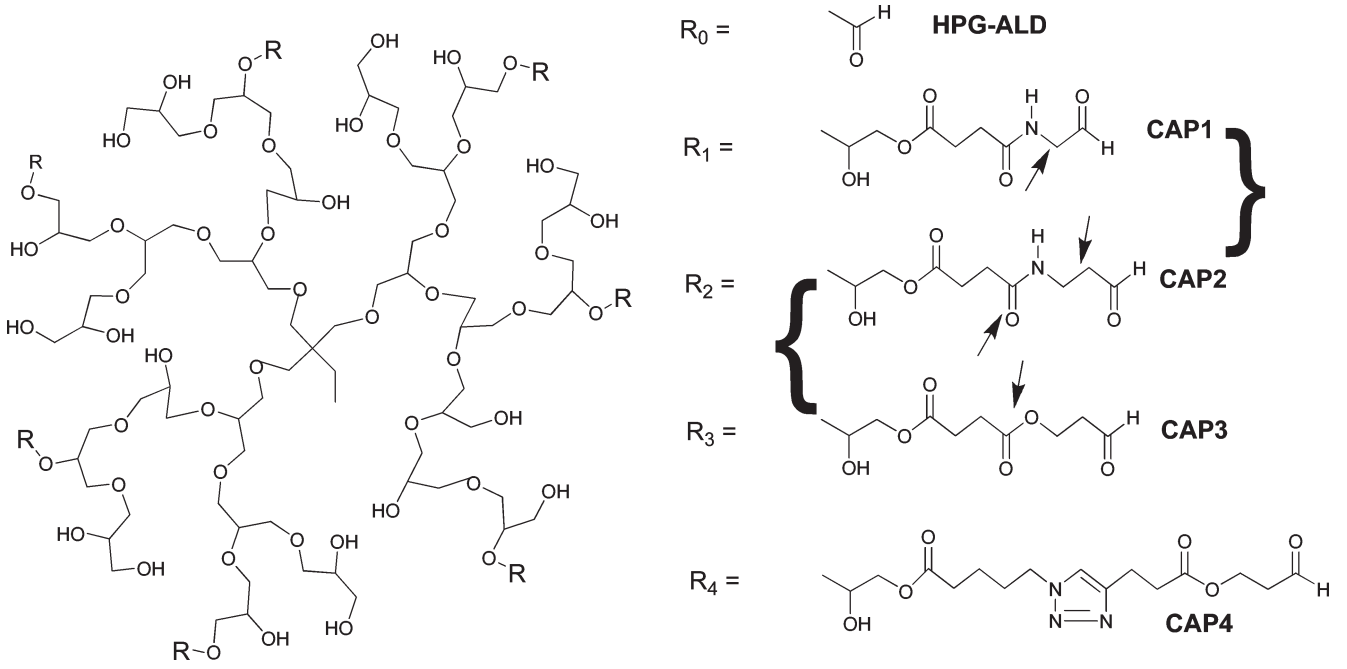


Figure 2. Structure of polyglycidol and the various soluble ester-linked cleavable aldehyde polymer (CAP) supports. Structural representation of polyglycerol (HPG), a dendritic and water-soluble polymer. The hydroxyl groups have been modified with each of the four cleavable aldehyde (R) groups and a noncleavable group shown on the right. The brackets shown in the figure highlight the polymers which were compared for reactivity and cleavage analysis.

Table 1. Characteristics, Binding Capacity, and Reactivity of the Polymers

			binding capacity	
name and structure[a]	no. of aldehydes/ polymer	affinity (slope)	150 nmol CHO (nmol peptide)	300 nmol CHO (nmol peptide)
HPG-ALD	124	0.104	7.88	7.92
CAP1	191	0.010	1.92	3.03
CAP2	99	0.067	7.25	8.62
CAP3	279	0.010	1.83	2.66

[a] In all the cases, HPG having M_n 444K and M_w/M_n 1.1 was used as starting polymer.

modified by a reaction of succinic anhydride to generate HPG acid followed by the coupling of 3-amino-1,2-propanediol. The limited conversion of terminal 1,2-diols generated the desired aldehyde-functionalized CAP1 support (Figure 2, Table 1). This synthetic scheme afforded a five-carbon spacer containing an ester group and an amide group between the polymer and the aldehyde group. There is also a one-carbon spacer (methylene group) between the amide group and aldehyde group. The aldehyde density on the polymer was varied by altering the ratio of periodic acid to the number of 1,2-diols present (see Table 1S, Supporting Information). The aldehyde polymer was stored as an aqueous solution at −80 °C under argon.

Synthesis of CAP2. In the case of CAP2, a different strategy was used to incorporate ester-linked aldehydes onto the polymer support (Scheme 2S, Supporting Information). In this synthetic scheme, HPG was reacted with a limited quantity of succinic anhydride to generate a carboxyl-functionalized HPG followed by coupling to 1-amino-3,3-diethoxypropane to generate an aldehyde precursor polymer, CAP2-acetal. The desired aldehyde polymer (CAP2, Supporting Information Scheme 2S, Figure 2, Table 1) was obtained by the acid hydrolysis of CAP2-acetal by trifluoroacetic acid. This synthetic scheme afforded a six-carbon spacer containing an ester group and an amide group between the polymer and the aldehyde group. The two-carbon spacer (ethylene group) between the amide group and aldehyde group (CAP2) allowed for a comparison to be made with the one-carbon spacer (CAP1) in terms of peptide capture and release.

Synthesis of CAP3. Another approach (Scheme 3S, Supporting Information) was used for the generation of CAP3 (Table 1). In this case, a six-carbon spacer was generated between the aldehyde group and the polymer by substituting the amide group of CAP2 spacer group with an ester group. Thus, the effect of ester (CAP3) and amide group (CAP2) (Figure 2) in the middle of the spacer group on peptide capture and release was determined. To synthesize CAP3, HPG was modified using 4-(3,3-diethoxypropoxy)-4-oxobutanoic acid followed by the deprotection of the acetal groups (see the Supporting Information).

Synthesis of CAP4. CAP4 was synthesized for probing the mechanism of dehydration of hydrolytically released peptides (see later sections) and was not used as a support for peptide enrichment. In the case of CAP4, a "click chemistry" approach was used (Scheme 4S, Supporting Information). HPG was modified using 5-azido valeric acid followed by reaction with 3,3-diethoxypropyl pent-4-ynoate in presence of Cu(I) catalyst to generate CAP4 (Figure 2, Table 1). 3,3-Diethoxypropyl pent-4-ynoate was synthesized by the reaction of 3,3-diethoxy-1-propanol with pentynoic acid (see the Supporting Information).

Effect of Linker Structure on the Peptide Reactivity and Binding Capacity of Polymer-Supported Aldehydes. Four aldehyde polymers (HPG-ALD, CAP1, CAP2, and CAP3) were

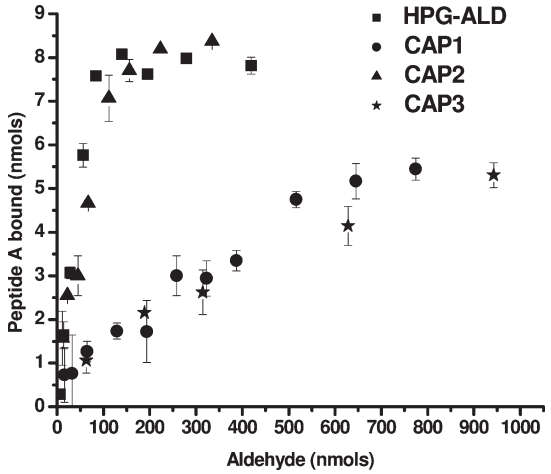


Figure 3. Reactivity of peptide-binding polymers and binding capacity. Ten micrograms (9.5 nmol) of peptide A was incubated with increasing amounts of the aldehyde polymers overnight: noncleavable aldehyde polymer HPG-ALD and three cleavable aldehyde polymers CAP1, CAP2, and CAP3. Plotted is the number of nanomoles of peptide A bound vs the number of binding sites (aldehyde groups) present.

evaluated with respect to reactivity toward the α-amine of a standard peptide A (H_2N−VWESATPLR−COOH, $[M + H^+]$ = 1058]) (Table 1). Peptides were covalently coupled to the polymer support using a reductive amination reaction in presence of $NaCNBH_3$. Figure 3 shows the relationship between the amounts of peptide A (in nanomoles) bound to an increasing amount of polymer added to solution; the concentration of peptide A added was fixed at 9.5 nmol. Since the number and type of aldehydes on the polymer support were varied, the value was normalized to the number of nanomoles of aldehydes present per reaction. HPG-ALD, the noncleavable aldehydes polymer, showed a high reactivity as indicated by the sharp increase in peptide binding with the increase in the amount of aldehyde groups. The HPG-ALD polymer was saturated at about 8 nmol of peptide A. Similar behavior is observed for CAP2, reaching a near plateau at approximately 8.3 nmol of peptide bound and 334 nmol of aldehyde. The data points for CAP1 have a much gentler slope, and the relationship between nanomoles of peptide bound and nanomoles of aldehyde in solution is more linear rather than the hyperbolic shape observed for HPG-ALD and CAP2. CAP3 shows an even more linear relationship, and neither CAP1 nor CAP3 reach a saturation plateau at the maximum concentration of aldehydes, the solubility limit in this assay. Table 1 gives a binding capacity for each at both 150 and 300 nmol of aldehyde. The slope of the curves between 0 and 150 nmol of aldehyde were calculated as a means for comparing the binding affinity of peptide A toward the peptide-reactive groups. A comparison between CAP1 and CAP2 (difference between these two supports is the spacer length between aldehyde and amide group (Figure 2)) showed that an ethylene spacer makes the support more reactive to the α-amine of the peptide compared to methylene spacer at similar experimental conditions. Also, the substitution of amide group in CAP2 with an ester group as in CAP3 (all other parameters kept constant) produced an aldehyde group that was less reactive toward peptides. On the basis of this data, HPG-ALD gave the highest reactivity, and among the new cleavable polymer supports, CAP2 exhibited highest reactivity toward primary-amine containing peptides.

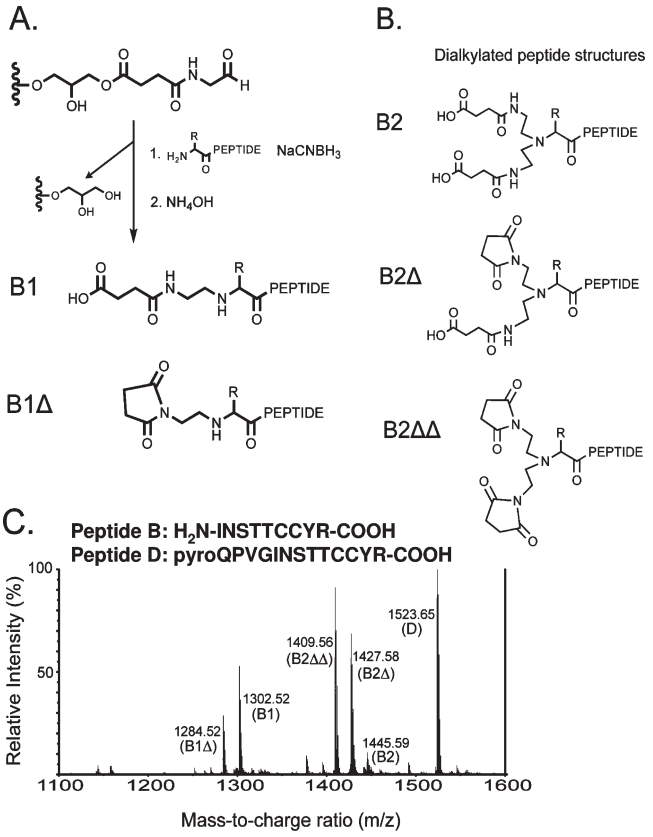


Figure 4. Binding and release of peptide B from polymer support (CAP1). (A) Peptide binds to the polymer through a reductive alkylation reaction between the peptide N-terminal and the polymer aldehyde. Treatment with 100 mM NH_4OH, pH 10, hydrolyzes the ester linker between the peptide and polymer, releasing the peptide. The peptide mass has increased due to the covalent attachment of the polymer linker group, forming species B1. Once hydrolyzed, this linker group can cyclize and dehydrate, forming species B1Δ. ("1" signifies monoalkylation; Δ signifies a loss of one H_2O molecule). (B) The structures of dialkylated peptide species with their corresponding mass shifts are shown. With two polymer-linked aldehyde groups reacted with a single peptide N-terminal, two separate dehydration events can occur to form the three structures B2, B2Δ, and B2ΔΔ. (C) MALDI-TOF MS spectrum of peptide B cleaved from CAP1 polymer support. Signals at 1302 and 1284 m/z are monoalkylated species B1 and B1Δ. Dialkylated peptide B species are present at higher abundance relative to the monoalkylated peptide B. Signals at 1445, 1427, and 1409 m/z represent B2, B2Δ, and B2ΔΔ, respectively. Peptide D lacks a primary amine and cannot react with the polymer aldehydes. Therefore, peptide D has not undergone the characteristic mass shift due to modification by the polymer linker.

Selective Peptide Binding and Release from the Polymer Support. An N-terminal (α-amine) free peptide B (INTSTTCCYR, $[M + H^+]$ = 1159) and N-terminal-blocked peptide D (pyroQPVGINTSTTCCYR, $[M + H^+]$ = 1523) were used for the evaluation of selective peptide binding and separation from a simple peptide mixture. CAP1 was used for initial studies. Following peptide binding in the presence of $NaCNBH_3$, the flow through was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). N-Terminal free amine was completely removed by the polymer

from the mixture as evidenced by the MALDI-TOF MS of the flow through. The unbound N-terminal-blocked peptides were also removed from the polymer support by repeated washing.

Following the coupling and separation, the polymer-bound peptides were hydrolyzed (Figure 4A) to release the bound peptides and were analyzed by MALDI-TOF MS. A number of signals were observed in the MALDI-TOF mass spectrum of the hydrolysate (Figure 4C). A signal at 1302 m/z corresponds to the anticipated mass shift of 143 Da for the linker modification (monoalkylation) of the peptide N-terminus (Figure 4A). Adjacent to this peak is another signal at 1284 m/z, representing a loss of 18 Da (dehydration) from the monoalkylated peak. A dialkylation of the peptide B N-terminus results in a mass shift of 286 Da, and the expected signal would be at 1445 m/z. Indeed there is an additional cluster of signals for peptide B present at 1445, 1427, and 1409 m/z, corresponding to the dialkylated product and losses of 18 and 36 Da, respectively. Figure 4B illustrates the dialkylated peptide structures. A peak for blocked peptide D is present at 1523 m/z. Its unmodified mass indicates that this blocked peptide did not react with the polymer-linked aldehyde groups but has been carried over through the separation and washing steps that sought to eliminate it.

All signals represent the same amino acid sequence information. As illustrated in Supporting Information Table 2S for peptide B, rather than one signal to select for sequencing by MS/MS, the peptide's signal intensity has been diluted across five peaks. High signal intensity for MS/MS is important for confident identification of a peptide in a bioinformatics analysis. A peptide recovered in low amounts as five separate m/z values has a lower likelihood of being correctly identified due to this reduction of intensity. Therefore, further development of the polymer and other strategies were employed to eliminate the redundant peptide signals. As the proposed dehydration reaction was an inherent property of the polymer's linker moiety, the dialkylation issue was given the foremost concern. Two strategies were designed to prevent this unwanted side reaction.

Strategies for Reducing Dialkylation of Bound Peptides. *Effect of Aldehyde Density on the Polymer.* The close proximity of amine-reactive aldehyde groups on the polymer surface was thought to play a role in promoting dialkylation. In separate reactions, three unblocked peptides (A, B, and C (H_2N–FYQLPSNQYQSTR–COOH, $[M + H^+] = 1631$)) and one blocked peptide (D) were incubated with two polymers, CAP1-II and CAP1-III, having 99 and 495 aldehyde groups per molecule, respectively (Table 1 and Supporting Information Table 1S). MALDI-TOF mass spectra (Figure 1SA, Supporting Information) of the hydrolysate from CAP1-III exhibit significant levels of the dialkylated peptide products. Indeed, for peptides B and C the dialkylated product represents the major species in solution. CAP1-II has 20% of the aldehyde density as CAP1-III, and in the MALDI-TOF mass spectrum of the hydrolysate the dialkylated peptide products are dramatically reduced in relative abundance to their monoalkylated counterparts. For all peptide species, both mono- and dialkylated versions exhibit high relative levels of the characteristic dehydration peaks at −18 and −36 Da. All signals are tabulated and identified in Supporting Information Figure 1SB. The data suggest that a reduction in aldehyde density decreased the possibility of dialkylation of captured peptides or increase in aldehyde density can generate predominantly dialkylated species.

Effect of Reaction Time. The reductive alkylation reaction responsible for peptides binding covalently to the polymer aldehydes proceeds via an intermediate Schiff base, a structure having a double bond between the peptide amine and the aldehyde carbon (Figure 2SA, Supporting Information). Subsequent reduction of the double bond to the single bond by $NaCNBH_3$ is an irreversible process that drives the reaction. Equilibrating the peptides with the polymer aldehydes in the absence of $NaCNBH_3$ means that this intermediate, a monoalkylated peptide–polymer conjugate, is as far as the reaction can advance. A short exposure to the reducing agent following equilibration completes the irreversible phase of the reaction, and the limited time window would inhibit the desired secondary amine of the monoalkylated peptide from reacting further at an adjacent aldehyde site to become dialkylated.

When the MS spectra of hydrolysate (Supporting Information Figure 2SB) for the normal 16 h reaction period ($NaCNBH_3$ throughout present at 20 mM) are examined, one sees major dialkylation of peptide B (approximately 1:1 with monoalkylated peptide B) and lower but still significant dialkylation of peptides A and C. The lower panel of Supporting Information Figure 2SB represents a reaction in which the reducing agent was introduced only for the final 30 min, after the peptides and aldehydes had equilibrated to form Schiff base intermediates for the previous 15.5 h. For peptides A and C, the dialkylated species have been reduced to the level of noise, whereas for peptide B the dialkylated species that dominated in the normal reaction has been dramatically reduced to less than 10% of its monoalkylated counterpart. For all of the polymer-conjugated peptide species MALDI-TOF spectra showed characteristic dehydration peaks at −18 and −36 Da. Thus, a reduction in reaction time dramatically decreased the dialkylation of captured peptides. Both strategies for reducing or increasing dialkylation showed promise in limiting the generation of this multiple species.

Effect of Polymer/Peptide Ratio. To further optimize the peptide binding to the polymer support, the effect of different ratios of polymer to peptide in the binding reaction was studied. The mass spectra of the hydrolysate (Figure 3S, Supporting Information) illustrate that increasing the amount of CAP1 results in differential recovery of the primary-amine peptides A, B, and C. At the 50 μg level, monoalkylated peptide B is the dominant product, while peptides A and C are at a level below 10% relative to peptide B. As the amount of CAP1 incubated with the peptides increased, so too did the levels of peptides A and C in the hydrolysate. However, the increasing concentration of aldehyde in the reaction did not lead to increased dialkylation of peptides, indicating that dialkylation is a result of intramolecular reactions. Peptide D is present in all spectra without modification, indicating carryover of this unbound peptide throughout the washing of this polymer batch.

Mechanism of Dehydration of Cleaved Peptides. We further investigated the possible dehydration mechanism observed during the MALDI-TOF analysis of cleaved peptides under optimized binding conditions. The characteristic loss of 18 Da from monoalkylated peptides and 18 and 36 Da from dialkylated peptides illustrated in mass spectra of hydrolytically released peptides from CAP1 correspond to the loss of one or two molecules of H_2O (18 Da), respectively (Figure 5). The proposed mechanism for this dehydration is a cyclization of the modified N-terminus under acidic MALDI-TOF MS conditions as shown in Figure 5A. CAP1, CAP2, and CAP3 have in common the succinic acid derived four-carbon unit as part of the linker moiety joining the peptide-binding site to the polymer backbone via an ester group. When a covalently bound peptide A is released

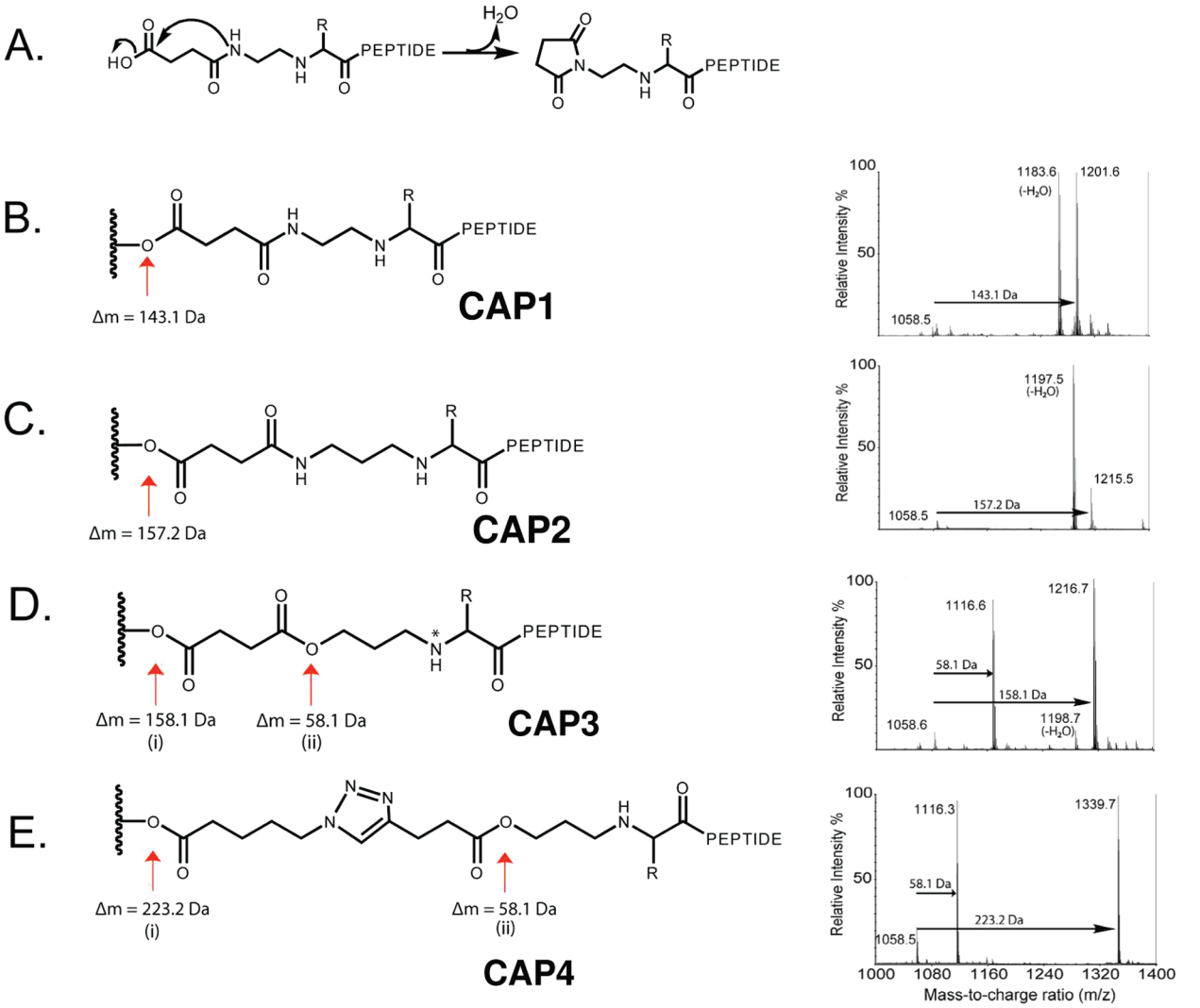


Figure 5. Comparison of the dehydration of peptide N-terminus with polymer linker covalently attached from different polymer supports. (A) Proposed mechanism for the dehydration of the linker-modified peptide N-terminus by cyclization. (B–E) Structures of the cleavable peptide-binding polymers with a peptide attached. Arrows indicate the hydrolyzable ester groups. Both CAP3 and CAP4 possess two base-cleavable esters. (B) Structure of CAP1 with peptide bound. MALDI-TOF mass spectrum showing the cleaved peptide at the expected mass shift due to monoalkylation (CAP1 linker modification) and another peak at 18 Da less (dehydration). (C) Second-generation aldehyde polymer CAP2. The spectrum shows the monoalkylated peptide at the expected mass shift and its dehydrated version. (D) Cleavable polymer CAP3. This polymer has two esters in its linker region and two possible hydrolysis products. Both products are observed in the spectrum, but only hydrolysis at the left ester yields a peptide undergoing dehydration, likely forming a nine-membered ring between the terminal carbonyl and the amide (*). (E) A cleavable aldehyde polymer CAP4 with peptide bound. This polymer's linker possesses two esters and two possible hydrolysis products. Both products are observed in the spectrum, and neither undergoes a dehydration.

by hydrolysis, the mass spectrum of this hydrolysate for all of these polymers exhibits a signal at 18 Da less than the expected mass shift due to the linker attachment. CAP3 possesses two base-hydrolyzable ester groups in the linker, i and ii (Figure 5D). Hydrolysis at group i generates a peptide species with this four-carbon unit with an adjacent dehydration peak at 18 Da less. Hydrolysis at ii removes this four-carbon unit from the peptide, and no dehydration is observed, suggesting that presence of the carboxylic acid in the linker group is playing a role in the dehydration mechanism. In the case of CAP3, it is predicted that dehydration would form a nine-membered ring between the amide and the terminal carboxylic acid. As this structure is less favored, the dehydration is less extensive and only accounts for about 10% of the monoalkylated peptide A signal (Figure 5D). To probe this dehydration mechanism we developed a fourth cleavable polymer, CAP4 (Figure 5E), that lacks the structural feature common to the other polymers. CAP4 possesses two base-labile ester groups in the linker. Peptide species corresponding to hydrolysis at both ester groups are observed, but in neither case are additional dehydration peaks associated, supporting the proposed mechanism.

Assessment of Nonspecific Binding to Polymers. Another important aspect of developing a soluble polymer support for proteomic applications is their low nonspecific peptide binding, and this was studied using amine-blocked BSA tryptic peptides. The BSA tryptic peptides with dimethylated tertiary amines display no nonspecific binding to the polymers HPG-ALD, CAP1, and CAP2. In the pooled 30 kDa molecular weight cutoff

dx.doi.org/10.1021/ac200419p |*Anal. Chem.* 2011, 83, 6500–6510

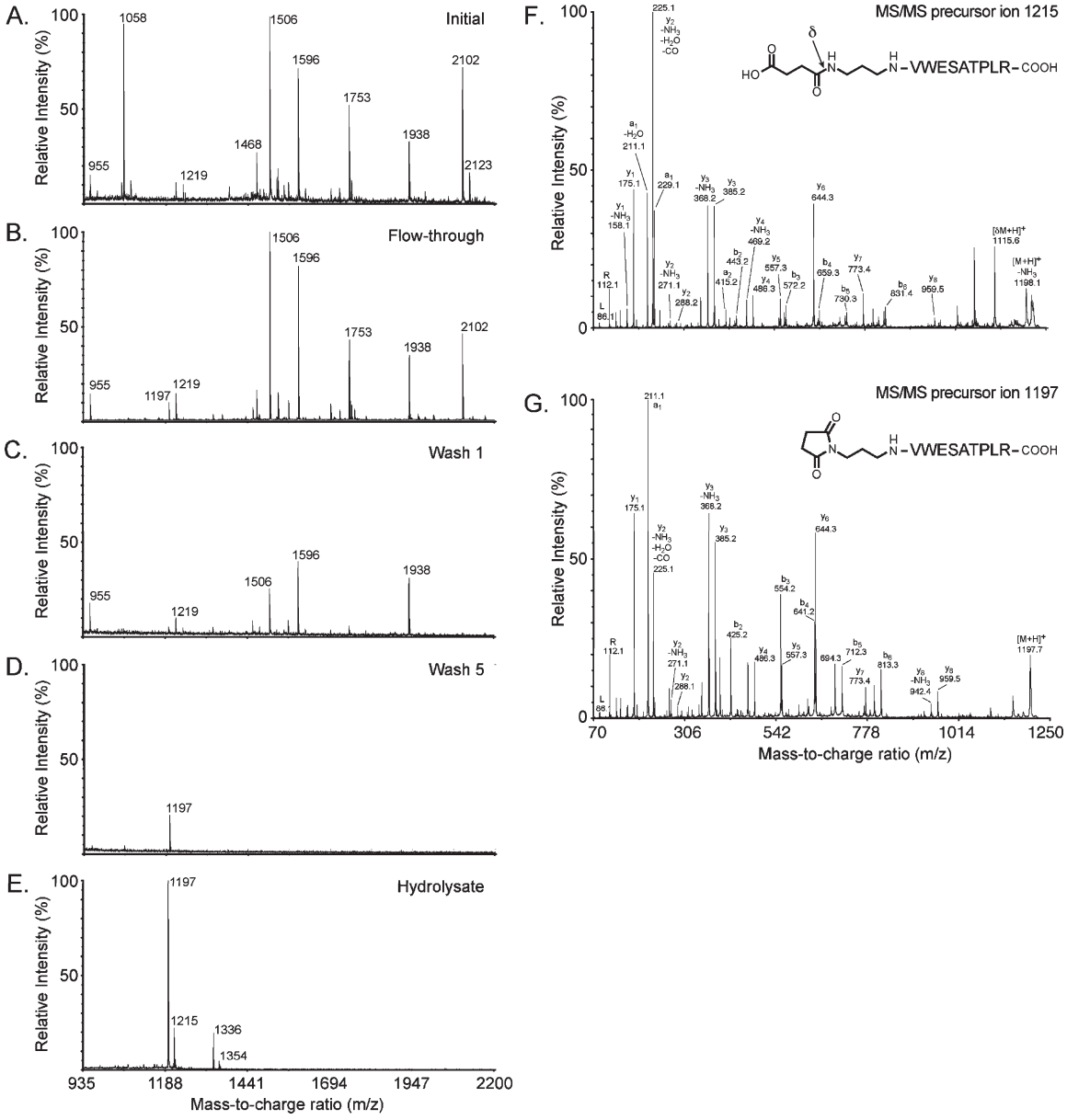


Figure 6. Proof of concept: selective separation of peptide A from a complex peptide mixture by MALDI-TOF analysis. Unblocked peptide A (2 nmol) and light-formaldehyde-blocked BSA tryptic digest (20 μg) were incubated overnight with cleavable aldehyde polymer CAP2 (1000 μg; CAP2 used for this study has ~99 aldehydes/polymer). (A) MALDI-TOF mass spectrum shows the initial conditions with no polymer added. (B) Following incubation, the 30 kDa MWCO flow through contains only a near-baseline signal for peptide A, indicating near-complete depletion from the solution. Unbound blocked BSA tryptic peptides are also observed. (C and D) Washes 1 and 5 demonstrate removal of the BSA tryptic peptides from the CAP2−peptide A complex. (E) Following washing of the CAP2−peptide A conjugate and hydrolysis of the linker's ester group, the MALDI-TOF spectrum of the hydrolysate shows recovery of the released peptide, present largely in its monoalkylated and dehydrated form. (F) MALDI-Q/TOF MS/MS spectrum of the monoalkylated peptide A precursor ion with m/z = 1215. The fragmentation site in the CAP2 modification at the peptide N-terminus is indicated with δ. (G) MALDI-Q/TOF MS/MS spectrum of the monoalkylated, dehydrated peptide A precursor ion with m/z = 1197.

(MWCO) flow though, heavy−light labeled peptide pairs show an equal intensity in the MALDI-TOF MS spectra, the light peptides having previously been incubated in buffer with 1000 μg of polymer while the heavy peptides were incubated without polymer. Peptide pairs are separated by 6 or 12 Da in the case of peptides with a lysine residue. For HPG-ALD the peptide pairs were of equal (1:1) intensity in the flow through (Figure 4SA, Supporting Information). For CAP1, the peptide pairs (Supporting Information Figure 4SB) are mixed between being of equal intensity and pairs where the light peptide has a higher abundance. The greater abundance of the light peptide relative to the heavy is more pronounced in the first wash fraction (100 μL of 50 mM HEPES). The opposite trend is observed when heavy peptides are incubated with polymer (Supporting Information Figure 4SB, bottom). Virtually all peptide signals have disappeared by the fourth wash (100 μL of 2 M NaCl/50 mM HEPES), and hydrolysis of CAP1 recovered from the incubation yields no further peptide signals. In the initial flow through for CAP2 (Supporting Information Figure 4SC), the light peptide is more abundant in each peptide pair, on average about 30%

dx.doi.org/10.1021/ac200419p |*Anal. Chem.* 2011, 83, 6500–6510

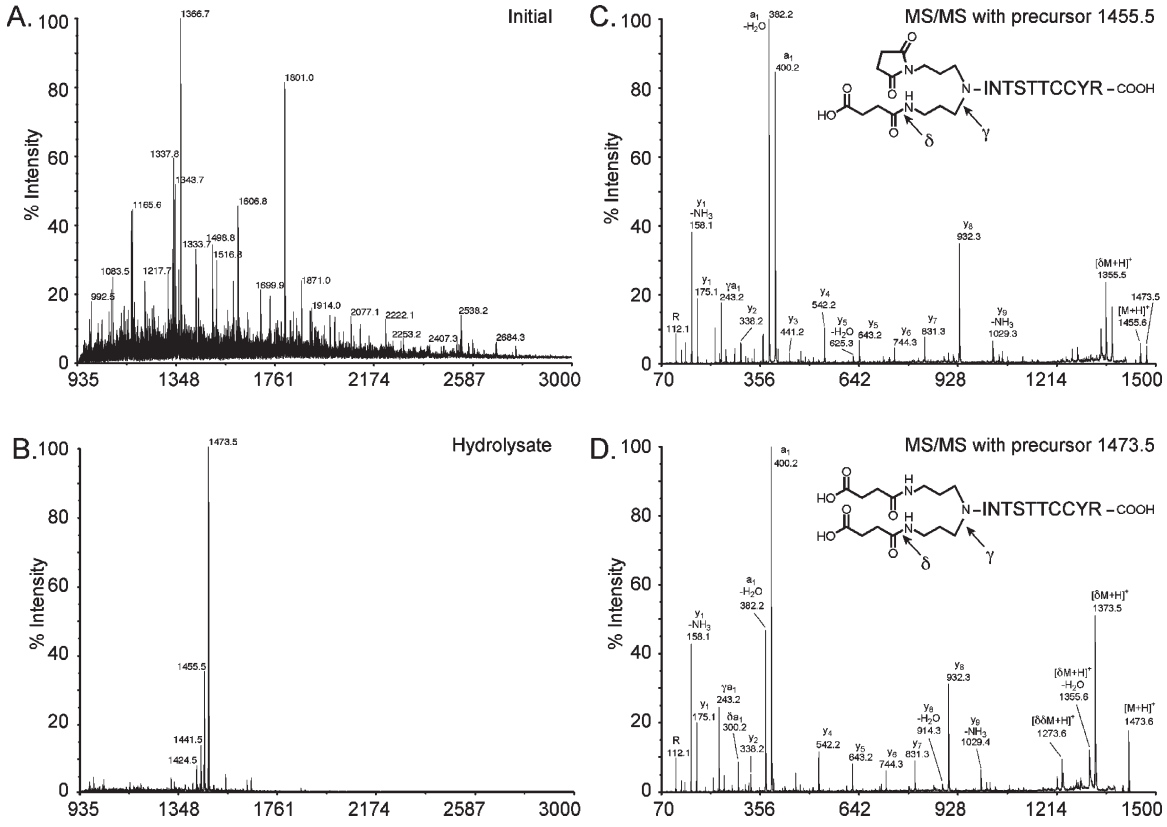


Figure 7. Proof of concept: selective separation of peptide B from a highly complex human proteome digest and identification by MALDI-TOF/TOF. Reactive peptide B (10 nmol) and blocked peptide D (10 nmol) were mixed with formaldehyde-modified human GluC-digested whole cell extract (200 μg) and incubated overnight with cleavable aldehyde polymer CAP2 (1000 μg; CAP2 used for this study has ~180 aldehydes/polymer). (A) MALDI-TOF mass spectrum showing the initial conditions with no polymer added. (B) MALDI-TOF spectrum of the eluate after extensive washing and hydrolysis of the polymer linker's ester group. Peptide B is selectively enriched and present largely in its dialkylated and dialkylated−monodehydrated form. (C) MALDI-TOF/TOF MS/MS spectrum of the dialkylated−monodehydrated peptide B precursor ion with m/z = 1455.5. The observed fragmentation within the CAP2 linker modification is indicated with δ. (D) MALDI-TOF/TOF MS/MS spectrum of the dialkylated peptide B precursor ion with m/z = 1473.5. Fragmentation sites within the CAP2 linker modification are indicated by γ and δ.

greater, with the exception of pair C where the light peptide intensity fully doubles that of the heavy. As with CAP1 this trend is again seen in the first wash fraction; the light−heavy peptide ratio is significantly greater than 1. Peptide signals have been reduced to baseline by the fourth wash, and hydrolysis of CAP2 yielded no peptide signal. No significant differences in nonspecific binding were observed for the three tested polymers despite their different structures and degrees of modification.

Selective Capture, Enrichment, and Sequencing of Peptides from Complex Peptide Mixtures. The goal of first part of this experiment was to demonstrate specific recovery of a peptide with a free N-terminal, an α-amine (peptide A) from a mixture of N-terminal-blocked BSA tryptic peptides and its sequencing. Results are shown in Figure 6. The peptide-binding procedure was carried out at optimized conditions using CAP2. An acetonitrile (ACN) precipitation of the CAP2−peptide conjugate and a more vigorous washing regimen using 2 M NaCl/50 mM HEPES as well as 20% ACN/50 mM HEPES were added in the protocol to remove unbound peptides. Peptide A (m/z = 1058) is seen at high intensity in the initial experimental conditions, prior to addition of the polymer. Following incubation to bind the peptides and ACN precipitation, the supernatant was evaporated and reconstituted and subjected to MALDI-TOF MS analysis (Figure 6A). Peptide A has been nearly eliminated from the spectrum, whereas the BSA tryptic peptides remain in flow through. As is evident from the spectrum of wash 1 and wash 5 these BSA peptide signals have disappeared. The hydrolysis of peptide-bound CAP2 yields the expected signals for cleaved monoalkylated peptide A ($\Delta m/z$ = 157; 1058 + 157 = 1215) from CAP2 and a prominent dehydration peak at −18 Da (m/z = 1197). MALDI-TOF/TOF MS/MS analysis confirmed the peak at m/z = 1215 as monoalkylated peptide A (Figure 6F) and the peak at m/z = 1197 as monoalkylated−dehydrated peptide A. Fragmentation of the amide bond in the polymer linker moiety (indicated by δ in Figure 6F) is observed by a characteristic shift of 100.2 Da of the unfragmented monoalkylated peptide A precursor ion. This fragmentation further contributes to a complex fragmentation pattern containing a and b series fragment ions with either intact hydrated, intact dehydrated and fragmented N-terminal CAP2 linker modification (Figure 6F). In contrast, unambiguous b and y ion series are observed for the monoalkylated−dehydrated peptide A, where the dicarbonyl-amide five-membered ring of the peptide modification is resisted to further fragmentation (Figure 6G). Dialkylated peptide A is also detected at relatively low intensity, with peaks at 1354 and 1336 m/z, representing the loss of one and two molecules of H_2O, respectively, from the hydrolysate. Notably, the dehydrated, monoalkylated peptide A is evident at low levels in the flow through and washes, indicating

dx.doi.org/10.1021/ac200419p |*Anal. Chem.* 2011, 83, 6500–6510

premature hydrolysis of the polymer linker's ester moiety under the current experimental conditions.

In the second part of the experiment, a peptide with a free N-terminal α-amine (peptide B) was selectively captured and separated from a GluC-digested, amine-modified human proteome (N-methylated using formaldehyde) containing an equal amount of blocked peptide D to demonstrate the applicability of the CAP polymers for complex proteomic experiments (Figure 7). Prior to addition of the CAP2 polymer, both peptide B and peptide D are masked by the complexity of the sample in a MALDI-TOF MS spectrum (Figure 7A). Peptide capture was carried out at overnight at 37 °C using CAP2, and the CAP2−peptide conjugate was vigorously washed with 20% ACN/50 mM HEPES, 2 M NaCl/50 mM HEPES, and finally 50 mM HEPES to remove unbound peptides. MALDI-TOF MS and MALDI-TOF/TOF MS/MS analyses of the hydrolysate showed highly selective recovery of peptide B (Figure 7B). Peptide B was present predominantly dialkylated (m/z = 1473.5; Figure 7D), but also in a monodehydrated dialkylated form (m/z = 1455.5; Figure 7C). In this experiment, CAP2 polymer with a higher aldehyde density (∼180 aldehydes/polymer) was used to drive the reaction predominantly to the dialkylated species to make the analysis simpler. Fragmentation of the amide bonds in the polymer linker moieties (indicated by δ) resulted in the loss of a neutral 100.2 Da fragments, which is observed by m/z shifts of the precursor ion and fragment ions (Figure 7, parts C and D).

DISCUSSION

We designed, synthesized, and optimized a novel polymer support useful in proteomics analyses for the enrichment and identification of primary-amine containing peptides from a complex mixture of amine-blocked peptides (Figure 1). A series of water-soluble and base-cleavable ester-linked aldehyde polymers were prepared to covalently bind peptides with a primary-amino group via reductive amination reaction. Although *N*-hydroxy succinimidyl (NHS) esters are common in peptide amine-labeling approaches, they are less stable and hydrolyze easily in aqueous systems; the aldehyde functionality offers long-term stability in aqueous conditions compared to NHS esters. The high molecular weight of the polymer facilitates easy and fast separation of the polymer with bound peptides from a complex peptide mixture using either 30 kDa molecular weight cutoff filtration or precipitation in acetonitrile. The ester groups facilitated release of bound peptides for MS analysis by a mild hydrolysis without affecting the chemical structure of the peptide. Attachment of the linker moiety to the N-termini results in a characteristic mass shift that can distinguish the covalently bound peptides, which is useful for categorizing bona fide bound peptides from any minor peptide contaminants present due to carryover. Another advantage of this approach that is being incorporated in future versions of the support is that isotopic labeling of the linker groups will enable relative quantitation techniques to be employed when comparing two samples. The predicted mass shift is also useful in polymer characterization to confirm the presence of the intended chemical functionality. Hence, this cleavable polymer is a useful reagent in proteomic workflows such as those aiming to identify the prime side of peptidic cleavage products generated by proteases. In addition, soluble polymer support restores homogeneous reaction conditions, enhancing the reaction efficiency compared to biphasic systems while still taking advantage of the large size of the polymer to enable separation from a complex mixture.

The general peptide catch-and-release protocol designed for the ester-linked aldehyde polymers has proven effective. A pH of 6.5 was selected to promote the reaction through protonation of the aldehyde carbonyl oxygen. As shown in Figure 6, the 16 h binding has fully depleted peptide A from a complex mixture, and postwashing hydrolysis yields only the desired hydrated and dehydrated signals for CAP2 linker-modified peptide A. The use of higher pH (>12) during the hydrolysis led to some peptide degradation, but no such degradation was observed for hydrolysis at pH 10 under the conditions used. There was no peptide degradation observed when tryptic BSA digest was subjected to cleavage conditions (data not shown). With respect to peptide binding and release behavior, several unexpected hurdles were presented during the development. The first was the MALDI-TOF MS observation of extensive peptide dialkylation in the hydrolysate (Figure 4C). The original polymer design called for complete polymer modification to maximize the number of peptide-binding sites (Figure 2), resulting in close proximity of surface aldehyde groups. This promoted the second alkylation reaction as the singly bound peptide with a reactive secondary amine nucleophile was constrained within close proximity to an adjacent aldehyde neighbor. Reduction of the number of aldehydes per molecule to increase the spacing proved to be a logical and effective solution to minimizing this undesired intramolecular reaction (Supporting Information Figure 1S). Limiting the reaction time was also effective but resulted in incomplete binding of the available peptides (Supporting Information Figure 2S). The type of linker groups may also influence the dialkylation reaction. The optimization of experimental conditions to drive the reaction to generate single species (predominantly monoalkylated or dialkylated product) was found to be advantageous in sequencing, and this can be controlled by fine-tuning the conditions (Figures 6 and 7).

In addition to the dialkylation reaction, the dehydration of the released peptides also complicated MS spectra. As with dialkylation, the same peptide sequence information was split over multiple peaks rather than concentrating the ion intensity on only one signal (Figure 4C). For CAP2, the cleavable polymer with the highest reactivity, the dehydration was very pronounced and indeed represented the abundant species observed in the MS spectrum. The suggested cyclization mechanism (Figure 5A), supported by the investigated panel of cleavable polymers, would not have been predicted without experimentation. The dehydration was indeed proved beneficial for sequencing in the current experimental setup as demonstrated in the MS/MS spectrum of dehydrated peptide A (Figure 6G). The dehydrated peptide A generated a less complicated MS/MS spectrum with a and b series ions only from fragments with dehydrated linker than the nondehydrated peptide A (Figure 6F). The dehydration-induced cyclization prevented the cleavage of the amide group in the linker (Figures 2 and 6F) during the MS/MS analysis. In general, the abundance of the dehydrated peptide signal relative to the hydrated peptide signal was dependent on linker length. The linker of CAP2 is one methylene group (CH_2) longer than for CAP1, and the dehydrated peak dominates the MALDI-TOF spectrum (Figure 5, parts B and C). The extra length will diminish steric hindrance effects and favor the cyclization into a five-membered ring. This dehydration mechanism is analogous to the cyclization occurring in the preparation of maleimide, a similar five-membered dicarbonyl amide ring.[28] This feature of

the polymer remains to be optimized, and alteration of the linker moiety is a critical factor.

Although dendrimers have been demonstrated for proteomic applications, their synthesis proceeds in a stepwise fashion and is tedious and very time-consuming, requiring expertise in organic synthesis methods. In contrast, HPG polymers described here possess many of the same benefits as dendrimers and can be prepared more readily, often in a single overnight reaction in high yield,[29−32] richly functionalized with reactive hydroxyl groups. It has a more flexible structure than the rigid architecture of many dendrimers, and this may further enhances the reaction kinetics in solution. The surface hydroxyls are easily converted into other chemical functionalities, such as amine-reactive aldehyde groups described here, proven to be highly valuable. Indeed we have demonstrated the synthesis of a working prototype HPG-based cleavable supports (Figure 2) in sufficient quantities as well as their application in capture, release, and the sequence identification of primary-amine containing peptides from simple peptide mixtures (Figure 6) to highly complex human proteome (Figure 7) with high efficacy, high recovery, and low nonspecific binding. Thus, in principle, CAP polymers can be adapted to proteomics workflows designed to identify proteolytic cleavage products.

In certain proteomic workflows, negative selection (bound peptides are not used for the analysis) was used reduce the sample complexity such as those described by our group recently.[25−27] However, in other approaches it is desirable to recover and analyze these bound peptides. For example, in PICS (proteomic identification of cleavage site specificity),[33,34] a peptide library prepared by tryptic digestion has all primary amines blocked by dimethylation. Treatment with the test protease generates two peptides, representing amino acid sequences C-terminal and N-terminal to the cleaved bond. As reported, the N-terminal peptides are captured by conjugation with a cleavable biotin reagent followed by streptavidin affinity pull-out. A cleavable aldehyde polymer would offer the advantage of increased capacity, a simplified workflow, greater recovery, and reduced cost and possibility of isotopic labeling of linker group for quantitative proteomics. Also in peptidomics experiments low concentrations of nontryptic peptides are encountered. Our cleavable amine-reactive polymers enable efficient capture of peptides with primary amines and fractionation of peptides with blocked amino groups from peptides with primary amines, so simplifying samples for searches and identification of the typically nontryptic peptides.[35]

■ ASSOCIATED CONTENT

Supporting Information. Experimental section and some results. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: chris.overall@ubc.ca (C.M.O.); jay@pathology.ubc.ca (J.N.K.).

■ ACKNOWLEDGMENT

P.B. acknowledges graduate scholarships from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Michael Smith Foundation for Health Research (MSFHR). J.N.K. acknowledges the funding from Canadian Institutes of Health Research (CIHR). The authors thank the LMB Macromolecular Hub at the UBC Centre for Blood Research for the use of their research facilities; the infrastructure facility is supported by the Canada Foundation for Innovation (CFI) and MSFHR. N.A.A.R. is a recipient of a CIHR/CBS postdoctoral fellowship in transfusion science. P.F.H. is supported by postdoctoral fellowships of the German Academic Exchange Service (DAAD) and MSFHR. J.N.K. is a recipient of a CIHR/CBS new investigator in transfusion science and MSFHR scholar award. C.M.O. is supported by a Canada Research Chair in Metalloproteinase Proteomics and Systems Biology. This research was supported by Grants from the Canadian Institutes of Health Research, the National Cancer Institute of Canada (with funds raised by the Canadian Cancer Association), a Special Program Grant from the Canadian Breast Cancer Research Alliance Metastasis.

■ REFERENCES

(1) Aebersold, R.; Mann, M. *Nature* **2003**, *422*, 198–207.
(2) Guerrera, I. C.; Kleiner, O. *Biosci. Rep.* **2005**, *25*, 71–93.
(3) Domon, B.; Aebersold, R. *Science* **2006**, *312*, 212–217.
(4) Zubarev, R. A.; Hakansson, P.; Sundqvist, B. *Anal. Chem.* **1996**, *68*, 4060–4063.
(5) Olsen, J. V.; Ong, S. E.; Mann, M. *Mol. Cell. Proteomics* **2004**, *3*, 608–614.
(6) Issaq, H. J.; Xiao, Z.; Veenstra, T. D. *Chem. Rev.* **2007**, *107*, 3601–3620.
(7) Wang, H.; Hanash, S. *Mass Spectrom. Rev.* **2005**, *24*, 413–426.
(8) Mann, M.; Jensen, O. N. *Nat. Biotechnol.* **2003**, *21*, 255–261.
(9) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994–999.
(10) Gaberc-Porekar, V.; Menart, V. *J. Biochem. Biophys. Methods* **2001**, *49*, 335–360.
(11) Chaga, G. S. *J. Biochem. Biophys. Methods* **2001**, *49*, 313–334.
(12) Ficarro, S. B.; McCleland, M. L.; Stukenberg, P. T.; Burke, D. J.; Ross, M. M.; Shabanowitz, J.; Hunt, D. F.; White, F. M. *Nat. Biotechnol.* **2002**, *20*, 301–305.
(13) Mahrus, S.; Trinidad, J. C.; Barkan, D. T.; Sali, A.; Burlingame, A. L.; Wells, J. A. *Cell* **2008**, *134*, 866–876.
(14) Butler, G. S.; Dean, R. A.; Tam, E. M.; Overall, C. M. *Mol. Cell. Biol.* **2008**, *28*, 4896–4914.
(15) Guo, M.; Galan, J.; Tao, W. A. *Chem. Commun. (Cambridge, U.K.)* **2007**, 1251–1253.
(16) Guo, M.; Galan, J.; Tao, W. A. *Methods* **2007**, *42*, 289–297.
(17) Iliuk, A.; Galan, J.; Tao, W. A. *Anal. Bioanal. Chem.* **2009**, *393*, 503–513.
(18) Gravert, D. J.; Janda, K. D. *Chem. Rev.* **1997**, *97*, 489–510.
(19) Gravert, D. J.; Janda, K. D. *Curr. Opin. Chem. Biol.* **1997**, *1*, 107–113.
(20) Tomalia, D. A.; Baker, H.; Dewald, J.; Hall, M.; Kallos, G.; Martin, S.; Roeck, J.; Ryder, J.; Smith, P. *Polym. J.* **1985**, *17*, 117–132.
(21) Iliuk, A.; Tao, W. A. *Methods Mol. Biol.* **2009**, *527*, 117−129, ix.
(22) Tao, W. A.; Wollscheid, B.; O'Brien, R.; Eng, K.; Li, X. J.; Bodenmiller, B.; Watts, J. D.; Hood, L.; Aebersold, R. *Nat. Methods* **2005**, *2*, 591–598.
(23) Doucet, A.; Overall, C. M. *Mol. Aspects Med.* **2008**, *29*, 339–358.
(24) Gevaert, K.; Goethals, M.; Martens, L.; Van Damme, J.; Staes, A.; Thomas, G. R.; Vandekerckhove, J. *Nat. Biotechnol.* **2003**, *21*, 566–569.
(25) Kleifeld, O.; Doucet, A.; auf dem Keller, U.; Prudova, A.; Schilling, O.; Kainthan, R. K.; Starr, A. E.; Foster, L. J.; Kizhakkedathu, J. N.; Overall, C. M. *Nat. Biotechnol.* **2010**, *28*, 281–288.
(26) Prudova, A.; auf dem Keller, U.; Butler, G. S.; Overall, C. M. *Mol. Cell. Proteomics* **2010**, *9*, 894–911.

(27) auf dem Keller, U.; Prudova, A.; Gioia, M.; Butler, G. S.; Overall, C. M. *Mol. Cell. Proteomics* **2010**, *9*, 912–927.
(28) Cava, M. P.; Deana, A. A.; Muth, K.; Mitchell, M. *J. Org. Synth.* **1961**, *41*, 93–95.
(29) Kainthan, R. K.; Muliawan, E. B.; Hatzikiriakos, S. G.; Brooks, D. E. *Macromolecules* **2006**, *39*, 7708–7717.
(30) Wilms, D.; Stiriba, S.; Frey, H. *Acc. Chem. Res.* **2010**, *43*, 129–141.
(31) Calderon, M.; Quadir, M. A.; Sharma, S. K.; Haag, R. *Adv. Mater.* **2010**, *22*, 190–218.
(32) Kizhakkedathu, J. N.; Creagh, L.; Shenoi, R. A.; Chan, T.; Lam, J.; Rossi, N. A. A.; Dendepally, S. R.; Haynes, C. A. *Biomacromolecules* **2010**, *11*, 2567–2575.
(33) Schilling, O.; Overall, C. M. *Nat. Biotechnol.* **2008**, *26*, 685–694.
(34) Schilling, O.; Huesgen, P. F.; Barre, O.; auf dem Keller, U.; Overall, C. M. *Nat. Protoc.* **2011**, *6*, 111–120.
(35) Menschaert, G.; Vandekerckhove, T. T. M.; Baggerman, G.; Schoofs, L.; Luyten, W.; Van Criekinge, W. *J. Proteome Res.* **2010**, *9*, 2051–2061.