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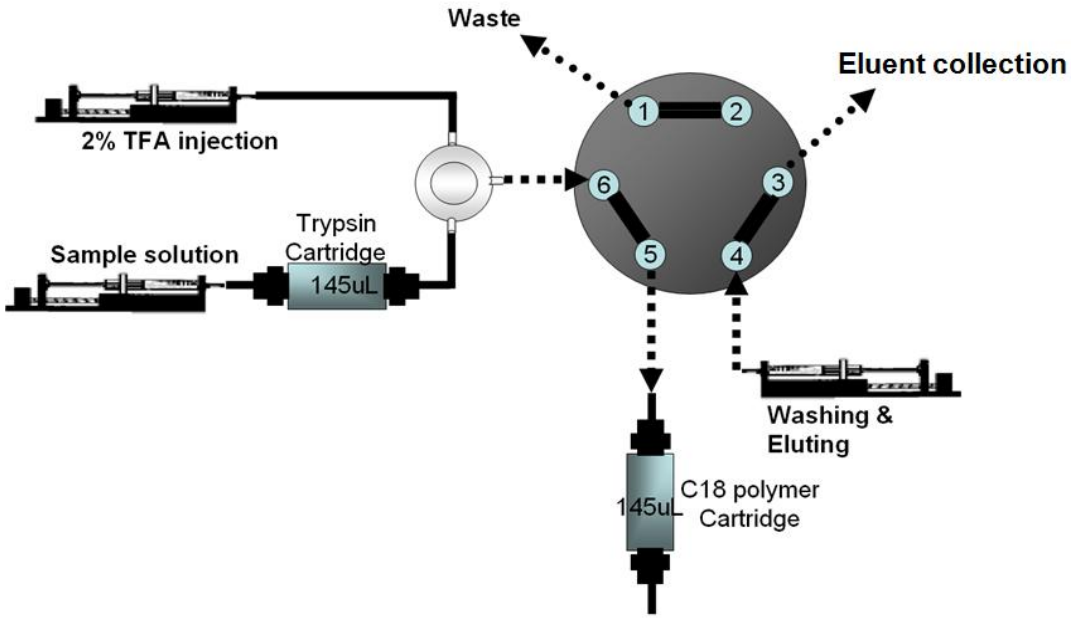
Figure S1. The setup for integrated trypsin digestion and peptide desalting/enrichment. For digestion only, the trypsin cartridge was not connected to the T valve (see text).



Note: The porosity and permeability of the packed bed were determined from the measurement of the void (solvent elution) volume and the pressure drop across the cartridge packed with our materials using Di-water as the mobile phase (Journal of Chromatography A, 1182 (2008) 161–168). The total porosity was determined to be around 0.88 and for poly-C18 and poly-MAA materials, respectively. The column permeability was determined to be around 2.8×10^{-8} cm^2 and for poly-C18 and poly-MAA materials, respectively. Although the porosity is comparable to some polymeric monolithlic materials, the permeability of our materials is higher than polymeric monolithlic materials by one order of magnitude (Journal of Chromatography A, 1182 (2008) 161–168). Thus, the cartridge packed with our materials exhibited low back pressure which may be handled by hands instead of syringe pumps shown above.

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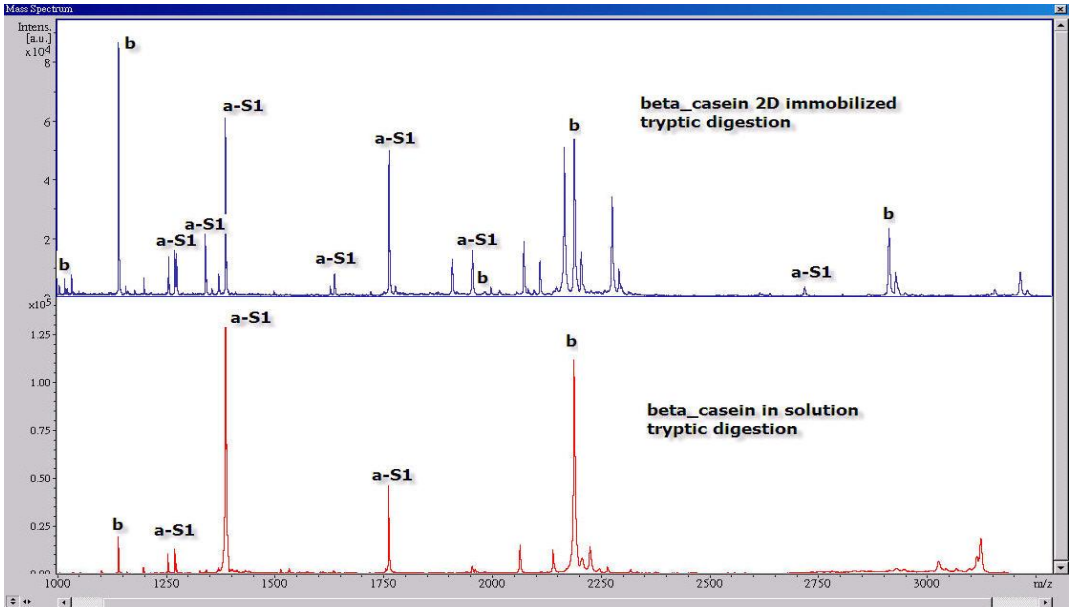
Figure S2. Comparison of immobilized digestion and the over-night liquid digestion using beta casein as the substrate.



There are several commercial trypsin columns from different brands and their performance varies. The trypsin cartridge provided by Promega was reported to exhibit a comparable efficiency compared to the over-night solution digestion (Engel, L., Flemming, R., Johnson, T., Simpson, D. and Urh, M. Conveniently Perform In-Solution Digestions With Immobilized Trypsin. 2009: http://www.promega.com/resources/articles/pubhub/conveniently-perform-in-solution-digestions-with-immobilized-trypsin/). Thus, we compared the solution digestion using Promega trypsin and immobilized digestion (Figure 4B) using our material which was immobilized with the same enzyme (Promega trypsin). As shown in Figure S2, the MALDI spectra indicated that the percentage of digestion using our immobilized method is superior to the solution digestion. Thus, we believe the performance of our material is comparable or superior to the 'standard' column material.

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2. Experimental

2.1. Materials—

Ethylene glycol dimethacrylate, methacrylic acid (MAA), 2,2-dimethoxy-2-phenylacetophenone (DPA), dithiothreitol (DTT) and iodoacetamide were purchased from Fluka (Buchs, Switzerland). The β-casein protein, 1-ethyl-3-(3-dimethylaminopropyl)-carbodimmide (EDC), sodium dodecyl sulfate (SDS), ammonium persulfate (APS), and N, N, N, N'-tetra methylethylene diarmine (TMEDA) were provided by Sigma (St. Louis, MO, USA). Trypsin was bought from Promega (Madison, WI, USA). Methanol, ethanol (EtOH), acetone, N, N'-methylene bisacrylamide and acetonitrile(ACN) were purchased from J. T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid (TFA) was obtained from Riedel-de Haen (Seelze, Germany). N-hydroxysuccinimide (NHS), ammonium hydrogen carbonate (NH_4HCO_3), stearyl methacrylate (StMA), mineral oil, sodium hydrogencarbonate, and α-Cyano-4-hydroxycinnamic acid (α-CHCA) were purchased from Aldrich (Milwaukee, WI, USA). Water was deionized to 18.2 MΩ using Barnstead NANOultrapure water system. β-casein was used as the standard protein to produce the tryptic peptide for the performance test. A 10000 ppm protein solution was prepared in 50 mM ammonium hydrogen carbonate buffer (pH 8.3).

2.2. O/W/O emulsion monomer polymerized by heat—

The emulsion monomer solution contains methacrylic acid (MAA) 800μL, N, N'-methylene bisacrylamide 0.16g, sodium dodecyl sulfate (SDS) 0.3g, ammonium persulfate (APS) 0.03g, N, N, N, N'-tetra methylethylene diarmine (TMEDA) 100μL, pure water 2000μL and mineral oil 2000μL were mixed by vortexing. The emulsion solution was then spilled through a syringe needle with 0.5mm internal diameter to form droplets dripping into a glass cup that contains hot mineral oil keeping at 95℃ beforehand. The hot mineral oil was kept rotating. After radical-induced polymerization, the hot mineral oil was left to cool and then was decanted. The polymer macro-spheres were collected, washed with methanol, ethanol, acetone, and detergent to remove mineral oil, and then with pure water. Finally, the macro-porous spheres were baked in an oven under 60 ℃.

2.3. Trypsin immobilization—

The poly-MAA macro-porous spheres were activated by immersing the spheres into an activation solution containing 1 mL pure water, 13 mg EDC and 5 mg NHS for 3 hours and were repeated again with fresh solution. The activated spheres were then immersed into the trypsin solution (1000 ppm, 1 mL) dissolved in ammonium hydrogen carbonate buffer (pH 8.3) for 6 hours at room temperature. After immobilization, the solution was centrifuged to remove the un-immobilized trypsin and the trypsin-immobilized poly-MAA macro-porous

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spheres were kept in ammonium hydrogen carbonate buffer (100 mM) under 4 ℃ until use. To quantify the amount of trypsin which is covalently bound or adsorbed to the surface, two batches of materials were prepared with and without covalent activation by adding EDC/NHS reagents, respectively. The amount of immobilized trypsin under either condition was calculated by subtracting the trypsin intensity obtained from the input solution from that obtained from the flow-through solution using HPLC-UV. The results indicated that about 34% of the input trypsin was immobilized into the surface without surface activation and about 88% of the input trypsin was immobilized into the surface with EDC/NHS activation. Thus, we concluded that about 38% (34/88 x100%) of trypsin was adsorbed on the surface and about 62% of trypsin was covalently bound to the surface.

2.4. W/O/W emulsion monomer polymerized by UV-light—

The emulsion solution was prepared by mixing 600μL 10% ammonium hydrogen carbonate, 700μL 1-decanol, 300μL stearyl methacrylate (StMA), 80μL ethylene glycol dimethacrylate, 20mg sodium dodecyl sulfate (SDS) and 20mg 2,2-dimethoxy-2- phenylacetophenone (DPA) in a 2-mL eppendorf tube and shaked vigorously to become emulsion. The emulsion solution was poured slowly along the wall of a microtube (15 mL) which was filled with 8 mL pure water. The microtube was fixed on a vortexer and photo-polymerization was initiated by UV light (365 nm). Ethanol and water were used to remove un-reacted monomers. Finally, the poly-C18 porous spheres were baked in 60 ℃ oven. The hydrophobic spheres were further sieved by 2 mm screen to remove large spheres.

2.5. On-line integrated setup—

The setup of the on-line integrated flow system for sample digestion and enrichment was depicted in Supplement Figure S2. Trypsin-immobilized Poly-MAA and poly-C18 macro-porous spheres were packed into two 145-μL cartridges by a 500-μL syringe, respectively, and integrated by a T valve and a six-port valve. For digestion only, the trypsin-immobilized Poly-MAA cartridge was not connected to the T valve and β-casein solution (20 μL, 1000 ppm) dissolved in ammonium hydrogen carbonate buffer (180 μL) was infused into the cartridge through a syringe pump at a flow speed of 1 μL/min and digested *in-situ*. After sample injection, ammonium hydrogen carbonate buffer (200 μL) was injected into the immobilized trypsin bed for washing. The digested products were then eluted with 200 μL 50% ACN in 0.1% TFA and the eluent was collected in a microtube. For on-line integrated digestion and enrichment, the digested products were eluted with 200 μL 50% ACN and on-line mixed with 2% TFA solution delivered by another syringe pump through the T-valve. The acidified digests were swept to the cartridge packed with poly-C18 macro-porous spheres through the 6-port switching valve at a flow speed of 3 μL/min. The washing (0.1% TFA) and eluting (90% ACN in 0.1% TFA) buffer delivered by

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another syringe pump was also connected to the 6-port valve and the enriched digests were eluted at a flow speed of 3 μL/min and collected from the outlet port as indicated.

2.6. Instrument—

MS spectra were acquired from a MALDI-TOF spectrometer (MALDI, Bruker Daltonics, Auto Flex Ⅲ L200, Bremen, Germany) equipped with a 337 nm N2 laser under positive linear mode. A volume of 2μL of the collected digest was mixed with 2 μL matrix which was prepared by dissolving 4-cyanohydroxysuccinic acid (10 mg) in EtOH/ACN (1:1, 1 mL) solvent containing 0.1% TFA. The Scanning electron microscopy (SEM) image was acquired from a JEOL JSM-6700F (Tokyo, Japan) microscope. The macro-porous spheres were fixed and pasted with carbon tape (2.5 cm diameter) *prior to* SEM detection. Fourier-transform Infrared spectroscopy (FT-IR) was acquired from Perkin-Elmer Spectrum RX-1 spectrometer.

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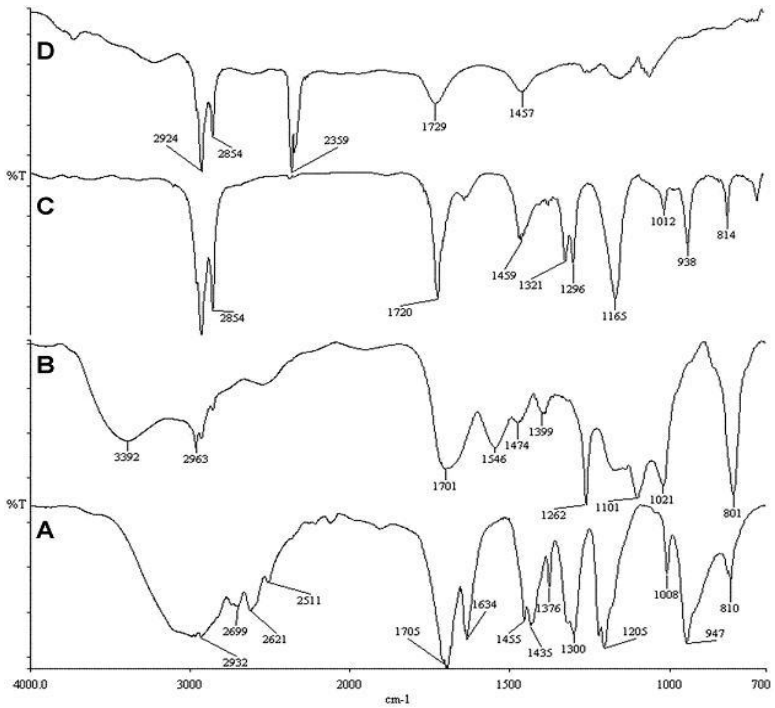
FT-IR spectra (shown below) of the MAA monomer (A) shows a board and strong band around 2500-3300 cm^{-1} which is attributed to the hydroxyl group, and the peak at 3392 cm^{-1} detected from the polymerized MAA surface (B) is attributed to the N-H group of N, N'-methylene bisacrylamide of of poly-MAA. The sharp peaks at 1700-1720 cm^{-1} are for the carboxylic group and the ketone group (C=O stretching) for both the monomer and the polymer. It must be mentioned that poly-C18 and poly-MAA were polymerized through free radical activation to connect alkene ($-CH=CH_2$) bonds. The peaks ranging from 780-995 cm^{-1} are due to =C-H and $=CH_2$ out-of-plane bending. However, the monomeric bands of MAA detected at 810 and 947 cm^{-1} can be differentiated from its polymeric band detected at 801 cm^{-1}. The monomeric peaks of StMA detected at 814 and 938 cm^{-1} (C) were not detected after polymerization (D). The peak at 1634 cm^{-1} belonging to the symmetric C=C bonding was detected in MAA monomer (A), but could not be detected in StMA monomer (C). The band ranging from 1720 to 1729 cm^{-1} (C and D) are C=O group stretching, and the peaks 2854 and 2924 cm^{-1} are attributed to C-H group stretching. The medium peak at 1296 and 1300 cm^{-1} could be assigned to the $-CH_2$ twisting from the monomer of MAA (A) and StMA (C). In general, the IR spectra indicate that both MAA and StMA were polymerized on the sphere surface.



FT-IR spectra of (A) Methacrylic acid (MAA) monomer, (B) polymerized MAA ,(C) Stearyl Methacrylate (StMA) monomer, and (D) polymerized StMA.