**Fabrication of a Poly (GMA-co-EDMA) trypsin Nanoreactor for High Throughput Proteomics**

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**Introduction**

Plasma proteomics represents an enormous challenge. Current estimations report the human proteome to contain around 100,000 proteins with up to 20,000 expressed at any time. Some of the most interesting proteins, however, are low in abundance in extremely complex mixtures. Protein digestion into peptides using a proteolytic enzymes such as trypsin, and the separation of the resulting fragments by mass spectrometry are critical steps both in their isolation and further identification steps by mass spectrometry. The most frequently used digestion strategies are based on liquid phase approaches using high enzyme to substrate ratios.

This classical approach has some drawbacks that may limit high-throughput protein identification such as, long digestion times (up to 24 h) and enzyme autoxidation. While the digestion time can be shortened by lowering the enzyme to substrate ratio, the free trypsin digestion often decreases enzyme specificity, promotes autodigestion subproducts, produces undesirable formation of additional peptides, which may lead to ion suppression in the MS analysis and complicate data interpretation. Some of these issues can be solved by immobilizing the proteolytic enzyme to a solid support, eliminating by this means autoxidation issues, as two enzyme molecules cannot interact. More importantly, by using solid phase supported enzymes, it is possible to significantly increase the enzyme to substrate ratio, and re-use the enzyme, yielding short digestion times and reduced enzyme cost. The present work reports on optimization studies to achieve a robust and high throughput enzyme reactor for proteomics.

**Methods**

**Monolith Fabrication**

The poly(GMA-co-EDMA) monolithic columns were prepared in 75 µm ID / 360 µm OD fused silica capillaries. The internal surface of the capillary was first sterilized with 3-(trimethoxysilyl)propyl methacrylate in dry DMF and 2,2-Diphenyl-1-oxirynhydrayl as free radical, enabling covalent attachment of the monolith to the capillary wall. The monolith (poly(GMA-co-EDMA) or GMA), the cross linker (ethylene glycol dimethacrylate or EDMA), the solvent (cyclohexanone) and the porogen (either dodecanol, 1-octanol or 2-octanol) were mixed in a wide with the free radical (2,2-Dimethoxy-2-phenylacetophenon or ABIN) and thermally polymerized at 60°C for 14 hrs, to obtain the desired surface. Then the prepared monolith was washed with methanol and dried under N₂. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

**Enzyme Linking Chemistry**

Since the dry methacrylate-co-ethylen dimethacrylate (GMA-co-EDMA) has epoxy groups which can directly react with the amine groups of the enzyme, direct linking was possible. 2 mg/ml trypsin in 50 mM carbonate buffer (pH 10.5) was pumped through the capillary in the presence of 0.2 mg/ml benzamidine (inhibitor). This approach has reportedly given better immobilization yields than the static protocol, where the capillary is filled with the solution to react, probably due to the improved mass transfer ratio. Figure 1 shows the schematic diagram of the reaction.

**Model protein, Digestion and Mass spectrometry**

A solution of 0.25 mg/ml Cytochrome C in 20% methanol containing 10 mM ammonium bicarbonate at pH 8.5 was used for digestion. The presence of the organic solvent substituted the traditionally needed denaturing steps prior to the digestion step.

The denatured Cytochrome C solution was directly pumped at approximately 200 nL/min flow-rate through the monolith for two hours at room temperature. The collected fraction was then analyzed by a chipLC-MS system (Agilent 1100 with a chip-cube and ion trap MS). 2 µL of sample was injected using an autosampler; the sample was desalted by the enrichment column of the LC chip (40 nl trap 75 µm x 150 mm) and separated by a typical water - acetonitrile gradient in the analytical column part of the chip (stationary phase: 5 µm C-18 SB-ZX).

The collected fraction was further analyzed on our Chip Cube LC-MS. Three different reactors were fabricated using the following GMA/EDMA percentage ratios: 24/14, 23/17 and 22/18. Further to substrate optimization experiments were done with the goal to identify the optimal 2-octanol concentration to attain the best monolith combination in terms of its back pressure, homogeneous structure and attachment to the capillary wall. These last three factors were empirically evaluated by observing the generated monoliths under microscope (Figure 4.).

Some important aspects should be pointed out about the monolith back pressure data presented in Figure 3. As previously mentioned, changes in the porogenic concentration can cause significant differences in monolith porosity and back pressure. Apparently, not only the back pressure determines the reactor performance, but also the mass transfer. Since the back pressure of the reactor tends to increase when the monolith is grafted with the enzyme, we decided to select a minimum back pressure yielding homogeneous porogen. This was achieved by using 8 % of 2-octanol during monolith fabrication. This assured the required good mass transfer and low back pressure at the same time. To optimize the mass transfer, the monoliths were fabricated with this composition. Once the type of porogen and its concentration were both optimized, it was possible to fabricate a set of enzymatic reactors in order to study their performance in terms of sequence coverage of the model protein.

**Results**

**Porogenic Solvents**

Factors like, column length, pore size and back pressure were carefully considered during the fabrication of the monolith. The ideal column should be long enough to assure appropriate residence time, low back pressure and small pore size. Therefore, monoliths with a small mass transfer rate. Since these properties are somewhat contradicted each other, a good compromise has to be established. Picking the right porogenic solvent is a crucial part of monolith fabrication. Usually, long-chain alcohols are used for this purpose. By changing the porogenic and its concentration in the polymerization mixture, it is possible to control the pore size and back pressure of the resulting monolith. Optimization studies were conducted in our laboratory with this goal. The different porogenic solvents initially considered were dodecanol, 1-octanol and 2-octanol. Figure 2 shows the back pressure measurements in 3 cm long monoliths with 200 nL/min flow-rate (methanol).

Apparently, 2-octanol was the best porogenic among the ones tested based on back-pressure values. Increasing 2-octanol concentrations were evaluated in order to determine its optimal value (Figure 3). Please note that apparently no polymer-inorganic flocs were observed when 1-octanol was used as the porogenic material, yet, further to substrate optimization experiments were done with the goal to identify the optimal 2-octanol concentration to attain the best monolith combination in terms of its back pressure, homogeneous structure and attachment to the capillary wall. These last three factors were empirically evaluated by observing the generated monoliths under microscope (Figure 4.).

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**Monomer / cross linker (GMA/EDMA) Ratio**

Optimization experiments were also done to evaluate the influence of monomer-cross linker ratio on reactor performance. Three different reactors were fabricated using the following GMA/EDMA percentage ratios: 24/14, 23/17 and 22/18 (%), while keeping the porogenic concentration at its optimal value of 8 %. Figure 5 shows the back pressure measurements and as one can see the optimal GMA / EDMA ratio was fixed at 24/16 %, which provided minimum back pressure.

The optimal monolith for trypsin nanoanalyzer was fabricated by following the protocol described above (see methods) using the optimized polymerization mixture of 24/16 % GMA / EDMA ratio, 8 % 2-Octanol, 4.5mg ABIN and 12 % cyclohexanol.

Cytochrome C samples (denatured with methanol) were digested using the fabricated enzymatic reactor and following the protocol previously described, and further analyzed on our Chip Cube LC-MS. Three different runs were carried out and the obtained data was analyzed by the Spectrum Mill® software. We got good reproducibility and with sequence coverage of 57 +/- 0.8%.

**Conclusions**

- Fabrication and optimization of a nano poly (GMA-co-EDMA) trypsin enzymatic reactor was implemented.
- Monolith characteristics like, porosity, monomer / cross linker ratio were studied as key factors in achieving optimal fabrication parameters.
- Further optimization experiments are in progress to understand the temperature dependency on digestion yield (sequence coverage).
- Methanol denatured Cytochrome C was successfully digested with 57 +/- 0.8% sequence coverage at room temperature, without any detectable self digestion products.

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