MINIATURIZED HPLC COLUMN WITH NANO-PORE BEADS FOR PROTEIN SEPARATION ON FLEXIBLE MEDICAL IMPLANTS

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ABSTRACT

This paper reports a miniaturized HPLC (High Performance Liquid Chromatography) column packed with nano-pore beads for flexible medical implants applications. The all-flexible-polymer column capable of integrating with micro/nano-devices on a chip separates biological entities to relax stringent requirements on subsequent detectors and to enable parallel detection in a detector array. The fabricated device has successfully separated a protein mixture using nano-pore beads in a miniaturized column. According to HPLC analysis, the intensity ratio of large to small protein varies significantly, more than a factor of 300, over sample collecting time, ~ 12mins, suggesting very high separation performance.

Keywords: HPLC Column, Size Exclusion Chromatography (SEC), Protein separation, Medical implant

INTRODUCTION

Among a number of medical implants one of the most attractive applications is healthcare monitoring [1]. With a small implant near the skin, patients can monitor their health routinely with minimal hospital visits. Most medical implants are typically made of stiff materials which can exert pressure on living tissues and thus may retard or even inhibit interbody fusion [2]. A flexible implant that alleviates stress from muscle movements and minimizes damage to surrounding tissues will be extremely beneficial in preventing re-surgery processes due to a failed implant. Micromachining technologies can offer highly biocompatible, flexible and very small size systems. A conceptual flexible micro-chip for a medical implantation is illustrated in Figure 1. Although extensive research activities on detectors and fluid control devices have continued for more than 20 years, the importance of preparation stage of mixed samples has been greatly overlooked [3]. This is partially because scientists and engineers often use pre-treated samples for biochips or prepare them outside the chip. There are many required sample preparation stages including mixing, synthesizing, separation, etc.; among them we focus on sample separation. Figure 2 illustrates an operating principle of our proposed on-chip separating stage. We mimic a column of HPLC to generate phase delay of biological/chemical entities from mixed samples using nano-pore beads. Several miniaturized HPLC columns have been developed in the past [4-7]; however none is for medical implant applications, which prefers to be mechanically flexible to avoid unwanted stress and is made of fully biocompatible materials. The mixed analyte goes through a series of nano/micro-beads and is separated in time domain. Among a number of separation techniques, we choose Size Exclusion Chromatography (SEC) since it fits the best for medical implant applications, fully biocompatible mobile phase and no need of high performance pump; SEC uses isocratic mobile phase and demands much lower flow rate than other techniques. Although HPLC has numerous implementations of preparative system, very few applications are feasible for medical implantation [4]. Also, the size reduction of HPLC column made of flexible materials allows great potential on portable HPLC equipment [5, 6]. In this paper, we present a chip-scale flexible and biocompatible separation column that is capable of separating a group of protein. We describe the proposed separation column design and fabrication using biocompatible and flexible material. The fabricated device is extensively tested for artificial sample and mixture of proteins both qualitatively and quantitatively.

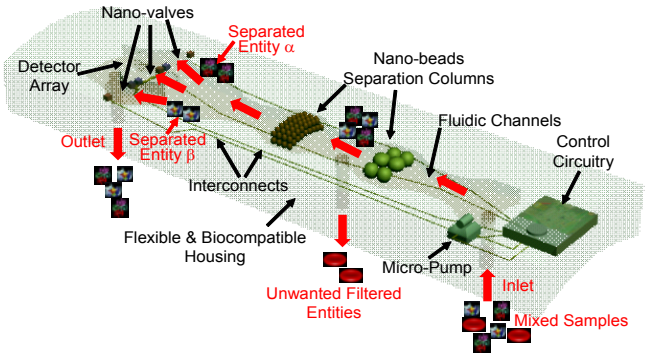


Figure 1: The proposed flexible medical implants integrated with micro/nano-devices.

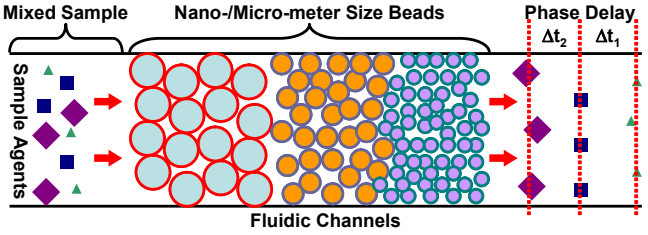


Figure 2: Operating principle of a separation column.

SEPARATION COLUMN DESIGN

Figure 3 depicts the miniaturized column design. The separation column is all made of PDMS thus biocompatible, simple to fabricate, and mechanically flexible. Nano-pore beads are packed at the center chamber and the column is sealed with a PDMS customized housing with only two ports; inlet and outlet. Inlet and outlet are connected from the customized housing to chambers via fluidic channels. The top PDMS layer is made by soft lithography technique

and the housing is cast from a customized aluminum mold. We used two different beads; one is polymeric and the other is silica-based beads [8, 9]. Both nano-pore beads are 5-20μm (10μm mean) in diameter and pore size is 10nm (polymeric) and 100nm (silica).

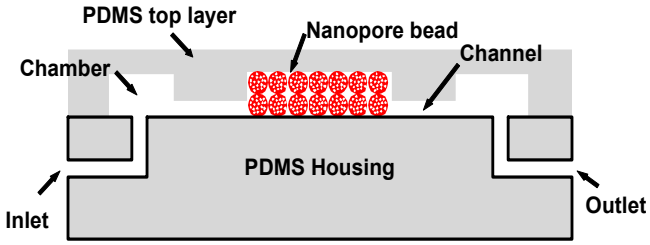


Figure 3: Schematic of miniaturized HPLC SEC column.

FABRICATION

The fabrication of the all-flexible column is similar to multi-level soft-lithography [10]. Figure 4 illustrates PDMS top layer process flow. First, photoresist, AZ4330, is spin-coated on a silicon wafer and patterned for chambers and channels. Then, the pattern is transferred to silicon by Deep RIE, etched ~ 7μm. PDMS is poured and cured on the silicon wafer and we peel off the molded PDMS.

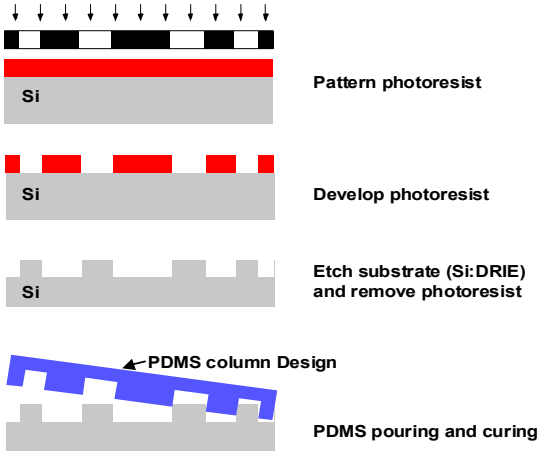


Figure 4: PDMS top layer process flow.

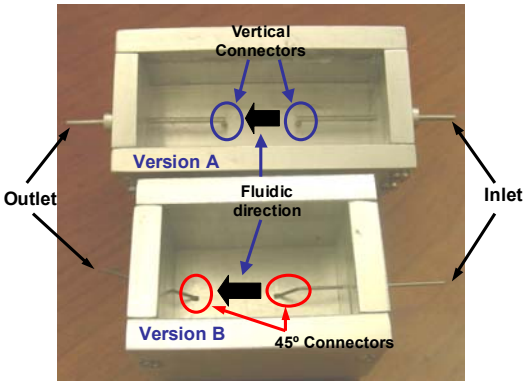


Figure 5: Customized aluminum molds.

The customized housing mold is made of aluminum and two versions of molds are shown in Figure 5. Two aluminum rods, perpendicular to each other, connect inlet and outlet in version A. Inlet and outlet chambers to the 1/16-inch conduits are also vertically formed; this scheme causes a high pressure to fail flowing analyte. Version B is modified to accommodate drawbacks of the version A, by using 45° angled connectors. Also, the size of the rod is reduced to 1/32-inch to minimize dead volume of the sample analyte.

Nano-pore beads are placed accurately at the center chamber. The density of the beads is one of the critical factors to make separation occur. The nano-beads are immersed into a solvent and poured in a 31 gauge dermatic syringe. After boding two PDMS structures using O_2 plasma surface treatment, the syringe needle is inserted from the top layer to the center chamber. Nano-beads are immobilized inside the chamber because the fluidic channel is smaller than the size of beads. After packing the beads inside the chamber additional O_2 plasma treatment is performed to help PDMS channels be hydrophilic.

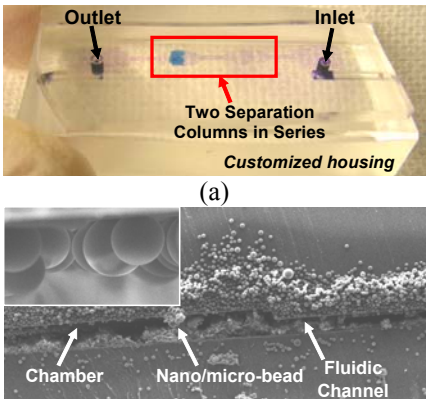


Figure 6: Optical photographs of the fabricated column (two columns in series) packed with micro-/nano-beads (a) and SEM images (b).

Figure 6 shows fabricated all-flexible separation column packed with nano-pore beads. HPLC often requires multiple columns to achieve better separation capability and higher resolution [7]; however multiple columns require much more analyte and mobile phase, thus increase complexity and dead volume. Micromachining technology enables series columns without any compromise as shown in Figure 6 (a).

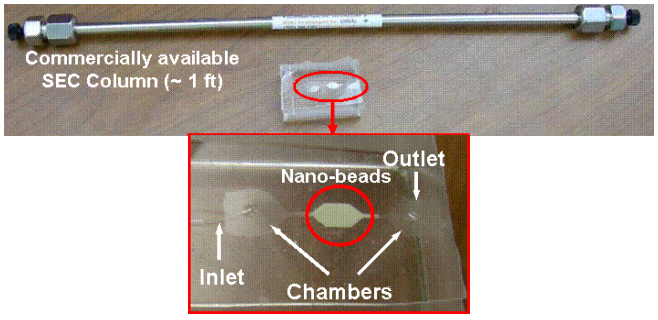


Figure 7: Commercially available SEC column (SRT SEC-300, 4.6×300mm^2) and fabricated separation column.

For comparison, Figure 7 shows a commercially available SEC column (SRT SEC-300, SEPAX Technologies) and the fabricated SEC column. The column

size of the fabricated device is only 5×5×0.01mm^3, a factor of ~ 20,000 reduction from the macro-scale column.

TEST RESULTS

Proof-of-concept

We first tested the fabricated column using artificial polystyrene beads with different sizes; 3μm (red) and 0.19μm (blue) as shown in Figure 8. The beads packed at the center chamber are made of polystyrene and is 45-90μm in a diameter, providing inter-beads pore size of 20-40μm. Clearly, mixed beads at inlet become separated at the outlet.

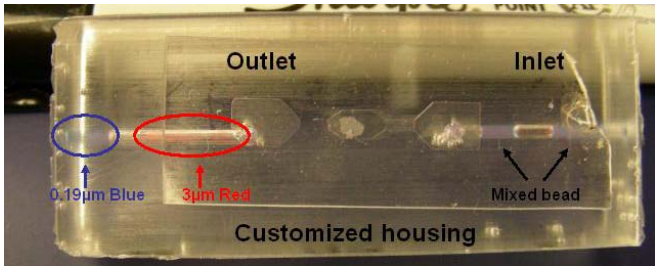
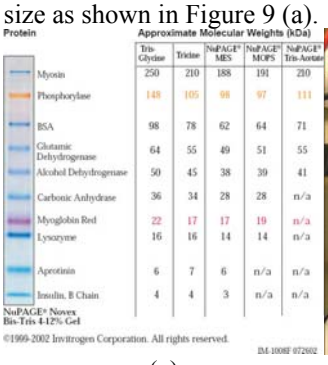


Figure 8: Proof-of-concept separation using stained polystyrene beads.

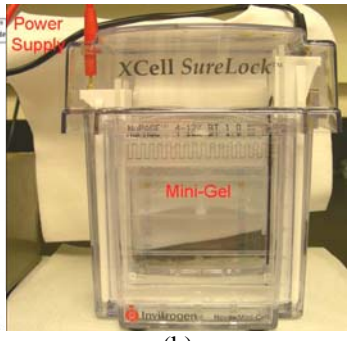
The proof-of-concept data suggests the fabricated column purely separates sample analyte by size exclusion mechanism; almost perfect separation of two colored beads.

Qualitative measurement using electrophoresis

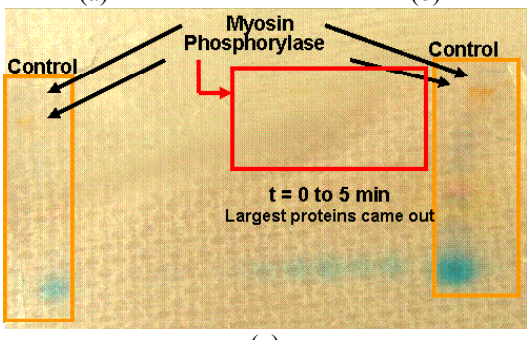
For protein separation, we use nano-pore beads to separate a mixed protein sample. The beads are silica-based, 5-20μm in size and the size of pore is ~100nm. The mixed protein sample contains 10 denatured pre-stained proteins ranging from 3 kDa (2.8nm) to 188 kDa (18nm) in size as shown in Figure 9 (a).



(a)



(b)



(c)

Figure 9: Separation results (electrophoresis plots) using denatured pre-stained proteins; (a) protein molecular weight on the electrophoresis, (b) electrophoresis, (c) electrophoresis result after protein separation.

The mixed proteins are inserted into the microfluidic channel first and then mobile phase pushes them through the column. The flow is controlled by a syringe pump at 10 μl/min. At the outlet, we collect the eluted analyte samples periodically and analyze them by electrophoresis and HPLC. Figure 9 (c) shows the electrophoresis result of the protein separation. The very left and right columns show the control group, and the four columns from the left in the middle show the timed outputs collected from t_0 (0 min) to t_5 (5 min) at the outlet. Phoshporylase spectrums, orange color band, are shown at the electrophoresis mini-gel, indicating half of large proteins seem to come out at the first 5 minutes. Because the raw analyte is largely diluted by a mobile phase and electrophoresis running buffer, each spectrum of protein is subdued very much. We believe small-size proteins are separated since we don't see any spectrum for the first 5mins sample; however it is very hard to verify if it is the case.

Quantitative measurement using HPLC

In order to verify qualitative data obtained by electrophoresis, timely collected samples are analyzed by a commercial HPLC using SEC mechanism. Figure 10 shows analytical results of three different groups of samples (time vs. normalized intensity); one is control group, 10 proteins diluted with a mobile phase and running buffer, the other is collected from 0 to 3mins, and the last one is collected from 10-12mins.

The control group shows many sharp peaks for the first 10mins, indicating large proteins such as Myosin and Phoshporylase. Peaks at ~ 27 mins represent small proteins, on the other hand, such as Aprotinin and Insulin B Chain. Table 1 shows numerical data of the HPLC analysis. Peak number starts from the earliest detected protein, which is the largest protein; this is because SEC mechanism separates analyte only by its size; larger the protein earlier the detection time. Therefore, we can map the peaks to individual protein. Figure 10 (b) shows the HPLC analysis of t_0 (0 to 3min). Both the peak intensities of large and small proteins are reduced by more than a factor 2.5. This could be due to low sample volume (~0.1μl) compared to that of the control group (~1μl). We additionally dilute t_0 and t_5 samples due to the minimum sample volume requirement of HPLC. It is clear that the intensity ratio of large to small proteins is very high (6.85% for peak #1), which indicates we have more large-size than small-size proteins in 0 to 3min sample.

Figure 10 (c) shows HPLC analysis for t_5 sample, collected from 10 - 12mins. The peak intensity of large proteins is significantly smaller (more than a factor of 6) than that in t_0, indicating very little large protein exists inside the sample. On the other hand, the peak for small protein remains almost identical. This clearly represents t_5 sample has small proteins much more than large ones. The intensity ratio drops from 6.85% to 0.02%, more than a factor of 300; very good separation performance.

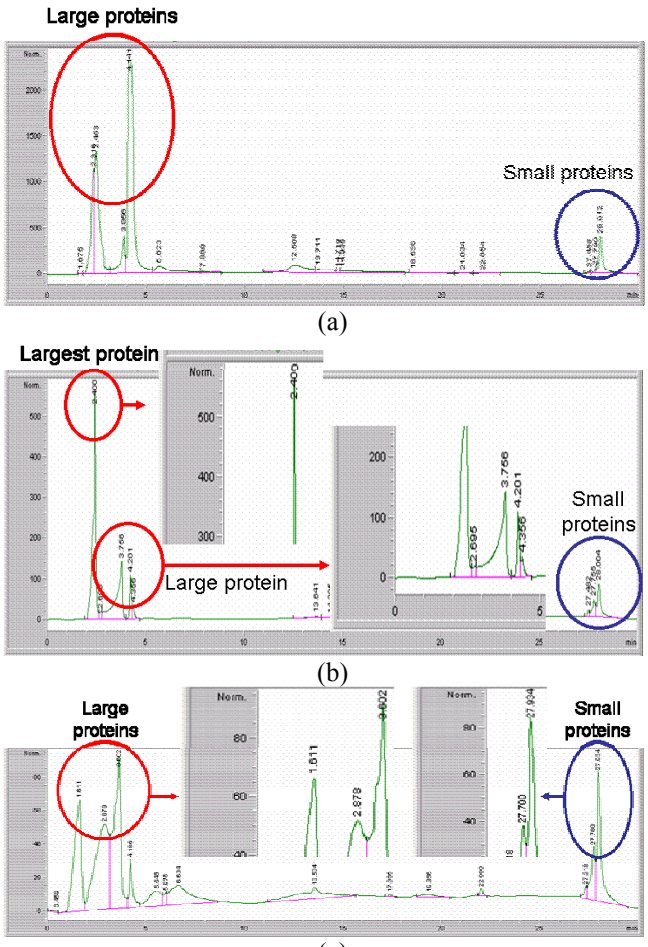


Figure 10: HPLC analysis data (a) control group (ten protein diluted with a mobile phase), (b) collected sample at t_0 (0 to 3 min), (c) collected sample at t_5 (10 to 12 min)

Table 1: Numerical data of HPLC analysis (a) control group; (b) collected sample at t_0 (0 to 3 min) (c) collected sample at t_5 (10 to 12 min)

(a)

1:9 diluted protein sample		
Peak	Intensity ratio (large/small proteins)(%)	Area ratio
1	2.83	2.37
2	3.33	3.88
3	1.02	1.03
4	5.82	8.52
5	0.15	0.403
6	0.19	0.902
18	1.00	1.00

(b)

t_0 (0-3min)		
Peak	Intensity ratio (%)	Area ratio
1	6.85	4.74
2	0.22	0.14
3	1.72	2.70
4	1.31	0.67
5	0.41	0.20
9	0.21	0.10
10	0.52	0.43
11	1.00	1.00

(c)

t_5(10-12min)		
Peak	Intensity ratio (%)	Area ratio
1	0.02	0.02
2	0.84	1.42
3	0.65	2.07
4	1.15	1.88
5	0.37	0.29
15	1.00	1.00

Although the collected samples are diluted much more than the control group due to the required minimum sample volume for HPLC, it is clear that t_0 sample has more large proteins than t_5 sample. Figure 11 shows peak number *vs.* peak intensity of t_0 and t_5 samples. Obviously t_0 sample has mostly large-size proteins whereas t_5 sample holds mainly small-size proteins. We believe we can separate individual proteins if the minimum required volume for HPLC is small enough to allow analyzing extremely minute sample amount. This remains future challenges; however it is possible to minimize analyte sample volume if on-chip detectors are sensitive enough for the small volume.

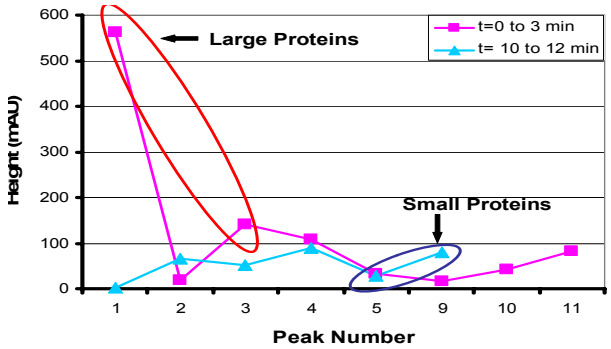


Figure 11: Intensity of t_0 and t_5 samples

CONCLUSIONS

We have miniaturized HPLC column packed with nano-pore bead on the flexible polymer enclosure for human body implantation. By miniaturization of column, we achieve to use smaller volume for a mobile phase, analyte, and minimize the required flow rate. Fabricated nano-pore column was tested by electrophoresis and HPLC SEC column with ten protein mixture. The result clearly shows that the fabricated column performs very well using SEC separation mechanism. Future development on in-situ on-chip detectors allows high efficiency, high resolution, and high plate number, etc.

ACKNOWLEDGMENTS

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REFERENCES

[1] *G. Amato, et al.*, "Health Care Monitoring of Mobile Patients," ERCIM News, No.60, 2005

[2] *T. Smit, et al.*, "Changes in bone architecture during spinal fusion: three years follow-up and the role of cage stiffness," Spine, vol. 28, pp. 1802-8, 2003.

[3] *M. Handal et al.*, "DNA mutation detection and analysis using miniaturized microfluidic systems," Expert Rev Mol Diagn. pp. 29-38, 2006

[4] *D. Reichmuth, et al.*, "Microchip HPLC of Peptides and Proteins," Anal Chem, 77, pp. 2997-3000, 2005.

[5] *J. Xie, et al.*, "Complete gradient-LC-ESI system on a chip for protein analysis," MEMS 2005, pp. 778-81, 2005.

[6] *M. Nelson, et al.*, "A development of a Portable Immunoextraction/RPLC System for Field Studies of Herbicide Residues," Anal Chem, 76, 805-813, 2004.

[7] *H. Anderson, et al.*, "Micromachined flow-through filter-chamber for chemical reactions on beads," Sensors and Actuators B, pp. 203-208, 2000.

[8] *U D. Neue*, HPLC Columns, Wiley, New York, 1997

[9] *B. Cidlingmeyer*, Practical HPLC Methodology and Application, Wiley, New York, 1992.

[10] *B. Jo, et al.*, "Three-dimensional micro-channel fabrication in polydimethylsiloxane elastomer," JMEMS, pp. 76-81, 2000.