UV Embossed Plastic Chip for Protein Separation and Identification

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***Abstract*—this report demonstrates a UV-embossed polymeric chip for protein separation and identification by Capillary Isoelectric Focusing (CIEF) and Matrix Assisted Laser Desportion/Ionization Mass Spectrometry (MALDI-MS). The polymeric chip has been fabricated by UV-embossing technique with high throughput; the issues in the fabrication have been addressed. In order to achieve high sensitivity of mass detection, five different types of UV curable polymer have been used as sample support to perform protein ionization in Mass Spectrometry (MS); the best results is compared to PMMA, which was the commonly used plastic chip for biomolecular separation. Experimental results show that signal from polyester is 12 times better than that of PMMA in terms of detection sensitivity. Finally, polyester chip is utilized to carry out CIEF to separate proteins, followed by MS identification.**

***Index Terms*— Capillary isoelectric focusing, MALDI-MS, UV-embossing.**

I. Introduction

With the completion of Human Genomic Project, protein mapping becomes the current target of biomedical investigation, which is mainly driven by diagnose and drug discovery purpose [1]. The most promising approach to identify protein is using mass spectrometer for peptide mass fingerprinting, followed by database search [2]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is one of the techniques for protein detection with excellent sensitivity, simplicity and throughput [3-6].

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However, ion suppression usually occurred in ionization process, raised the necessity of the coupling of separation techniques with MS [1][10]; a variety of separation techniques [7-9] was implemented to interface the matrix-assisted laser Desorption/Ionization mass spectrometry (MALDI MS) for protein identification. Particularly, *Newman* et al has demonstrated a pseudo-cover micro channel [12] to perform capillary isoelectric focusing (CIEF) prior to MS identification of proteins. CIEF separation relies on the amphoteric property of proteins in a pH gradient under electric field and is able to separate and concentrate proteins simultaneously [11] [34], which has the merit of high-throughput, small amount of consumed analyte and high resolution (up to 0.05[34]).

The chip fabricated with PMMA by hot embossing suffers from several drawbacks, such as channel non-uniformity. Moreover, PMMA is not a good material for MALDI analysis because the surfaces suppress the protein ionization.

UV-Embossing is a plastic replication method based on the resin polymerization under UV irradiation, offering the advantages of high-throughput, low-cost and mild fabrication condition, which all facilitate the mass production. *Chan-park*'s group has demonstrated fabrication of high aspect ratio (up to 14) structure using UV-embossing. [13] Particular worthy to note is varieties of polymer options for UV-embossing, which usually include polyurethane, polyester, epoxy, polyethylene glycol (PEG) and polypropylene glycol (PPG), as shown in table 1; these polymers have different chemical, mechanical and physical properties so that UV-embossing could tailor to different applications.

The polymer as bulk material used in CIEF coupled with MS could generate strong ion signal, as it has been demonstrated that different polymer support would gives rise to ion signal with different strength and resolution. [14]

In this paper, we investigated the feasibility of UV-Embossing to fabricate the plastic chip for CIEF-MS protein identification. The best material from five families of polymer has been chose to meet the requirement of both CIEF separation and MS detection. The signal from the UV-embossed polymer is 12 times stronger than that of PMMA. Finally, the proteins are separated using CIEF from mixture solution and detected using mass

Table 1. The polymers and their components used for UV-Embossing.

Components \ Polymer		Polyurethane (PU)	Polyester (PE)	Epoxy (EP)	Polyethylene Glycol (PEG)	Polypropylen Glycol (PPG)
Oligomer	EB 270	68	-	-	-	-
	EB 80	-	68	-	-	-
	SR 349	-	-	68	-	-
	PEGDA 700	-	-	-	88	-
	PPGDA 900	-	-	-	-	88
Diluent	SR 508	20	20	20	-	-
	TMPTA	10	10	10	10	10
Additives	EB 350	2	2	2	2	2
	Irgacure 651	0.3	0.3	0.3	0.3	0.3

The amount of the each components (by weight) is indicated by the percentage of the value in the table; "-" represents not being included in the polymer.

spectrometer.

II. Experimental Section

A. Materials and Chemicals

The bovine serum albumin (BSA), horse heart myoglobin with isoelectric point (pI) of 6.8 and 7.2, trypsin inhibitor (pI=4.5), Pharmalyte (carrier ampholyte to generate pH gradient ranging from 3 to 10), 3,5-Dimethoxy-4-Hydroxycinnamic acid (also known as sinapinic acid, SA), potassium hydroxide, phosphoric acid, methylcellulose were all purchased from Sigma-Aldrich (Singapore). The molecular mass of BSA, myoglobin and trypsin inhibitor was 66.8 K Da, 17.6 K Da, and 20.3 K Da, respectively. The plastic micropipette tips (200µl) were purchase from Axygen Scientific (Singapore) with brand name Maximum Recovery. The matrix consisted of saturated sinapinic acid dissolved in the solution consisting of 70% acetonitrile, 29.9% D.I. water and 0.1% trifluoroacetic acid. The protein/ampholyte solution was a mixture of horse heart myoglobin (0.25 mg/ml), trypsin inhititor (0.25 mg/ml) and 1% of Pharmalyte. Catholyte and anolyte used in the CIEF were 100mM potassium hydroxide mixed in 1.5% methylcellulose and 50mM phosphoric acid in 1.5% methylcellulose respectively. The electrolyte tips were made from plugging the tips of the micropipettes with small amounts of methylcellulose gel, which was prepared by dissolving 3% methylcellulose into water at 90 degree. The platinum wire of 0.2mm in diameter with 99% purity was purchased from Quorum Technologies (Singapore). The <100>, P type, 4 inches silicon wafer of ~450 um thickness was purchased from local vendor (J. A. A, Singapore) to fabricate the silicon mold.

The mass spectrometer used is Kratos Axima CFRplus (Shimadzu Biotech, Manchester, U.K.) in linear mode. The home made stainless steel plate was used to hold the plastic chip. The UV lamp to irradiate prepolymer is provided by I & J Fisnar Inc with area-averaged intensity of 13mW/cm^2 at 365 nm. Scanning Electron Microscopy (SEM) is Joel SEM 5600.

The mask aligner in photolithography is Karl Suss MA6 with 10mW/cm^2 intensity at 365nm. The deep RIE machine is Multiplex ICP tool from Surface Technology System. Photoresist is AZ 9260 from Clariant. The PDMS is RTV 651 manufactured by GE Silicones.

The oligermeric acrylates were EB 270(UCB chemicals), EB 80(UCB chemicals), SR 349(Sartomer Chemicals), PEGDA 700(Aldrich Chemicals) and PPGDA 900(Aldrich Chemicals). EB 270 is an aliphatic urethane diacrylate; EB 80 is a polyester tetracrylate; SR 349 is ethoxylated (3) bisphenol A diacrylate; PEGDA 700 is polyethylene glycol diacrylate with average molecular weight of 700; PPGDA 900 is poly(propylene glycol diacrylate) with average molecular weight of 900. Tripropylene glycol diacrylate triacrylate (TPGTA) is supplied by Sartomer Chemicals with product name SR 508; trimethylolpropane triacrylate (TMPTA) is ordered from Aldrich; EB 350 is a silicone polyacrylate from UCB chemicals; photoinitiator 2,2-Dimethoxy-1, 2-diphenylethan-1-one is supplied as Irgacure 651 by Ciba Chemicals. All resins are stirred 24 hours in ambient yellow room environment.

B. Fabrication of Polymeric Channels

The fabrication of polymeric channel consists of 3 steps: silicon mold fabrication, PDMS soft rubber mold replication, polymer UV irradiation and demolding, as illustrated in Fig 1.

For the silicon mold, the photolithography was carried out to define patterns illustrated in Fig. 2, which is the top view of the channel, by AZ 9260 (~9µm in thickness) coated on silicon wafer, followed by deep RIE etching [15] resulting in 200 µm deep channel; the photoresist was removed by acetone and then the silicon wafer was processed in the Deep RIE machine again for passivation

process [16]. The soft lithography [17] was followed to replicate soft rubber (PDMS) mold [18] from the silicon mold: after casting the PDMS prepolymer (the mixture of PDMS base and curing agent) on silicon mold and degassing in vacuum for 30 mins [13], the PDMS prepolymer was cured with the silicon mold in the 80 °C oven for 12 hours and then peeled off. The resultant PDMS mold has the opposite relief to that in silicon mold.

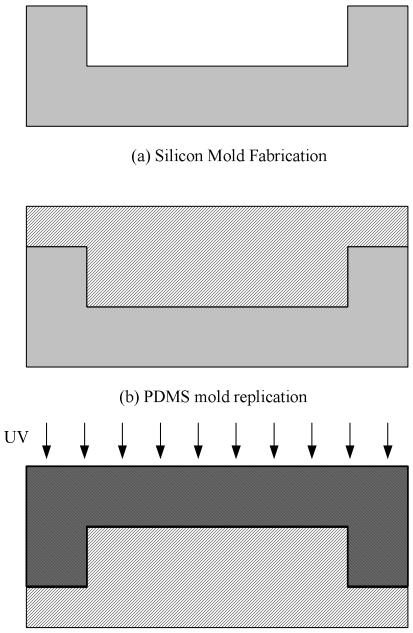

Fig. 1. The Plastic chip fabrication process. (a) the silicon mold fabrication using photolithography and deep reactive etching. (b) soft lithography replication of PDMS soft rubber mold. (c) UV irradiation of UV-embossing process.

The plastic chip was generated by UV irradiation: after casting the polymer resin onto the PDMS followed by degassing 30 mins in vacuum, UV shined onto the resin in order for polymer to perform the polymerization [13]. Finally, demolding was carried out directly by peeling off the soft PDMS from rigid plastic chip.

C. CIEF Experiment

Capillary isoelectric focusing was carried out using the

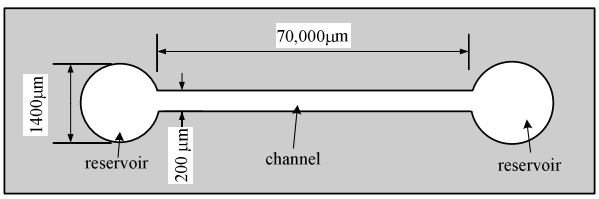

Fig. 2. Illustration of the CIEF chip. The top view consists of two round reservoir and a channel; the depth of the chip is 200 μm.

experimental setup shown in Fig 3. The flat PDMS cover was clamped with the chip to overlay the channel, leaving two reservoirs open. A drop of protein/ampholyte solution was placed into the reservoirs; due to capillary action, the solution filled up the channel. Here, the PDMS was chosen due to its conformity [26] to the contacted material if certain force was applied preventing the solution from leaking out.

Two electrolyte tips, which were filled with catholyte and anolyte, were fixed downwards into the reservoirs in the plastic chip. By inserting the 200μm platinum wire connected to a high power supply into the electrolyte tips, electrical field was introduced along the channel. The multimeter was connected to measure the current in the

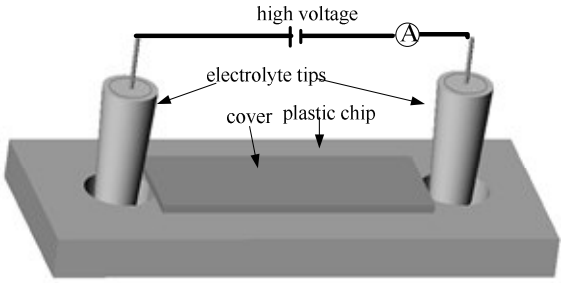

Fig 3. Illustration of CIEF setup to separate proteins. The high voltage is adjustable ranging from 0~6.5kv. The channel on the plastic chip is beneath the cover.

channel.

D. Mass Spectrometry Analysis

MALDI-TOF-MS experiments were performed with a Kratos Axima CFRplus (Shimadzu Biotech, Manchester, U.K.) operating in positive ion mode. Desorption/ionization was achieved with a 337-nm nitrogen laser with 3-ns pulse width. Accelerating potential was set to 20 kV. Acquisitions were accumulated with 2 laser shots at each location, and the number of laser shots used to obtain each spectrum was 100. The mass calibration was performed with an external standard. The intensity of laser shot was maintained at 78μJ/pulse. The external calibration was performed using standard sample prior to experiment.

III. Results and Discussion

A. Fabrication of Plastic Chip

Five families of resin all consists of oligomer, diluent and additives; due to the predominant weight of oligomer in the resin, the final polymer's property is generally determined by the oligomer; the diluent is added in order to tailor the mechanical property of the resin-usually decrease the viscosity of the resin in order to dissolve the photoinitiator efficiently. EB 350 is used as the demolding agent by decreasing the surface energy of the polymer.

The passivation process after silicon etching is necessary to decrease the surface tension of the structure; the C4F8 plasma deposits a thin layer of –CF2-CF2- polymer, which functions as release layer on the silicon mold, due to –CF2-CF2- extremely low surface energy. Otherwise, during peel-off of cured PDMS from silicon mold, the PDMS sticks to the silicon mold and delaminate. The passivation process by C_4F_8 plasma is also superior to the vacuum

deposition of release agent, such as 1,2,2-(tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane [37], since the plasma generated release layer is more uniform and last longer during replications. The crossing section of the channel is illustrated in Fig 4 (a).

The soft mold replication is an essentially intermediate step for the polymeric chip fabrication; otherwise, the rigid polymer chip is difficult to demold from the rigid silicon wafer. The soft mold replication step does not result in the change of the dimension of the final plastic chip caused by the intermediate step, due to the high fidelity of the PDMS replication [17]. The cross-section structure of the PDMS is illustrated inFig 4 (b).

The UV irradiation time depends on the thickness of the chip and the property of the polymer; usually, T_{13} is measured in order to estimate the polymer's curing time needed [13]. The final demolding process is usually performed by peel-off the soft rubber mold from rigid plastic chip. After UV-curing, the acetone, ethanol and D.I. water respectively wash the plastic chip with ultrasound in order to remove the uncured polymer. The final fabricated polymeric channel is illustrated by Fig 4 (c) and (d).

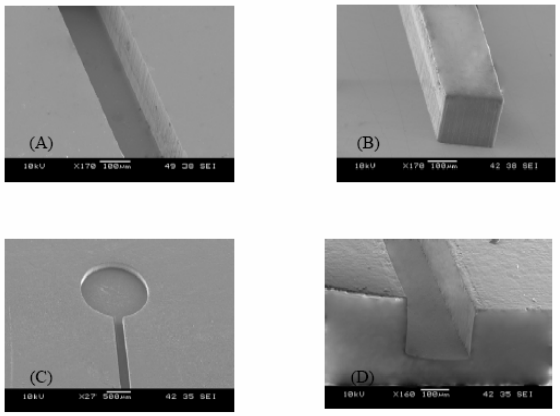

Fig.4. SEM pictures of fabrication results. (a) The channel fabricated by Deep RIE on silicon. (b) Replicated PDMS structure from silicon mold. (C) Fabricated polymeric chip (reservoir and channel) from PDMS mold. (D) Cross section of polymeric

B. Ion Signal from Different Plastic Support

It has proved that different sample supports generate the ion signal with different intensity, signal/noise ratio and reproducibility during MS analysis [29][30][31]; due to the topology of the support, chemical interaction between matrix and polymer support [32]. From experiments, we find out that polyester generates best signal during ionization. Herein, the signal from UV-embossed polyester is compared with that from PMMA and PEG, using detection of high mass protein, BSA. Usually, ion strength from the certain laser intensity is implemented to evaluate the suitability of sample support [30][31], because ion strength is related to the ionization efficiency. The S/N ratio is associated with the sensitivity of the mass spectrometry, because the matrix also could give rise to the ion signal which belongs to the noise in this case. As shown in Fig. 5, the signal strength measured from polyester is 12 times stronger than that of PMMA and also 2.5 times better than that of PEG. In terms of S/N ratio, polyester is 5 times better than PMMA, which indicates that polyester could improve the sensitivity of high mass protein identification. Besides, in terms of the relative standard deviation, signal from polyester is 30% lower than that from PMMA, which means, to certain extent, the polyester alleviates the "sweet spot" problem significantly, since the ionization is more uniform upon the sample. In addition, due to the superior ion strength and S/N using polyester, at concentration of 0.9pmol/μl of BSA, the signal using polyester is still striking while there is no signal at all from PMMA, as in Fig 6.

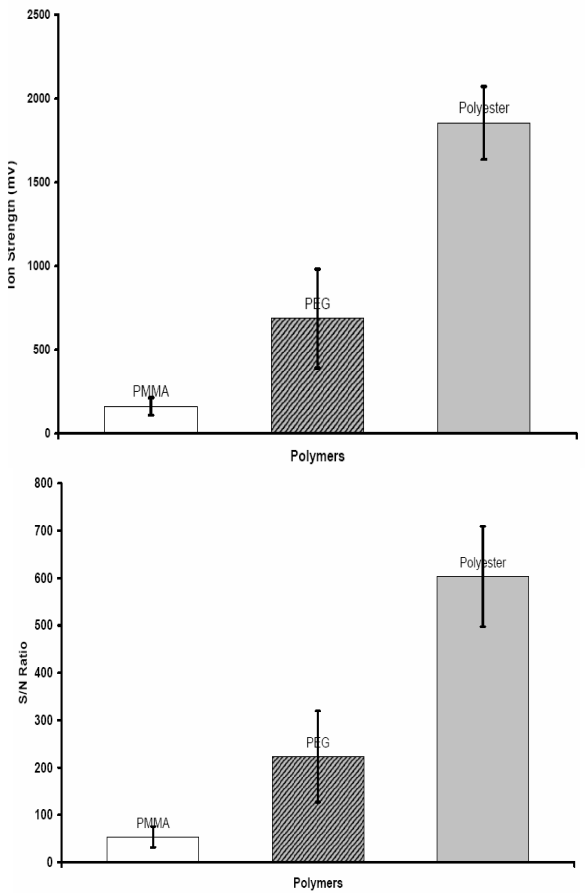

Fig 5. Comparison of the MS ion signal of BSA using PMMA, PEG and Polyester as sample support. The ion strength and signal-to-noise (S/N) ratio of each polymer are averaged by signal of 20 samples onto 4 pieces of each polymer. Each sample is scanned by 200 laser shots randomly. The concentration of BSA solution is 1.8pmol/μl; 1μl protein solution is deposited onto 1 mm^2 polymer surface. The all ion measured is from the single (A). The polyester is 12 times superior to PMMA, in terms of ion strength. (B). The S/N ratio of polyester is nearly 5 times stronger than that of PMMA.

C. Capillary Isoelectric Focusing

The pH gradient (3-10) in the protein solution is generated by carrier ampholyte, which consists of thousands of small molecular buffered ampholytes[34]. Under electric field, due to their small molecular weight,

these ampholytes quickly move to their specific pI points ranging from 3~10, where they function as pH buffer to maintain the relatively constant pH value in their neighbor environment; because of the highly electrical field, these concentrated ampholytes tend to overcome the diffusion effect and stay still; thus a stable pH gradient is generated.

The concentration of carrier ampholyte in the protein solution is critical of two aspects: it is related to the current during CIEF, and it is also associated to the noise during mass spectrometry. If the concentration is very high (>10%), large noise is generated from MS by the ionization of these ampholyte; besides, the high concentration causes the high current, heat and bubble in solution, according to Joule's law; thus we hope low concentration of carrier

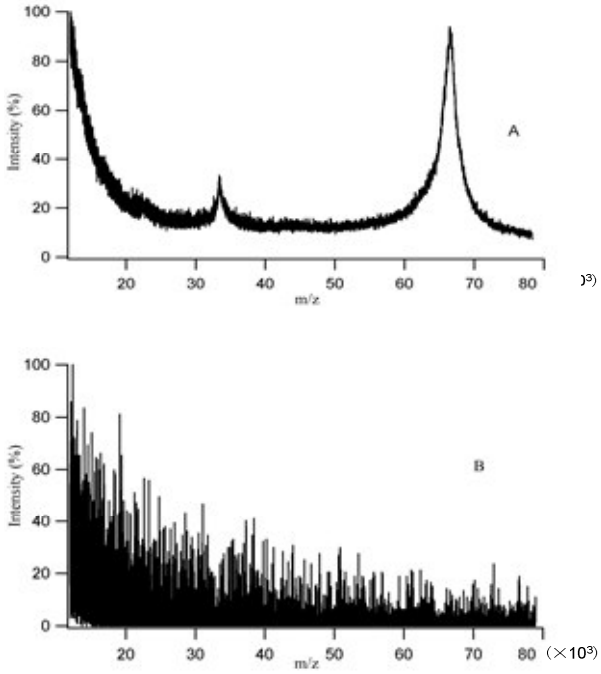

Fig 6. MALDI mass spectra of 0.9 picomole/ul bovine serum albumin (BSA, ~66.4 kDa) using different sample support. The matrix was deposited using dried-droplet method. Both spectra scanned 300 profiles. (A) The sample support is polyester. (B) The sample support is PMMA. The optimized mass range in MS is set at 65k to 72k.

ampholyte. However, if the concentration is too low (<1%), we find that it takes as long as 20 mins for the protein to concentrate, due to the week buffer effect of the carrier ampholyte. Therefore, in the experiments, we used 1% pharmalyte in the protein solution [12].

The voltage applied is not constant throughout the experiment, due to the generated current in the channel during CIEF. In the initial period of CIEF, the increase of voltage results in the proportional increase of current. This is because the carrier ampholytes do not reach their equilibrium so that they still carry charge. If the voltage further increases, the resultant current is so high that the Joule heat generates lots of bubbles in the channel which disturb the stability of pH gradient in the channel. Therefore, in initial period of our experiments, we control the voltage relatively low (<2.3kv) and the electric field is around 300v/cm so that the current is usually below 0.15mA. As the pharmalyte gradually reach their pI points, the current is declining steadily. Then, we could continue to increase the voltage and the current maintains at 0.12mA, until the voltage reaches 5.6kv and the electric field is 800v/cm. Usually after a 3 mins, the current in the channel is stabilized, since the protein concentrated. We continue to maintain the voltage until the current reaches zero, due to the evaporation of the solution. The total time for CIEF is around 6 minutes. Eventually, at the bottom of the channel, we can see the concentrated protein, if it has the color, as shown in Fig 7.

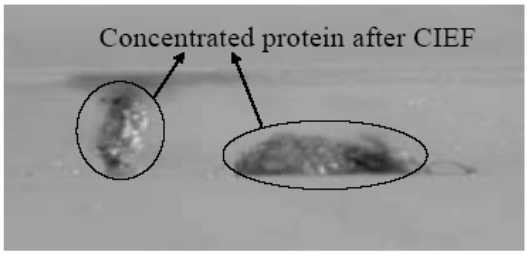

Fig. 7. Concentrated proteins after CIEF.

The electroosmotic force is very helpful for some IEF applications where dynamic injection by electroosmotic force is necessary to transfer the solution to the observation points to detect the protein by fluorescence [19][20].However, electroosmotic force also could make the concentrated protein move under electric field; in our CIEF experiment, we observed the very week EOF mobility of $3.5\times10^{-6}\,\mathrm{cm}^2\cdot\mathrm{v/s}$ at $25^\circ\mathrm{c}$ and pH=7, which is in agreement with the research by Caslavska[35] that acrylic material has relatively small electroosmotic mobility.

D. Mass Spectrometry

The spectra from mass spectrometer are illustrated in Fig 8. The peaks represent the ion signal from the proteins. For the trypsin inhibitor, its peak is at around m/z=20.3; we also see a small peak at m/z=10.15, which also represents the trypsin inhibitor due to the double charge effect. For the myoglobin, the peak is at 17.6 (m/z). The peak corresponding to myoglobin was also detected, as shown in Fig. 8.

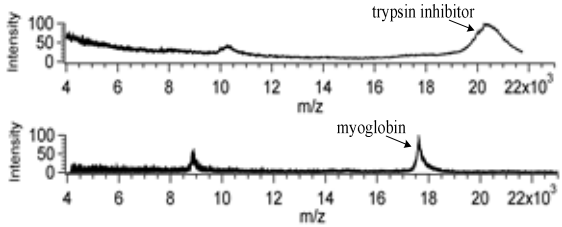

Fig. 8. Mass spectra of trypsin inhibitor and myoglobin by mass spectrometry.

IV. CONCLUSIONS

In this report, we demonstrate the UV-embossing as a fabrication method to develop plastic chip for the purpose

of protein separation and identification-CIEF and MALDI-MS. The plastic chip is successfully replicated from the soft PDMS rubber mold by UV irradiation. In order to enhance the protein ion signal from MS, polyester based polymer has been chose from 5 types of widely used resin, due to its generated superior intensity of ion signal and signal/noise ratio. From our experiments, we found that the ion signal and ion/noise ratio from polymer support are 12 times and 5 times stronger than that from previously used PMMA. CIEF to separate the protein has been performed, followed by mass spectrometry identification. From our experiments and preliminary investigation, we demonstrate that UV-Embossing polymeric fabrication with high throughput has the merit to use in the biological application.

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