Aqueous and non-aqueous microchip electrophoresis with on-chip electrospray ionization mass spectrometry on replica-molded thiol-ene microfluidic devices

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ABSTRACT

This work describes aqueous and non-aqueous capillary electrophoresis on thiol-ene-based microfluidic separation devices that feature fully integrated and sharp electrospray ionization (ESI) emitters. The chip fabrication is based on simple and low-cost replica-molding of thiol-ene polymers under standard laboratory conditions. The mechanical rigidity and the stability of the materials against organic solvents, acids and bases could be tuned by adjusting the respective stoichiometric ratio of the thiol and allyl ("ene") monomers, which allowed us to carry out electrophoresis separation in both aqueous and non-aqueous (methanol- and ethanol-based) background electrolytes. The stability of the ESI signal was generally ≤10% RSD for all emitters. The respective migration time repeatabilities in aqueous and non-aqueous background electrolytes were below 3 and 14% RSD (n= 4-6, with internal standard). The analytical performance of the developed thiol-ene microdevices was shown in mass spectrometry (MS) based analysis of peptides, proteins, and small molecules. The theoretical plate numbers were the highest ($1.2\text{-}2.4\times10^4$ m^{-1}) in ethanol-based background electrolytes. The ionization efficiency also increased under non-aqueous conditions compared to aqueous background electrolytes. The results show that replica-molding of thiol-enes is a feasible approach for producing ESI microdevices that perform in a stable manner in both aqueous and non-aqueous electrophoresis.

Keywords: Microchip electrophoresis, Non-aqueous capillary electrophoresis, Electrospray ionization, Mass spectrometry, Replica-molding, Thiol-enes

1. INTRODUCTION

Microchip capillary electrophoresis (MCE) is the gold standard of microfluidic separation systems. MCE in combination with electrospray ionization mass spectrometry (ESI-MS) is a promising tool for modern bioanalysis, especially in proteomics and metabolomics.[1] Although numerous approaches for interfacing separation microdevices with MS *via* ESI exist, the implementation of on-chip ESI emitters as an integral part of the separation chip is feasible for only a few microfabrication methods and materials. One important limitation is that fabrication typically requires expensive cleanroom instrumentation.[2, 3] Integration of a sharp-pointed, on-chip ESI emitter directly with the separation microchannel outlet eliminates the dead volume at the ESI interface and the need for manual post-processing required to attach off-chip emitters. In addition, a sharp-pointed tip reduces sample spreading at the channel outlet and facilitates producing the small Taylor cone that is required for efficient ionization (small droplet size) and stable spraying. Integrated MCE devices with on-chip emitters have been made from glass by manual pulling the ESI emitter [4], by sawing a sharp corner at the microchannel outlet [5, 6] and by isotropic etching [7] techniques. Silicon [8] or silicon-glass hybrid materials [9] have also been used for fabricating on-chip ESI emitters onto chromatographic separation chips. The semiconductive properties of silicon, however, render it unfeasible for electrophoresis applications.

The lowest-cost approach for fabrication of sharp-pointed ESI emitters appears to be achieved *via* polymer microfabrication. Electrophoresis separation chips with sharp-pointed, on-chip ESI emitters have been implemented on SU-8 by standard photolithography [10], on organically modified ceramics by sawing [11], on polycarbonate by laser micromachining [12] and on cyclo-olefins by hot embossing [13]. However, high-precision fabrication and bonding of the above mentioned materials require expensive instrumentation or special facilities, such as a cleanroom environment. This constraint inevitably hinders the wider adoption of the microchip technology to routine laboratory

63 analyses. Thus, non-cleanroom polymer processing methods, such as the replication of polydimethyl
64 siloxane (PDMS) [14], have also been introduced as an approach to achieve the low-cost fabrication
65 of on-chip emitters. In addition to its straightforward replication, PDMS also allows for the easy
66 sealing of microchannel by adhesive bonding of two cross-linked layers. The drawbacks to PDMS,
67 however, is that it is susceptible to swelling and severe monomer leaching upon exposure to organic
68 solvents [15, 16] and it also undergoes significant nonspecific adsorption of biomolecules unless the
69 surface is physically or chemically treated prior to use [17]. Moreover, the elasticity of PDMS
70 prevents fabrication of very thin layers, which is often desired as a prerequisite to reproduce three-
71 dimensionally sharp ESI emitters.
72
73 We describe a new, low-cost method for the fabrication of MCE-ESI microdevices with fully
74 integrated, thin and sharp on-chip ESI emitters using the approach of replica-molding of thiol-ene
75 polymers. In addition, thiol-enes enable adhesive bonding similar to that of PDMS, but show much
76 better stability against organic solvents. The mechanical stiffness and rigidity of thiol-enes can also
77 be tuned by altering the respective quantities of the thiol and allyl ("ene") monomers in the bulk
78 material.[18] The use of off-stoichiometric monomer ratios results in excess of free thiol or allyl
79 functional groups on the polymer surface [19-21] which have been exploited for numerous
80 biofunctionalizations [22-25] and for aqueous MCE in combination with fluorescence detection. [19,
81 22, 26] Thiol-ene channels generally maintain high cathodic electroosmotic flow over a wide pH
82 range (pH 3-12) [19, 22] and show little nonspecific adsorption of peptides in native, allyl-rich
83 microchannel walls. [19] Polyacrylate copolymer coatings can be used to eliminate protein adsorption
84 in thiol-rich microchannels. [22]
85 Thanks to their inherent good stability against organic solvents, thiol-enes as chip fabrication
86 materials also provide greater flexibility in terms of analytical method development than most other
87 microfabrication polymers. In this study, we exploit the good solvent compatibility to carry out

microchip electrophoresis in non-aqueous conditions. The study demonstrates how the selection between aqueous and non-aqueous background electrolyte affects not only the separation efficiency and selectivity, but also sensitivity and repeatability. Thus far only a very few on-chip NACE applications (in combination with any detector) have been reported. [27-30] The fabrication of sharp, on-chip emitters that use solvent-compatible fabrication materials (such as glass) is challenging [2] and thus the combination of microchip NACE and on-chip ESI is less common than its aqueous-phase counterpart despite the inherently good technical compatibility between NACE and ESI-MS. The replica-molding of thiol-enes presented in this work achieves fabrication of an integrated, sharp emitter at low-cost and theproduced MCE-ESI devices show good analytical performance in both aqueous and non-aqueous electrophoresis.

2. EXPERIMENTAL

2.1. Materials and Reagents

Acetic acid, methanol, ethanol, propanol and acetonitrile were purchased from Sigma-Aldrich (Steinheim, Germany). Ammonium acetate and hydrochloric acid were purchased from Riedel-de Haën (Seelze, Germany). Formic acid was purchased from Merck Millipore (Darm-stadt, Germany). Angiotensin I human acetate salt hydrate (≥90 %), angiotensin III (≥98 %) and cytochrome c from bovine heart (12327 Da ≥95 %) were from Sigma-Aldrich. Angiotensin II acetate salt (96.2%) was from Bachem (Bupendorf, Swizerland). Verapamil hydrochloride was from ICN Biomedicals (Aurora, OH). Stock solutions of peptides (each 1 mg/mL in milli-Q water), cytochrome c (5 mg/mL in water) and verapamil (1 mM in MeOH) were diluted before analysis in respective solvents. All reagents and solvents used were of HPLC or LC-MS grade (≥99.0%) unless otherwise stated. Water was purified with a Milli-Q water purification system (Millipore, Molsheim, France).

Trimethylolpropanetris(3-mercaptopropionate) ('trithiol') (≥95.0%), pentaerythritoltetrakis(3-mercaptopropionate) ('tetrathiol') (≥95.0%) and 1,3,5-triallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione ('triene') (≥98,0%) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Poly(dimethyl siloxane) (PDMS) was prepared from Sylgard 184 base elastomer and curing agent (Down Corning Corporation, Midland, MI, USA). SU-8 negative photoresist (Microchem Corporation, Newton, MA, USA) were purchased from Micro Resist Technologies GmbH (Darmstadt, Germany).

2.2. Microchip fabrication

Thiol-ene chips were fabricated by mixing commercially available trithiol or tetrathiol monomers with triallyl ("triene") monomer in stoichiometric or off-stoichiometric ratios (50 mol-% excess of allyls, trithiols or tetrathiols). No photoinitiator or other additives were used during the thiol-ene crosslinking process. First, a PDMS negative mold was prepared from a 4-inch SU-8 master. This wafer-scale mold featured 12 parallel MCE-ESI units, each incorporating the separation microchannel with an integrated electrospray emitter.. The thiol and allyl monomers were mixed and poured onto the PDMS mold (Figure 1A-D). The thiol-ene mixture was cured without any cover plate or photomask by exposing it to UV from a Dymax 5000-EC Series UV flood exposure lamp (Dymax Corpo-ration, Torrington, CT, USA, nominal power of 225 mW/cm^2). The UV exposure times were chosen based on our earlier study [19] and were 10 min for all thiol-ene compositions. The bottom layer of the thiol-ene chip, featuring only the outer edges of the chip, was prepared in a similar manner and laminated against the microchannel layer. The thiol-ene layers were preheated to 70ºC before lamination to gently soften the polymer and thus obtain uniform sealing between the layers. The lamination was done under a stereomicroscope to ensure precise alignment of the two thiol-ene layers at the emitter area. Last, the bonding was completed by additional UV exposure of 5 min similar to that described in earlier work [19, 26].

The masters for the PDMS molds were made from SU-8 negative photoresist under cleanroom conditions and were separately prepared for the microchannel and the bottom layers (Figure 1A). The microfabrication protocols for the SU-8 master and the PDMS mold are described in detail in the Supplement material.

The fabricated microchips featured a 20-mm-long separation channel (effective length) that incorporated a simple cross injection channel and was intersected by a 10-mm-long makeup liquid channel just behind the emitter tip (Figure 1E). The cross-section dimensions of the separation channel were 50 μm×50 μm (w×h), of the injection channel30 μm×50 μm (w×h) and of the makeup liquid channel 200 μm×50 μm (w×h). The inlets were 1 mm in diameter and the thickness of the emitter tip was approximately 200 μm (Figure 1F)

2.3. Solvent exposure tests

The stabilities of the stoichiometric and various altered off-stoichiometric thiol-ene compositions were tested against selected organic solvents (methanol, ethanol, propanol and acetonitrile), acids (10 % formic acid, 10 % acetic acid, 2 M hydrochloric acid) and bases (10% ammonium hydroxide) commonly used in MS applications. Thiol-ene slabs (thickness 0.5 mm, A=1 cm^2) that had been cured for 10 min were used as test pieces. The pieces were immersed in 1 mL of each solvent for 1 h or for 4 days after which they were visually monitored for any mechanical damage, e.g., swelling, degradation, or defects on the surface.

2.4. Microchip electrophoresis-electrospray ionization mass spectrometry

The thiol-ene microchips were coupled to an Agilent 6330 iontrap mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with a modified nanospray frame (Proxeon Biosystems, Odense, Denmark), which featured an xyz aligning stage and a CCD camera (Figure S1). The ion

trap was operated in positive ion mode with a capillary voltage set at -1200 or -1500 V and end plate offset at -500 V. Nitrogen produced from compressed air by a Parker nitrogen generator (Cleveland, OH) was used as the drying gas with a flow rate of 4.0 L/min at 70°C. The MS data were acquired by averaging two cycles over a mass range of m/z 100−2200 with maximum accumulation time of 200 ms. Data Analysis 3.4 was used for data acquisition and processing.

Before use, thin PDMS sheets with 2 mm inlet holes were attached on top of the inlets to increase the sample volume and to limit spreading of the sample and buffer aliquots over the chip surface. Since PDMS was only used as passive support structures, no PMDS monomer leaching to the MS was observed. An external high voltage power supply (Micralyne, Edmonton, AB) was used to apply the ESI and the separation voltages through platinum wires placed in the microchannel inlets. The samples were introduced through a simple injection cross by applying an injection voltage of +800 V to the sample inlet (SI) and grounding the sample outlet (SO) for 20.0 s. The nominal injected sample volume (V=75 pL) was defined by the injection cross geometry, which was 30 μm×50 μm×50 μm (w×L×h). The make-up liquid inlet (MLI) was floating during injection. The MCE separation was performed by applying a separation voltage (typically 4900-4700V) to the buffer inlet (BI) and antileakage voltages (typically 4500-4000V) to the SI and SO. The ESI voltage, which also served as the counter voltage for the MCE separation was applied to the MLI and was between 2000 and 3500 V (see Supplementary material Figure S1). The separation current was typically between 30−40 μA, and the electrospray current less than 200 nA. Thanks to the laminarity of flows, the make-up liquid did not much dilute the sample flow prior to ESI-MS. The excess current from the separation channel was grounded through a 50 MΩ resistor coupled in parallel with the ES voltage supply. The distance between the tip and the MS orifice was typically between 5 and 10 mm.

3. RESULTS AND DISCUSSION

3.1. Fabrication and material stability aspects

This work describes simple and low-cost fabrication of microfluidic electrophoresis chips with fully integrated on-chip emitter tips *via* UV replication of thiol-ene polymers under standard laboratory conditions. We used PDMS (negative) molds replicated from SU-8 masters as the templates for replica-molding of thiol-enes. Each SU-8 master (featuring 12 parallel MCE-ESI units) could be re-used for PDMS molding for at least 5-10 times and each PDMS mold (also featuring 12 parallel MCE-ESI units) for thiol-ene replication for at least 5 times. This totals minimum of 300 thiol-ene MCE-ESI chips reproduced out of a single SU-8 master. Thus, the materials cost of a single thiol-ene chip becomes very low. Also the infrastructure needed for thiol-ene replication included only low-cost, standard equipment such as flood exposure lamp and oven. Only the fabrication of the SU-8 master was carried out using established cleanroom techniques (see Supplementary material) and thus, the cost of the fabrication and the need for cleanroom processing were significantly reduced compared to fully cleanroom-microfabricated glass [4-7] or SU-8 [10] electrospray microchips, for example. Therefore the ease of replica-molding and bonding of thiol-enes significantly promotes the use of microchip based techniques in routine MS analyses by providing new technical solutions to chip fabrication that are accessible to all. The only critical step of the thiol-ene chip fabrication was the bonding of the two cured layers together with high precision in alignment at the emitter tip. However, if misalignment occurred, it was possible to re-do the bonding step before the bond was finalized by additional UV curing. Since the PDMS molding and thiol-ene replication steps were carried out in a laminar flow hood, the particles in the regular laboratory air mainly landed on the chip surface and had thus negligible influence on the device performance (e.g., the flow rate or the migration of the analytes inside the microchannel).

Only PDMS of the other commonly used polymer materials allows an equally straightforward adhesive bonding as that of thiol-enes, but the elasticity of PDMS prevents the replication of thin emitters. Instead, the good mechanical strength and rigidity of the thiol-ene compositions used in this study allowed the fabrication of relatively thin microchips (ca. 200 μm at the tip, Figure 1F), which enabled the reproduction of three-dimensionally sharp tips. Comparison of the tensile strengths of the different thiol-ene formulations (see Supplementary material Figure S3) indicates how the mechanical properties of crosslinked thiol-enes are affected by both the monomer ratio and the selection of the precursor monomers (trithiol vs. tetrathiol). For example, off-stoichiometric compositions comprising excess quantities of trithiol monomers formed relatively elastic structures, which complicated thiol-ene-to-thiol-ene bonding and also hindered the fabrication of rigid and sharp emitters. However, replacement of the trithiol monomer with tetrathiol enabled the fabrication of sufficiently rigid MCE-ESI microchips while still having thiol-rich surfaces. Stoichiometric and allyl-rich compositions also resulted in sufficiently rigid structures.

Apart from elasticity, good chemical stability is essential for obtaining reproducible analytical performance. Good compatibility with aliphatic and aromatic organic solvents has been reported for thiol-ene polymers. [31-33] However, limited information exists about the stability against acids and bases that are commonly used in MS applications. The compatibilities of the different thiol-ene compositions used in this study with the selected organic solvents, acids and bases were thus determined and are summarized in the Supplementary material (Table S1) together with photographs and light microscope images of thiol-ene surfaces exposed to selected solvents (Figure S4). Briefly, all compositions tested showed good resistance to methanol, ethanol, and propanol during short-term exposure (1 h), whereas acetonitrile caused cracking and fragmentation of all the thiol-ene compositions. The thiol-rich (50 mol-%) composition prepared from trithiol underwent cracking upon long-term methanol exposure, but all other compositions, including the tetrathiol-rich (50 mol-%

excess) composition, tolerated the tested alcohols for extended periods of time (up to 4 days) without incurring any clear defects. Similarly, none of the acid or base solutions had any influence on the stoichiometric, allyl-rich (50 mol-%) or tetrathiol-rich (50 mol-%) composed devices during short or long-term exposure. Only the trithiol-rich composition underwent cracking during prolonged exposure (4 days) to acetic acid. Clearly, the lower crosslinking density of the trithiol-rich thiol-ene composition causes not only less stiffness [21], but also makes the composition more vulnerable to degradation upon exposure to organic solvents, acids and bases. Again, replacing the trithiol monomer with tetrathiol resulted in greater stiffness and improved solvent compatibility.

3.2. Electrospray performance

Monomer leaching (due to incomplete crosslinking) from the bulk polymer to the MS is a well-known drawback for many polymer based electrospray chips. Such leaching has detrimental effects upon the analytes' ionization efficiency and upon the quality of the MS spectra obtained. PDMS in particular has poor material stability regarding to leaching, although it has been reported that the cross-linking density (i.e., the curing time) plays an important role in reducing the monomer leaching from PDMS devices. [16, 34] Thiol-enes, on the other hand, are often used for chip fabrication in off-stoichiometric ratios in order to achieve the desired mechanical properties or surface chemistry (thiol- or allyl rich surfaces) that facilitates further biofunctionalization reactions.[21-26] To examine if the use of off-stoichiometric formulations cause leaching of the excess monomers to the MS, we compared the ESI-MS background spectra of microchips fabricated from each of the four different thiol-ene formulations, each of which had two different curing times (10 or 20 min). The MS background spectra were recorded by electrospraying sample solutions containing an antihypertensive drug, verapamil (m/z 455.4), as an internal reference of the ESI stability.

As expected, allyl (m/z 250.1), trithiol (m/z 399.1) and tetrathiol (m/z 489.0) monomers leached out of the chip whenever they were used in excess in the bulk composition and were observed as protonated ions at their respective m/z (Figure 2A, C and D). The extension of the curing time from 10 to 20 min did not significantly reduce the background interference that originated from the off-stoichiometric compositions. Despite this, the background interference was relatively low and could be effectively eliminated by rinsing the channels prior to experiments. Most importantly, the microchips that had been prepared from stoichiometric thiol-ene provided good quality spectra with no traces of uncured monomers even without rinsing before the experiments (Figure 2B).

The performance of the ESI emitters prepared from different thiol-ene compositions was also examined by comparing the total ion current (TIC) and the extracted ion current (EIC) stabilities of the test compound verapamil. The TIC stabilities of the ESI emitters that had been fabricated from allyl-rich (50 mol-%), tetrathiol-rich (50 mol-%) or stoichiometric thiol-ene compositions typically ranged between 4.6 and 7.0% RSD, whereas their EIC stabilities ranged between 6.1 and 8.9% RSD (n=3 chips, over 2 min range). As stated above, the fabrication of thin emitter tips from the trithiol-rich composition (50 mol-% excess) was difficult due to its high elasticity and lack of rigidity. Thus, the trithiol-rich emitter tips bent during electrospray and stable ion current was hardly obtained (Figure 2C).

Apart from the trithiol-rich emitters, stable ion current could be easily maintained for as long as 20 min with an overall stability of 10.4% RSD (Figure S2). The chip-to-chip repeatability of the average total ion current obtained by direct infusion was 13.3 % (n=4 chips). Each chip could also be re-used for multiple analyses for several days. In addition to small molecule analysis, the feasibility of the thiol-ene emitters was shown for direct infusion of a protein sample (cytochrome c), which showed no interfering background originating from thiol-enes even in the high m/z range and thus good

accuracy (12230.5±0.5 Da, 0.004% accuracy) in terms of molecular weight determination (Figure 3A).

These results suggest a high level of feasibility of thiol-ene replica-molding for fabrication of sharp ESI emitters producing stable electrospray. The performance of the developed thiol-ene emitters in ESI-MS was generally similar to those of the previously reported state-of-the-art microfabricated emitters made of, e.g., glass, SU-8, or organically modified ceramics (see Table S3 for details). [2] However, in comparison to other common microfabrication materials, the thiol-ene chemistry provides greater flexibility in terms of chip fabrication (mechanical stiffness/rigidity and possibility for low-cost, non-cleanroom replication), improved material stability against alcohols (methanol, ethanol and propanol tested in this study), and more opportunities to tune both the surface chemistry and the bulk properties toward the desired applications, without affecting the ESI-MS performance much. Finally, the feasibility of the design to MCE-ESI-MS analysis was examined with help of excitatory neuropeptides, Orexin A and B (Figure 3B). These peptides, however, suffered from nonspecific adsorption to the native thiol-ene surface (as evidenced by pronounced peak tailing) and did not resolve from each other within the short separation distance used (effective separation length 20 mm), leaving a place for further separation method development.

3.3. Capillary zone electrophoresis in aqueous and non-aqueous conditions

In addition to ESI-MS, we addressed the separation performance of the thiol-ene devices in both aqueous and non-aqueous electrophoresis. For this purpose, we chose angiotensin II, a peptide hormone that affects vasoconstriction, and its biologically inactive precursor, angiotensin I, the ratio of which is an important biological indicator of angiotensin-converting enzyme (ACE) activity. The MCE-ESI-MS analysis of the two angiotensins in aqueous conditions is shown in Figure 4A. The repeatability of the migration time for angiotensin I and II were 4.6 and 4.7% RSD (2.7 and 2.4%

with angiotensin III as the internal standard, n=4-5) and the theoretical plate numbers 0.77×10^4 and 0.80×10^4 m^{-1}, respectively. However, even if MS detection could distinguish between the two forms based on their different *m/z* values, the two peptides did not resolve electrophoretically from each other within the short separation distance of 20 mm (similar to the Orexin peptides). Although these peptides can be separated electrophoretically [35], the resolution poses a challenge because of the similarities of their pI values (i.e. 7.70 v.s. 7.54 [36]) and their electrophoretic mobilities in aqueous buffers. [37] In non-aqueous background electrolytes, the change in the solvents' ε/η ratio (see Supplementary material, Table S2) may have a favorable effect on the resolving power and separation selectivity. Moreover, the electroosmotic flow of non-aqueous background electrolytes is typically slower than that of aqueous electrolytes leaving more time for the compounds to resolve electrophoretically even in relatively short separation channels.

Therefore, we chose to study the possibility to use methanol and ethanol based, non-aqueous background electrolytes to improve the resolving power of the two angiontensins,. Acetonitrile is another commonly used solvent in NACE, but it was excluded due to its poor compatibility with thiol-enes as described in the Supplementary material (Table S1). The analytical performance of on-chip NACE in the analyses of the angiotensin peptides was compared to that obtained under aqueous conditions using angiotensin III as the internal standard. As expected, the apparent mobilities of the angiotensins decreased from ca. 4.5×10^{-4} $cm^2s^{-1}V^{-1}$ in aqueous electrolytes to 1.2×10^{-4} $cm^2s^{-1}V^{-1}$ in organic background electrolytes (Figure 4F) as a result of the decreased EOF and the change in the ε/η ratio of the solvent and thus decrease in electrophoretic mobilities of the two peptides. On the basis of the comparison of the migration times between aqueous and methanol solutions (on the average between 20 and 30 s) and ethanol solutions (on the average between 70 and 90 s), the EOF remained somewhat similar in aqueous and methanol based background electrolytes, but slowed

down significantly in ethanol based background electrolytes (Figure 4C). Depending on the background electrolyte, the linear flow rates varied within 0.2-1 mm/s corresponding to volume flow rates between 30 and 150 nL/min.At the same time, the electrophoretic mobilities were differently affected (due to the ε/η ratio), which eventually resulted in better resolving power (R_S=0.9) in acidified ethanol than in aqueous or methanol based electrolytes (Figures 4A and B). The absolute migration time repeatability was generally better in aqueous conditions (Figure 4C), whereas non-aqueous electrolytes clearly increased the ionization efficiency (Figure 4D) and improved the plate heights (Figure 4E). On the average, the peak areas increased 2-fold in ethanol-based electrolytes compared to those in aqueous electrolytes and showed sufficiently good repeatability from run to run (6.6% and 16.2% RSD for Ang I and Ang II, n=6, with Ang III as the internal standard). The theoretical plate numbers also increased 2-fold, to 1.2×10^4 m^{-1} (Ang I) and 2.4×10^4 m^{-1} (Ang II), in ethanol-based electrolytes.

In general, the ability to carry out microchip-NACE-ESI-MS with overall performance similar to or better than those obtained in aqueous conditions broadens out the applicability of thiol-ene microdevices to encompass a variety of bioanalytical purposes. Even if NACE is commonly used in the analyses of water-insoluble or sparingly soluble analytes, it may also provide improved resolution of the separation of water-soluble, charged analytes, because of the differences between the electrophoretic mobilities in non-aqueous and aqueous electrolytes. In addition to this work, increased resolving power in peptide separation by NACE has also been reported elsewhere. [38-41] In general, microchip NACE is also a good fit to ESI-MS due to the similarity of the flow rates and the solvents required. The low surface tension of organic background electrolytes improves and stabilizes the electrospray and thus increases the ionization efficiency over those of aqueous background electrolytes. On the other hand, the possibility to perform separations in either aqueous or non-aqueous conditions by using the same chip, provides greater practical flexibility in analytical method

development and has potential for improving resolution between compounds that do not sufficiently resolve in aqueous conditions, such as angiotensins I and II. Thiol-enes as chip fabrication materials play a key role in facilitating the analyses in organic solvents without degradation and thus, leaching of the monomer residues to the MS.

4. CONCLUSIONS

The inherent good solvent compatibility of thiol-enes was exploited to carry out microchip electrophoresis in non-aqueous conditions in addition to more commonly applied aqueous background electrolytes. We found that NACE-ESI-MS improved particularly the sensitivity and selectivity of angiotensin peptides over MCE-ESI-MS in aqueous conditions. In addition, the replica-molding of thiol-enes was shown to be a versatile tool for low-cost fabrication of MCE chips with integrated sharp-pointed ESI emitters. The fabrication process proceeds from a single lithographically fabricated SU-8 master but after that, numerous microfluidic chips can be fabricated using replica molding and bonding techniques under standard laboratory conditions. The materials' properties and the surface chemistry of the thiol-ene chips can be tuned simply by changing the precursor chemicals or adjusting the stoichiometry of the monomers. The material stability and the electrospray experimental data suggest that both stoichiometric and off-stoichiometric thiol-ene compositions (from 50 mol-% excess of allyls to 50 mol-% excess of tetrathiols) are feasible for the replication of sharp emitter tips. High quality spectra with negligible background interference were obtained when using the stoichiometric composition. However, off-stoichiometric thiol-ene compositions resulted in the leaching of the excess monomer into the MS, but the monomer background could be easily eliminated by carefully rinsing the channels prior to use. The results suggest that replica-molding of thiol-enes provides a simple, low-cost and flexible approach to the fabrication of microchips under standard laboratory conditions, which significantly promotes the adaptation of the microchip technology for routine analyses.

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APPENDIX A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

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FIGURES AND CAPTIONS

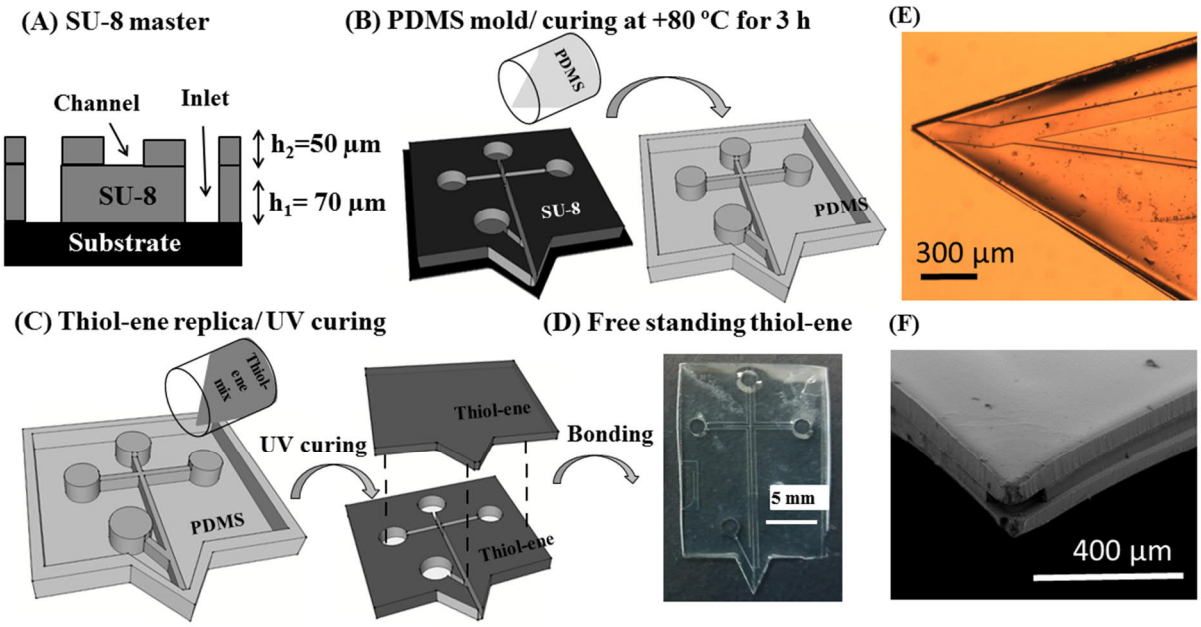
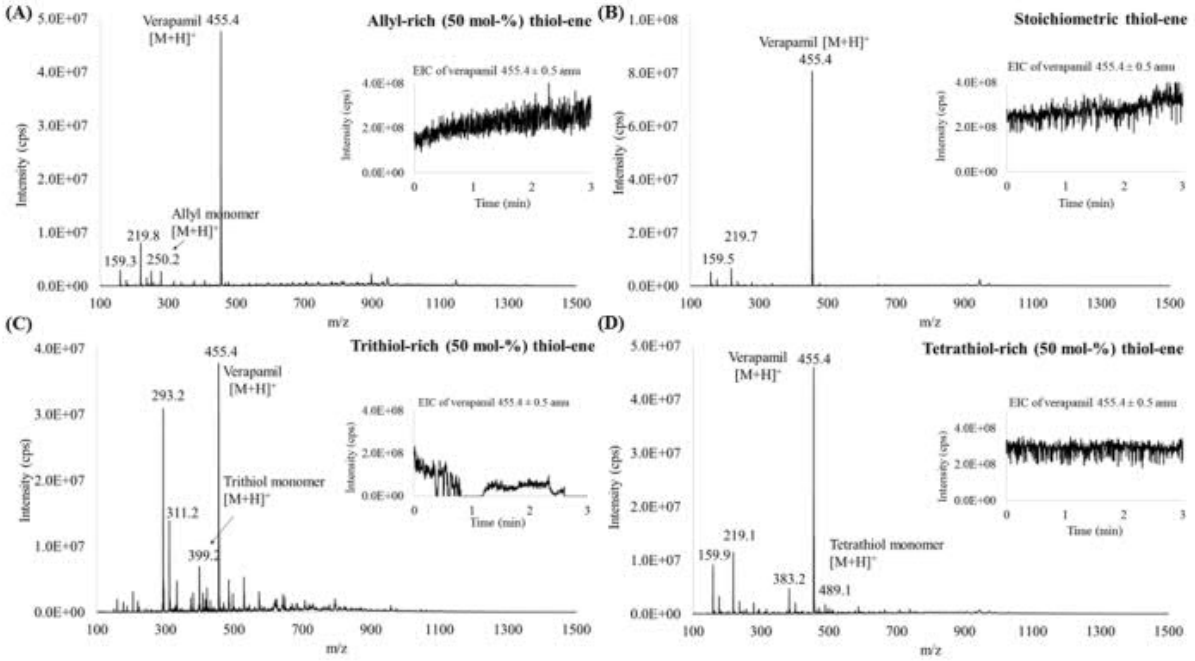


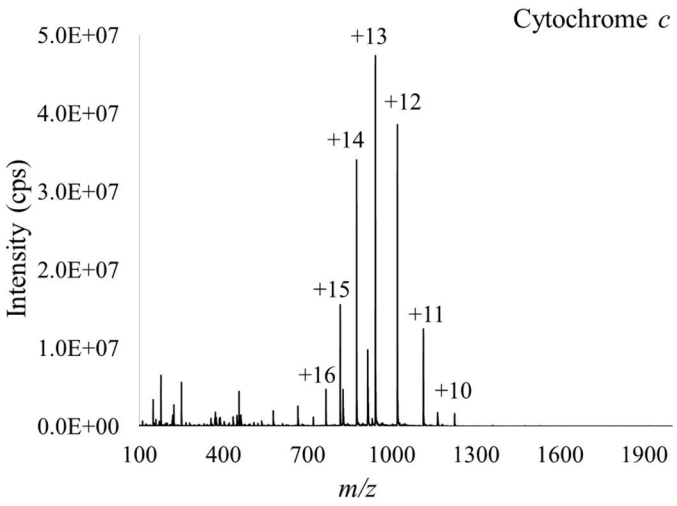
Figure 1. (A-D) Schematic presentation of the fabrication steps of thiol-ene MCE-ESI-chips (not in scale): (A) SU-8 master fabrication in cleanroom by spincoating two sequential layers of SU-8 over silicon substrate (h1=70 µm inlets and h2=50 µm channels), (B) casting of the PDMS mold and curing by heat, (C) replication and UV curing of the thiol-ene top and bottom layers followed by tip alignment and bonding, (D) photograph of a bonded free-standing thiol-ene chip. (E-F) Optical and scanning electron micrographs of an ESI emitter tip after bonding.



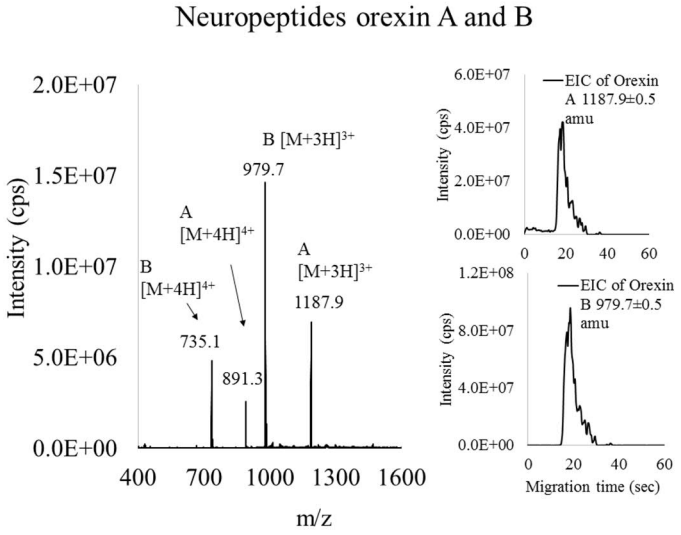
516

517 **Figure 2.** ESI-MS spectra obtained by direct infusion from thiol-ene chips that had been fabricated
518 from (A) allyl-rich (50 mol-%), (B) stoichiometric, (C) thiol-rich (50 mol-%, trithiol) and (D) thiol-
519 rich (50 mol-%, tetrathiol) compositions. The curing times used were 10 min in each. The sample
520 solution was 5 µM verapamil in methanol-water 80:20 containing 1% acetic acid. The ESI voltage
521 applied was 3kV.

522

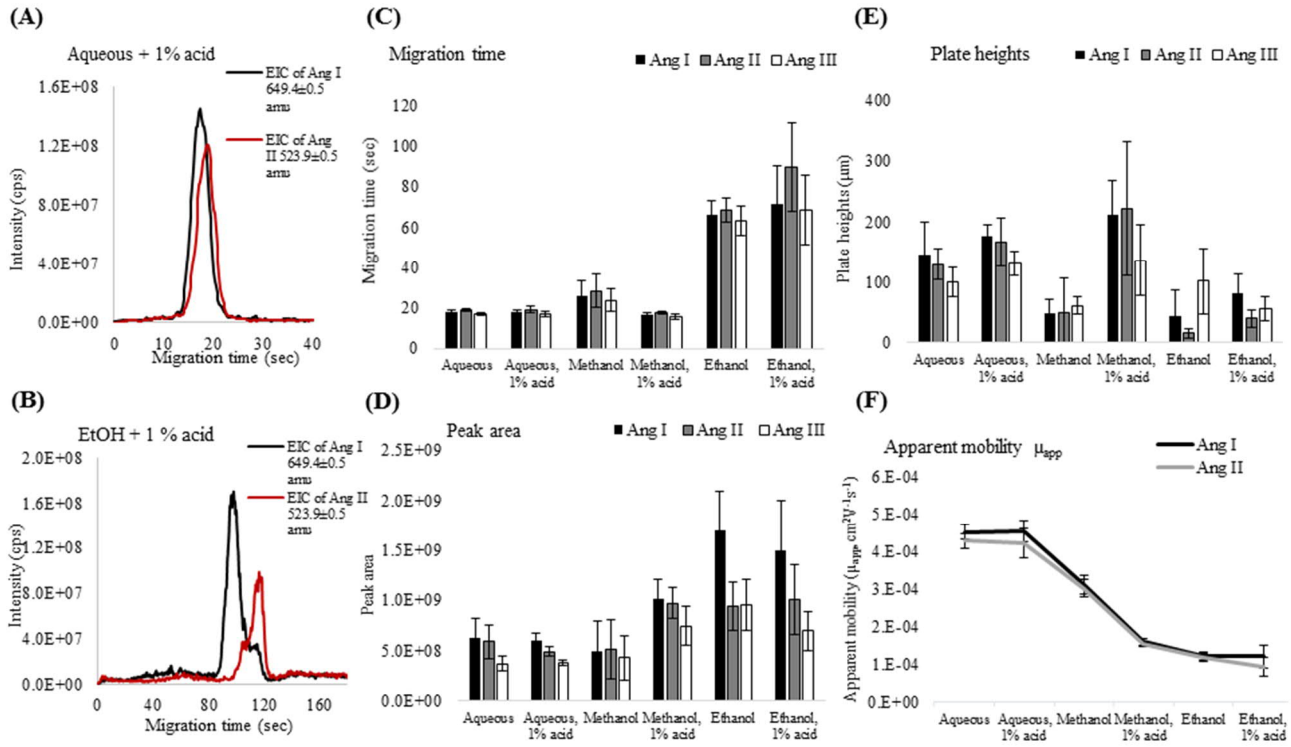


A)



B)

Figure 3. (A) The direct infusion mass spectrum of 5 µg/mL cytochrome c in 20 mM ammonium acetate containing 50% methanol was obtained by using the allyl-rich chip and electric field strength of 750 V cm^{-1} (between the BI and the MLI). (B) The mass spectra and extracted ion electropherograms of orexin A (356 µg/mL) and orexin B (294 µg/mL) injected for 20.0 s and separated in 20 mM ammonium acetate containing 40 % methanol. The analysis was carried out by using the stoichiometric chip and electric field strength of 500 V cm^{-1}. (B) In both analyses, makeup liquid was methanol–water 80:20 containing 1% acetic acid and the ESI voltage was 3.5 kV



532

533 **Figure 4.** (A-B) Extracted ion chromatograms of angiotensin peptides (each 100 μg/mL) in 20 mM
534 ammonium acetate containing 40% methanol and 1% (v/v) acetic acid (A) and in 20 mM
535 ammonium acetate in ethanol containing 1% (v/v) acetic acid (B). (C-F) Comparison of the aqueous
536 and non-aqueous MCE-ESI-MS analyses by means of migration time (C), peak area (D), plate
537 heights (E) and apparent mobility (F). The aqueous electrolyte used was 20 mM ammonium acetate
538 containing 40 % methanol with or without 1% (v/v) acetic acid. The non-aqueous electrolytes used
539 contained either 10 mM (methanol) or 20 mM (ethanol) ammonium acetate in pure organic solvent,
540 with or without 1% (v/v) acetic acid. The apparent pH ranges of the BGEs were 4.5-6.4 (with acid)
541 and 7.1-8.1 (without acid). In all runs, the electric field strength was 250 Vcm^{-1} with ESI voltage of
542 3.5 kV and the makeup liquid was methanol-water 80:20 containing 1 % (v/v) acetic acid. The error
543 bars (C-F) represent the standard deviations of n=4-5 repeated runs.