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Technical Note

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Multichannel Open Tubular Enzyme Reactor Online Coupled with Mass Spectrometry for Detecting Ricin

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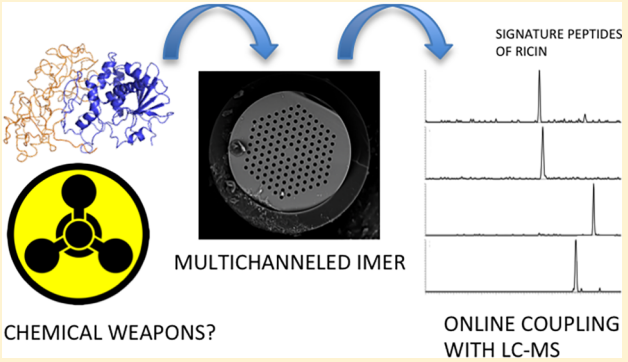
ABSTRACT: For counterterrorism purposes, a selective nano liquid chromatography–mass spectrometry (nanoLC-MS) platform was developed for detecting the highly lethal protein ricin from castor bean extract. Manual sample preparation steps were omitted by implementing a trypsin/Lys-C enzyme-immobilized multichannel reactor (MCR) consisting of 126 channels (8 μm inner diameter in all channels) that performed online digestion of proteins (5 min reaction time, instead of 4–16 h in previous in-solution methods). Reduction and alkylation steps were not required. The MCR allowed identification of ricin by signature peptides in all targeted mode injections performed, with a complete absence of carry-over in blank injections. The MCRs (interior volume $\approx$ 1 μL) have very low backpressure, allowing for trivial online coupling with commercial nanoLC-MS systems. The open tubular nature of the MCRs allowed for repeatable within/between-reactor preparation and performance.



Ricin is a type II ribosome-inactivating protein (RIP II), present in the beans of the castor plant *Ricinus communis*, consisting of two disulfide-coupled amino acid chains (A-chain and B-chain).[1] Ricin enters the cells by the B-chain binding to galactose-containing glycoproteins/lipids at the surface of the cell wall.[2−5] After binding, ricin is brought into the cell via endocytosis, through the Golgi and endoplasmic reticulum, before being sent into the cytosol.[6] In the cytosol, the toxin A-chain inactivates 1.8e3 ribosomes per minute,[7] eventually blocking protein synthesis, and cell death pathways are activated. Hence, ricin inactivates ribosomes faster than the cell can produce new ones, resulting in organ failure and death.[6]

Due to its extreme toxicity, ricin is known to be a concerningly potent agent for use in terrorism and in chemical warfare. Ricin powder was found in letters sent to Mississippi Republican Senator Roger Wicker[8] and U.S. President Barack Obama in April 2013.[9] There have been worries that terror groups, such as Al Qaida, may harness ricin to be used in bomb attacks against the United States.[10] In the United Kingdom, Mohammed Ammer Ali was in 2015 accused of trying to buy "enough ricin to poison 100 people" on the dark web.[11] To establish threat levels (e.g., call off false alarms) or aid medical evaluations in cases of exposure, it is essential to rapidly identify materials suspected of being toxic proteins.

A common approach to detect proteins, peptides and hormones, is to use enzyme-linked immunosorbent assays (ELISA). ELISA has been used to detect ricin in various matrices.[12,13] However, ELISA can not be used for multitarget screening, and there is a probability of nonspecific binding of antibodies and antigens which can lead to false positive results.[14,15] Moreover, ELISA can suffer from reproducibility issues.[16]

Liquid chromatography (LC) coupled to mass spectrometry (MS) can allow for fewer false positive/negative results compared to ELISA, and various MS based methods for ricin determination have recently been published.[17−25] Typical for these methods is the use of the "bottom-up" approach strategy, which involves protein denaturation and alkylation, followed by enzyme assisted protein digestion, that is, cleaving the protein to more MS detectable peptides. The total analysis time, including sample preparation and chromatography can be, depending on the protocol used, from 4 h and up to 16 h.[21−24,26] The sample preparation step is a major bottleneck, which is arguably the case for MS-based protein analysis in general.

The immobilized enzyme reactor (IMER) format is a tool for accelerating protein digestion,[27−37] and there has been a call for IMERs to be used for ricin online sample preparation.[18] Proteins are digested in columns containing particles or monoliths featuring covalently attached enzymes.[37−42] The IMER format has attractive kinetic features due to, for example, high enzyme-to-substrate ratios,[43] and can be coupled online with LC-MS/MS to reduce manual sample preparation steps.[27,28,44−50]

Unfortunately, packed/monolithic IMER formats are associated with poor reproducibility and carry-over and are rarely

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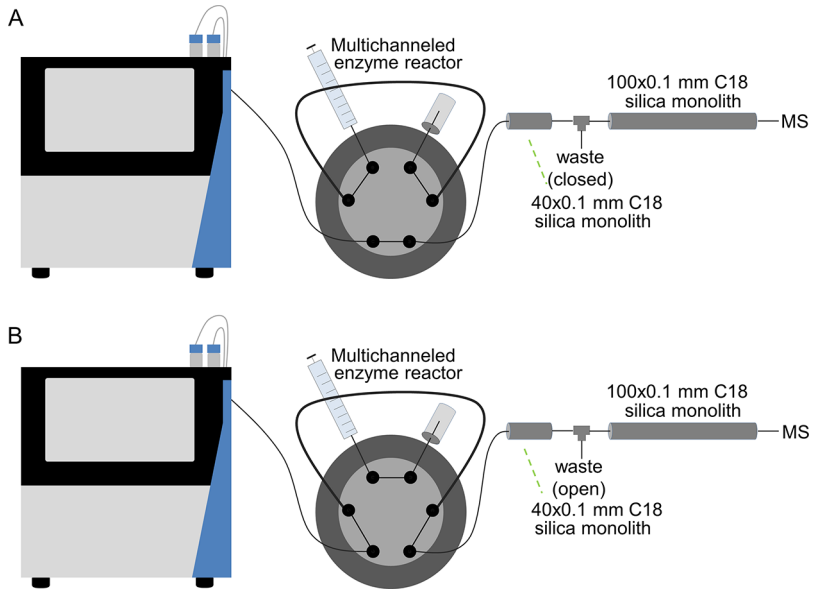

Figure 1. Valve position A illustrates the manual injection of the sample into the enzyme immobilized multireactor, while the precolumn and analytical columns are being conditioned (t-piece closed). Valve position B illustrates the elution of the digested peptides from the multireactor and into the precolumn (t-piece open). The external valve was switched to position B during the sample loading step of the Easy 1200 nLC pump. During gradient elution of the peptides from the precolumn and separation on the analytical column, the valve position was in B.

used for "real life" applications.[51] However, open tubular (OT) capillary IMERs,[27,28] where enzymes are attached to polymers coating the inner walls of a capillary, have low carry-over and allow very sensitive analysis.[27] Our group finds polymer layered OT columns to be generally simpler to (re)produce than, for example, monolithic columns, which can frequently feature cracking/incomplete polymerization, and so on (even when faithfully following published recipes[52]). We have successfully developed OT IMERs for comprehensive and targeted proteomics of limited samples/low abundant analytes.[27] A clear disadvantage of the OT IMER format is their narrow IDs (20 μm) requiring meter scale lengths in order to load sufficient sample for analysis.

One type of housing yet to be investigated for OT IMERs is multichannel capillaries, such as those used for optical fibers.[53] With dozens of parallel channels, they offer enhanced surface area/length, allowing OT reactors to be far shorter than their single channel predecessors. In addition, employing IMER units with multiple narrow channels (<10 μm) may also provide high enzyme efficiency due to increased enzyme-protein proximity. Multichannel reactors (MCRs) have previously been used in offline sample preparation prior to liquid chromatography[54] and in gas–solid chromatography.[55,56] We here describe a trypsin/Lys-C enzyme-immobilized MCR, online coupled with nanoLC-MS/MS for rapid ricin identification.

EXPERIMENTAL SECTION

Acetonitrile (ACN, LC-MS grade), formic acid (FA, LC-MS grade), trifluoroacetic acid (TFA, 98%), ammonium acetate (98%), acetone (99%), phosphate buffered saline (PBS) tablets, sodium phosphate monobasic (99%), *N*,*N*-dimethylformamide anhydrous (DMF), 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS, 98%), 1-heptanol (98%), sodium hydroxide (NaOH, 99%), 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH, inhibitor), 2-hydroxyethyl methacrylate (HEMA, 97%, containing 200–220 ppm monomethyl ether hydroquinone as inhibitor), and 2,2′ azobis(2-methylpropinonitrile) (AIBN, initiator) were all purchased from Sigma-Aldrich. Ammonia (28%) was bought from VWR (Fontenay-sous-Bois, France). Vinyl azlactone (VDM) was purchased from Polysciences Inc. (Warrington, PA, U.S.A.). Type-1 water was acquired from a Milli-Q Integral 5 water purification system (Merck Millipore, Billerica, MA, U.S.A.), while LC-MS grade water was bought from Fischer Scientific (Hampton, NH, U.S.A.). Trypsin/Lys-C mix was purchased from Promega Corporation (Madison, WI, U.S.A.). Fused silica capillary with ID ranging from 20 to 50 μm were obtained from Polymicro Technologies (Phoenix, AZ, U.S.A.). Nitrogen gas (99.99%) was obtained from AGA (Oslo, Norway).

The fused silica optical fibers with 126 channels á 8 μm ID was bought from NKT Photonics (Part number: LMA-25, Birkerød, Denmark). The fused silica optical fiber (20 cm long) was filled with 1 M NaOH by an in-house made filling system[57] and sealed with a rubber LB-2 septum from Supelco (Bellefonte, PA, U.S.A.) before being placed in an oven (Fisons Instruments, Ipswich, U.K.) at 100 °C for 2 h. The NaOH-treated fused silica optical fiber was flushed with water for 30 min, followed by ACN for 30 min and finally dried with N_2. Wall-anchoring sites for the polymer were prepared by silanizing the capillaries with γ-MAPS; the silanization solution was prepared by adding, in order, 5 mg DPPH, 313.5 mg γ-MAPS and 660.8 mg DMF. The solution was homogenized for 5 min by ultrasonic treatment before being filled into the NaOH-treated optical fiber using the in-house made filling system. The fused silica optical fiber containing the silanization solution was sealed by septa and placed in the oven at 110 °C for 6 h. Subsequently, the optical fiber was washed with ACN for 30 min and dried with N_2 for 30 min before proceeding with the polymerization step. The polymerization solution (0.1 mg AIBN, 600 mg 1-heptanol, 80 mg HEMA, and 20 mg VDM) was added to a glass vial and ultrasonicated for 5 min. The silanized optical fiber was filled with the polymerization solution and sealed with septa before being heated at 65 °C for 5 h, and then 80 °C for 5 h. After polymerization, the optical

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fiber was flushed with N_2 for 30 min, and was now ready for immobilization of enzymes. The immobilization solution, consisting of 20 μg Lys-C, 20 μg trypsin and 2.5 mg benzamidine in 1 mL of 20 mM phosphate buffer (pH 7.4), was continuously flushed through the polymerized fused silica fiber for 3 h at room temperature (23 °C). After immobilization, the MCR was filled with 50 mM ammonium acetate (pH 8) and stored at 4 °C until use.

The castor seeds were unshelled and grounded in a blender, with acetone added at a ratio of 4:1 (w/w). The pulp was allowed to stand for several hours before the acetone was filtered off under gravity in a coffee filter. For removal of castor oil remains, acetone was added again at the same amount, mixed and allowed to stand. After a second filtration, the mash was air-dried. An aliquot of the resulting powder was transferred to a 1.5 mL Eppendorf tube and added PBS solution (0.01 M phosphate buffer, pH 7.4) at a ratio of 10 μL/mg. The tube was closed, sealed with parafilm, and placed on a rocker platform for 2 h. Finally, the tube was centrifuged at 2000 g for 30 min and the extractant removed. Previous extractions from the seeds gave approximately 5 mg/mL ricin in solution (quantified by sandwich ELISA, in-house method). Aliquots of the extractant were diluted 1:40 with 10% ACN and 50 mM ammonium acetate (pH 8), and stored at −18 °C until use.

An Easy nLC 1200 pump from Thermo Scientific (Waltham, MA, U.S.A.) with a 6 × 8 autosampler was coupled to an external 6-port valve from VICI (Houston, TX, U.S.A.; Figure 1). The MS used was a Q Exactive Orbitrap from Thermo Scientific equipped with a Nanospray Flex ion source operated in positive mode. The silica-based monolithic column (0.1 × 40 mm) and analytical column (0.1 × 100 mm) were made as described by Miyamoto et al.[58] (comparable to Chromolith CapRod C18 capillary columns from Merck Millipore). The spray needle was a 30 μm ID electro-polished stainless steel LC/MS emitter from Thermo Scientific, coupled to the analytical column with a polyetheretherketone (PEEK) MicroTight adapter union (IDEX Health and Science, Oak Harbor, WA, U.S.A.). The fused silica tubing from the external valve to the precolumn had an ID of 20 μm. Mobile phase (MP) A consisted of 5% ACN in 0.1% FA and 0.02% TFA, mobile phase B contained 90% ACN in 0.1% FA and 0.02% TFA, and the loading solvent was 10% ACN in 50 mM ammonium acetate (pH 8). In all experiments, the sample was manually loaded into the MCR (15 cm long, ≈1 μL) by a glass syringe, and enzymatic digestion occurred for 5 min. Meanwhile, the precolumn and the analytical column were equilibrated with MP A (Figure 1A). A volume of 1 μL from a vial in the autosampler, containing 50 mM ammonium acetate (pH 8), was subsequently loaded over the MCR, allowing the flow stream to transfer the peptides to the precolumn (Figure 1B). After loading the generated peptides from the MCR to the precolumn, the external valve was switched back to the initial position (Figure 1A) to avoid the MCR to be subjected to the gradient program of the pump. The MCR was conditioned with 50 mM ammonium acetate (pH 8) immediately after each sample loading making it ready for a new injection.

A solvent gradient program for the LC-separation was initiated after the sample loading procedure. In the gradient program 5% MP B was kept for 2 min before gradually increasing to 45% MP B in 20 min, with a final washing step at 90% MP B for 8 min (total run time 30 min). The flow rate was 1 μL/min.

Acquisitions for the comprehensive studies were performed in data dependent tandem mass spectrometry mode ($ddMS^2$). Resolution was set to 70000 in full MS and 35 000 in $ddMS^2$. The automatic gain control (AGC) target was set to 3e6 and 2e5 for full MS and $ddMS^2$, respectively. In full MS, the maximum injection time (IT) was 100 ms and in $ddMS^2$ IT was set to 60 ms. A scan range of m/z 350−1850 was chosen for full MS, and an isolation window of 1.4 m/z was selected for $ddMS^2$. The collision energy in $ddMS^2$ mode was set to 25. A minimum AGC target of 2.00e2 with an intensity threshold of 3.3e3 was configured to the data dependent (dd) settings. Species with charges 1, 7, 8, and >8 were excluded from $ddMS^2$, while dynamic exclusion was set to 12.0 s.

Acquisition in targeted mode was done in parallel-reaction monitoring (PRM) mode. A resolution of 35000 was chosen and an AGC target of 2e4 with maximum IT of 200 ms. The isolation window was narrowed to 1.0 m/z. Charge 2 was set as default and collision energy of 25 for all monitored peptides was chosen. The inclusion list contained the tryptic fragments VGLPNIQR (448.7691^{2+}), HEIPVLPNR (537.8062^{2+}), LSTAIQESNQGAFASPIQLQR (753.7291^{3+}), and T_A24-ss-T_B1 (759.0140^{3+}) from ricin.

Scanning electron microscopy (SEM) images of 1 cm long dried enzyme immobilized MCRs were captured by an FEI Quanta 200 FEG-ESEM (FEI, Hillsboro, OR, U.S.A.). A large field detector in low vacuum mode was used during micrograph capturing.

Ricin is a highly toxic protein that inhibits the cell protein synthesis. Handling requires stringent safety measures, and all contact with the substance should be avoided, as well as aerosol or dust formation. Extraction from castor beans was performed at the Norwegian Defence Research Establishment (FFI), using dedicated equipment in a controlled access laboratory.

RESULTS AND DISCUSSION

The MCR was successfully prepared using procedures similar to single channel IMER preparation, and provides efficient protein cleavage and stability[27,28] (see Experimental Section). A 15 cm long MCR containing 126 channels (8 μm ID) had a total interior area of 4.7 cm^2 and a total internal volume of 0.95

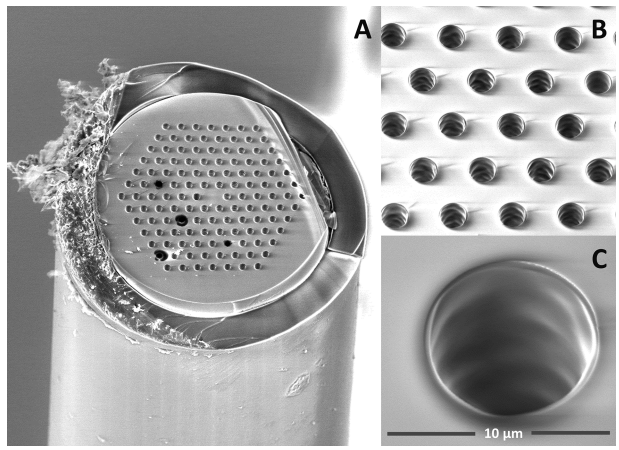

Figure 2. Scanning electron micrograph of the multireactor containing 126 channels (A). The HEMA-VDM polymer immobilized with enzymes covers the inside of the 126 channel multireactor (B). The inner diameter of each channel was 8 μm and the polymer thickness was about 0.4 μm (C).

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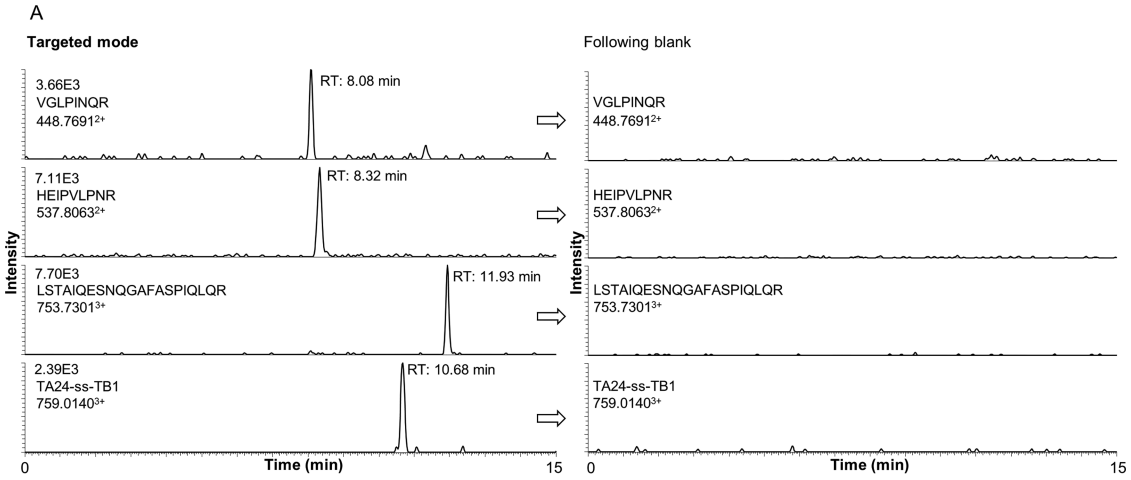

B

For signature peptide VGLPINQR	REACTOR 1 (n=4)	REACTOR 2 (n=3)	REACTOR 3 (n=4)	REACTOR 4 (n=6)	REACTOR 5 (n=4)
RT $\bar{x}$	7.91	8.09	8.11	8.17	8.12
RT st.dev	0.23	0.05	0.10	0.14	0.14
RT RSD%	2.86	0.62	1.22	1.65	1.78
$w_{0.5}$ $\bar{x}$	6.75	6.60	6.90	7.10	6.45
$w_{0.5}$ st.dev	0.30	0.30	0.57	0.45	0.57
$w_{0.5}$ RSD%	4.44	5.09	8.51	6.36	8.91

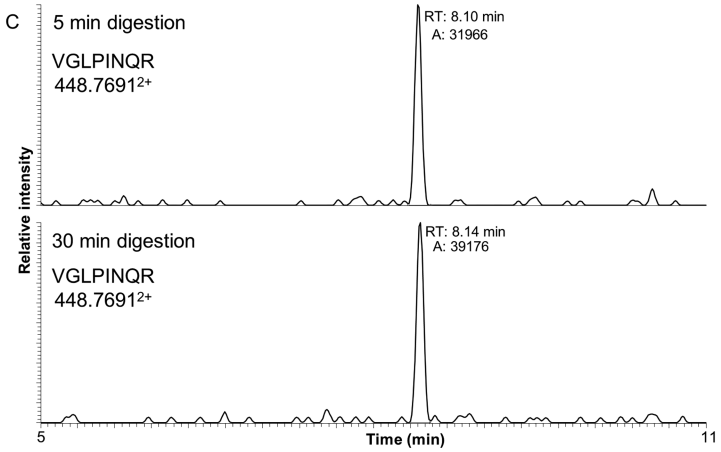

Figure 3. (A) Extracted ion chromatogram of peptides VGLPINQR (signature peptide for ricin only), HEIPVLPNR, LSTAIQESNQGAFASPIQLQR and TA24-ss-TB1 originating from ricin and agglutinin. Displayed to the right is the corresponding blank after each injection illustrating no memory effects after injection of a castor bean extract. (B) nanoLC-MS platform repeatability data including the retention time (RT) and peak width at half the peak height ($w_{0.5}$) repeatability. (C) Effect of allowing the castor bean extract to be digested for 5 and 30 min inside the multireactor.

μL. The ratio of the interior surface area to internal volume was 5000 cm^{-1}, which is a 50-fold increase compared to 252000 cm^{-1} of a 15 cm long 20 μm ID IMER that we previously published.[27,28] SEM revealed inner wall coating (HEMA/VDM polymer) of almost all 126 channels (Figure 2), without any globules or other irregularities observed. Using common nuts and ferrules for nanoLC systems, the MCR unit was straightforward to connect to a low dead volume 6-port LC injector. The back-pressure of the IMER was virtually negligible, and solution could be injected manually with a resistance similar to a conventional injection loop. This is in contrast to packed IMERs and several monoliths we have examined previously, which have required a high pressure pump for injection.

The low-pressure MCR/injection valve was straightforward to couple online with a commercial nanoLC-MS system (see Experimental Section and Figure 1). Resulting peptides could be transported from the MCR to an enrichment column and finally to the separation column before MS detection. The MCR-nanoLC-MS system was subsequently used for identifying ricin proteins in a castor bean extract: An MS/MS method was developed for targeting four peptides (VGLPINQR (position T_A7), HEIPVLPNR (position T_A6), LSTAIQESNQGAFASPIQLQR (position T_A20), and CAPPPSSQF(-SS-)ADVMDPEPIVR (position T_A24-ss-T_B1) originating from ricin. VGLPINQR (m/z 448.8^{2+}) is an unambiguous signature peptide for ricin,[59] whereas the other three peptides are present in both ricin and agglutinin (another protein only found in castor beans). In ricin's fairly complex native structure, these peptide sequences face outward, readily accessible to the enzymes. Hence, neither reduction nor alkylation of the toxic castor bean extract was needed prior to MCR digestion (5 min

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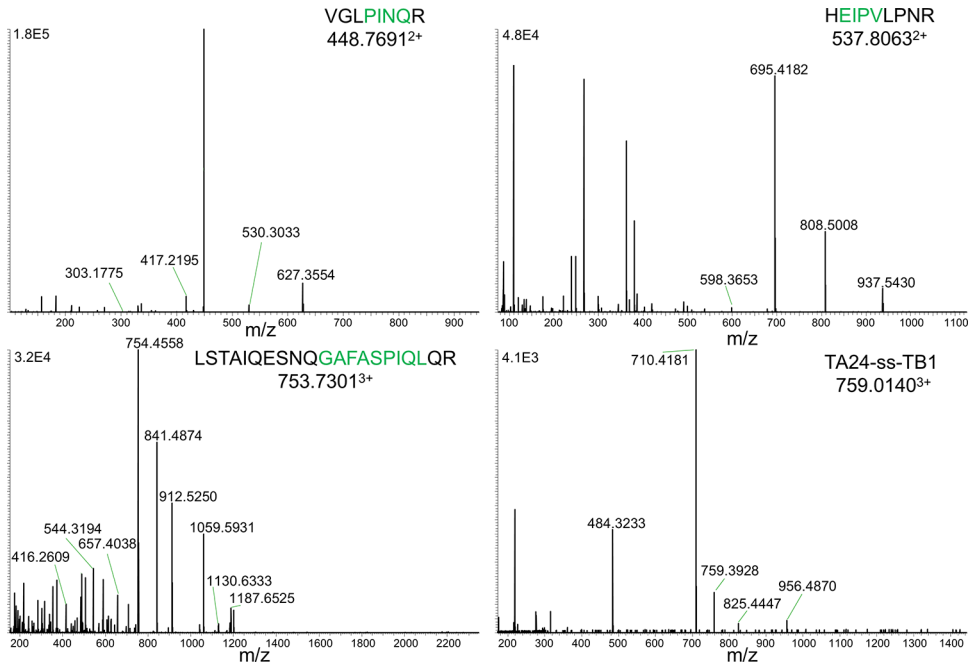

Figure 4. Fragmentation spectra of VGLPINQR (m/z 448.7691^{2+}), HEIPVLPNR (m/z 537.8063^{2+}), LSTAIQESNQGAFASPIQLQR (m/z 753.7301^{3+}), and T_A24-ss-T_B1 (m/z 759.0140^{3+}) originating from ricin. The fragmentation spectra are extracted from the peptides shown in Figure 3A. Amino acid sequence of each peptide is listed above each fragmentation spectra, and marked in green are the fragments identified (at least 4 amino acids in sequence per peptide).

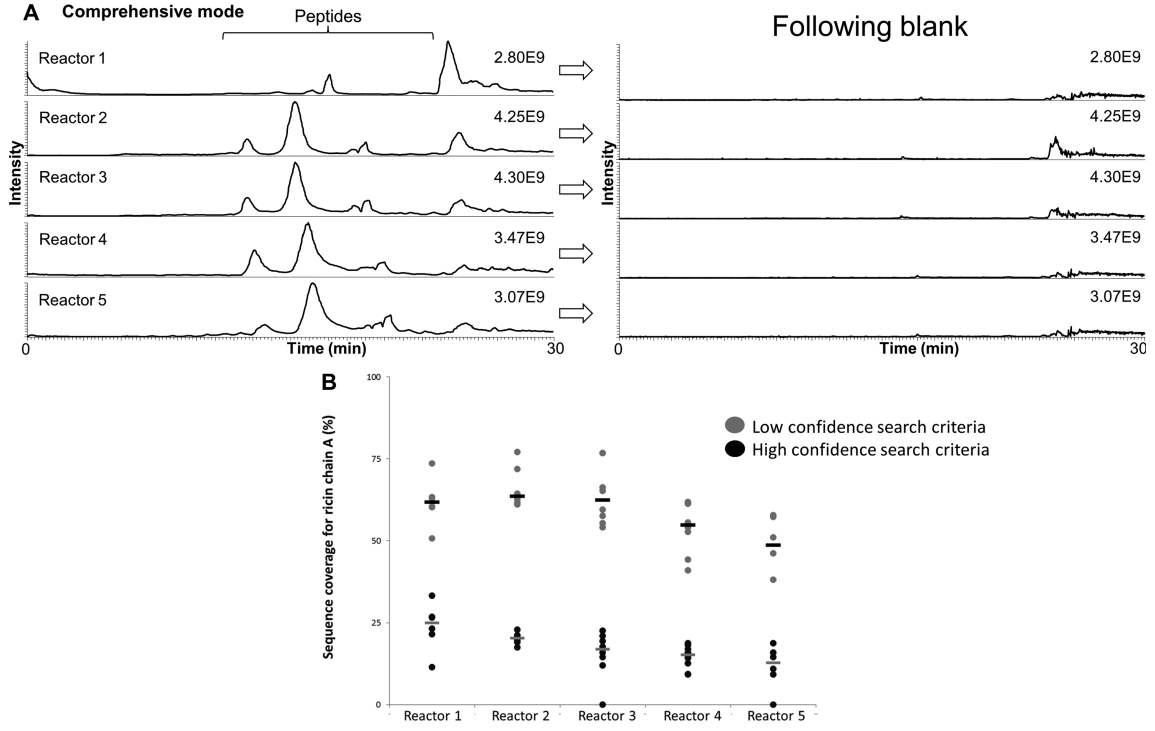

Figure 5. (A; left) Total ion chromatogram of the 30 min analysis of a diluted castor bean extract into the enzyme immobilized multireactor (≈1 μL) showing the peptide activity of reactor 1−5. The digestion time was 5 min. After digestion the peptides were trapped on a 0.1 × 40 mm C18 silica-based monolithic precolumn and separated on a 0.1 × 100 mm C18 silica-based monolith separation column. (A; right) Total ion chromatogram of the following blank after each comprehensive analysis of the castor bean extract illustrating a clean platform. (B) Amino acid sequence coverage of ricin chain A (%) for reactors 1−5.

reaction time, i.e. about the same time spent on column equilibration steps performed by the nanoLC instrument) to identify ricin. The targeted MS/MS method identified ricin, by its signature peptide, in all injections (MCRs N = 5, minimum 3 injections per reactor). The target peptides eluted between 8 and 12 min (Figure 3A), providing quality MS/MS spectra (e.g., at least four amino acids in a row were readily observed (Figure 4). The LC separation was advantageous as the

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coelution of other peptides in the sample caused signal suppression when coupling the MCR directly to the MS (the extract is complex[60]). In addition, the retention time serves as an additional identifier. When injecting blank samples (1 μL of 50 mM ammonium acetate, pH 8) after a targeted analysis, no traces of the four peptides were observed, illustrating an absence of carry-over (Figure 3). The average retention time of the VGLPINQR signature peptide for ricin ranged from 7.91 to 8.17 min for analysis with the five reactors with a relative standard deviation (RSD%) below 3%. The peak width at half the peak height ranged from 6.45 to 7.10 s for the five reactors with an RSD% below 9%, demonstrating robustness of the MCR-nanoLC-MS platform (Figure 3B). A 23% increase in the peak area of VGLPINQR was observed when the extract was allowed to be digested for 30 min compared to 5 min (Figure 3C). Longer digestion times were not pursued as this was not necessary for unambiguously detecting ricin. The RSD% for the area repeatability within each reactor ranged from 27 to 105%, which is considerable, but was acceptable in that the analysis was undertaken without internal standard correction of ng-scale levels of untreated ricin.

The diluted castor bean extract was injected into each MCR (N = 5) several times (between 6 and 10 injections per MCR), this time in data dependent (untargeted) MS/MS mode. As with the targeted mode experiments, the extract was not reduced and alkylated, and the total digestion time inside the MCR was again 5 min. Four out of five reactors produced qualitatively highly similar total ion current chromatograms (Figure 5A). When unbiasedly searching the spectra using SEQUEST towards the Mascot database, average sequence coverages of ricin were 18% and 58% (99 and 95% peptide confidence, respectively; Figure 5B). The signature peptide of ricin, VGLPINQR (m/z 448.7691^{2+}), was present in 75% of the comprehensive analysis (N = 5, n = 36) compared to 100% in the targeted analysis (N = 5, n = 21). The following blanks did not contain any peptide pattern implying that the system is thoroughly washed before the next injection. Importantly, blank injections did not contain any peptide residues (including nonsignature peptides) from ricin, nor agglutinin, also when searching the spectra using SEQUEST, confirming a low carry-over.

CONCLUSIONS

A novel IMER variant (open tubular multichannel reactor = OT-MCR) has been developed. The MCR allowed for unambiguous identification of ricin in castor bean extract. The MCR is simple to couple with LC-MS, has low carry-over, and has good between-reactor repeatability. This is encouraging, considering that many of these traits have been lacking in IMERs. The MCR-LC-MS platform will be further developed/validated for use in antiterror measures, also including a number of other protein toxins (security clearances pending) for simultaneous analysis. Due to the rather small format of the MCR (especially compared to our previous, meter-scale prototypes), it will also be incorporated into chip LC systems. In addition, the MCR will also be explored for global proteomics, which also is in need of less time and labor spent on sample preparation. Additional fine-tuning of the IMER will be performed, for example, exploring the effect of temperature and addition of porogens to further increase sample capacity.

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Notes

The authors declare the following competing financial interest(s): The enzyme immobilized multi-reactor technology is filed under patent application number 1706799.2 (Intellectual Property Office, South Wales, United Kingdom, 28.04.2017).

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