Towards automation in protein digestion: Development of a monolithic trypsin immobilized reactor for highly efficient on-line digestion and analysis

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immobilized reactor for highly efficient on-line digestion and analysis

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Abstract

Reducing experimental variability, limiting contamination and increasing automation are essential goals in the development of reliable analytical platforms for mass spectrometry (MS)-based proteomics. In this work novel trypsin-based monolithic immobilized enzyme reactors (tryp-IMERs), obtained by covalent immobilization on convective interaction media (CIMac^{TM}) analytical columns (5 mm x 5.2 mm I.D.), were developed. Notwithstanding the small dimensions, column format allowed the insertion in common high performance liquid chromatography (HPLC) systems, thus avoiding the use of expensive micro- or nano-platforms. Monolith pore diameter and surface chemistry were optimized to achieve high digestion efficiency even with high molecular weight proteins and to avoid protein/peptide adsorption, peak broadening and sample loss. A full characterization of the tryp-IMERs was undertaken to select the best protocol for preparation and type of trypsin. Optimization of the operational and storage conditions was carried out by an off-line approach. On-line studies were performed by setting a multidimensional analytical platform, which

included the trypsin-IMER, a trapping column, an analytical C4 column and a high resolution hybrid mass spectrometer (ESI-Q-TOF). In the optimized conditions rapid protein digestion (90 ± 9 s), high protein coverage (≥ 60%) and high score values were achieved for five selected sample proteins (cytochrome c, myoglobin and albumins from different sources) differing in molecular size, isoelectric point and accessibility to cleavage sites as well as for a protein mixture of 200 ng. The best performing tryp-IMERs showed high sensitivity down to the pmole level. The platform also resulted suitable for the analysis of high-molecular weight proteins such as a pool of human immunoglobulins G (hIgG) and the high molecular weight fraction of human plasma proteins, which were digested in less than two minutes to an extent similar to that achieved by overnight incubation in a classical in solution protocol. Finally, underestimated key procedural issues were also highlighted during the study. Such aspects are of general interest both for tryp-IMER users and tryp-IMER developers.

Keywords: Trypsin, immobilized enzyme reactor, monoliths, liquid chromatography-mass spectrometry, proteomics, IgGs.

1. Introduction

During the last decade, availability of new analytical tools, advances in micro- and nano-scale technology as well as introduction of high-resolution instrumentation have made possible an important step forward in the "omics" field. However, notwithstanding the great advances, most classical experimental workflows still suffer from several weaknesses, which require improved analytical strategies to be solved. In particular, sample preparation in mass spectrometry (MS)-based proteomics is still a limiting step. Indeed, irrespective of the specific research question, in shotgun proteomics (also known as bottom up proteomics) the experimental setup crucially relies on the

conversion of proteins into MS-friendly peptides, a step which usually involves a relatively slow in solution enzymatic protein cleavage by specific proteases. Even though alternative proteases or multi-protease digestion approaches have been recently suggested [1, 2], protein digestion is still almost uniquely performed using trypsin as a proteolytic enzyme (96% of the deposited data on the depository Global Proteome Machine Database - GPM DB - by November 2014, were obtained using trypsin) [1], mainly because of its high specificity, widespread availability and ease of use [3]. Indeed, trypsin cleaves at the C-terminal of Arg and Lys residues producing multi-charged peptides with a basic residue at the carboxyl terminus, thus leading to easily interpretable peptide fragmentation mass spectra [4-6]. However, long incubation times (9-18 h) and possible undesired covalent modifications induced by digestion conditions are common drawbacks of classic in solution protocols [7]. Thus, to obtain highly efficient proteolysis, in recent years, great efforts have been made to accelerate the digestion in solution [8, 9], and find alternative approaches. In particular, immobilized enzyme reactors (IMERs) have been proposed as an attractive alternative to in solution assays and have provided good promise for applicability in proteomics [10]. Indeed, other than general advantages related to immobilization, including a high turnover number, low reagent consumption, long term stability and enzyme reusability [11], trypsin-based IMERs (tryp-IMERs) feature larger enzyme to substrate ratio, low or negligible auto-digestion, even at high enzyme concentrations, and limited sample handling, which translate into lower sample contamination and higher data quality. Moreover, flow-through IMERs, i.e. chromatographic columns and capillaries, can be coupled to liquid chromatography (LC)-MS systems, thus increasing analysis throughput and allowing protein identification in complex matrix such proteomic studies on extracted proteins from human lung cancer cells [12], *Escherichia coli* [13] and cell lysates [14] with high sensitivity [15].

Among the different materials for tryp-IMER preparation, the use of monoliths has rapidly expanded thanks to the significant advantages they offer over conventional particle-based systems, such as

versatility, stability in most solvents and low back pressure [16, 17]. Additionally, mass transfer of target molecules within monolithic channels is exclusively governed by convective flow of the mobile phase, resulting in enzymatic conversion, which is not diffusion limited. Thus, reports dealing with monolith based tryp-IMERs have increased in last decade [10, 16-18].

For application in the proteomic field, sensitivity is a key issue. Hence particular interest has been recently focused on microscale reactors. The synergic combination of monoliths and miniaturization has been explored leading to μIMER-μLC-MS analytical platforms with high sensitivity [14, 15], which showed suitability for the analysis of complex samples [12, 18]. However, to achieve such sensitivity microscale or nanoscale IMERs are prepared and combined with expensive micro- or nano-analytical instruments such as capillary zone electrophoresis-electrospray ionization tandem mass spectrometry [14] or microchip technology [15] , which require well experienced operators to prevent or overcome technical difficulties [19]. Taken together, these requirements limit the extensive application of microscale systems in most laboratories.

Based on these considerations, aim of this work was the development of a monolith-based tryp-IMER that can be used with classic LC instruments and offer high efficiency and suitable sensitivity to achieve performances as close as possible to those obtained with microscale trypsin reactors. To achieve automation and short digestion time, tryp-IMERs were prepared using short bed (5 mm x 5.2 mm I.D.), high performance monolithic columns with micrometer pore diameter (CIMac Analytical columns) to allow digestion of large substrates. Low pressure drop and column format allow such columns to be inserted in traditional chromatographic systems, thus not limiting their use in conventional HPLC systems. A low pressure drop is also a preferable working condition when enzyme-based flow-through systems are used on-line.

Understanding key parameters for an optimal preparation and use is a prerequisite for an effective future applicability of this attractive technology at a larger scale. However, notwithstanding the great

number of reports on tryp-IMERs, a systematic and extensive evaluation of optimal preparation and working conditions is often missed. In this work, an extensive investigation on optimal fabrication and working parameters as well as a deep characterization of CIMac^{TM} based tryp-IMERs was undertaken. Indeed, different immobilization protocols were evaluated to achieve stable IMERs with high enzyme activity and limited nonspecific adsorption phenomena. Indeed, challenging issues of monolith-based IMERs are peak tailing, sample loss and carry over phenomena due to nonspecific adsorption of proteins and peptides. These phenomena are related to surface chemistry and, in most cases, to material hydrophobicity and different approaches have been explored to limit them [20-24]. Within this context, in this work residence time and carry over effects were evaluated to select the best performing tryp-IMER, enhance throughput and avoid sample loss. Furthermore, it has been shown that protein digestion efficiency depends on multiple factors including trypsin origin [25]. Thus, since trypsin origin cannot be considered a negligible factor, the digestion performance of IMERs containing trypsin from bovine pancreas and recombinant porcine were compared for optimal digestion yield.

Long-term stability is a further desirable feature of a new IMER. Denaturating and alkylating agents commonly employed in upstream protein digestion may seriously affect IMER stability. Therefore, sample preparation and working conditions were also investigated to ensure extensive IMER stability. Optimization was performed using five different proteins characterized by different molecular weight and isoelectric points as model substrates. Off-line and on-line studies were combined in the optimization phase. For on-line studies an integrated platform was set up coupling tryp-IMERs with a LC-electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) mass spectrometer. The optimized procedure was validated in terms of intra-day and inter-day reproducibility, IMER to IMER (batch-to-batch) reproducibility, ruggedness, stability and sensitivity. To assess the use of such devices for the efficient digestion of large substrates, the best performing tryp-IMERs were employed

for the analysis of a pool of human immunoglobulins (IgG), as challenging high-molecular weight proteins. Finally, to evaluate their applicability for a real sample profiling, the high molecular weight protein fraction of human plasma was isolated and successfully processed.

2. Materials and Methods

2.1 Materials

Aldehyde activated monolithic analytical columns (disc dimensions: 5.2 mm (I.D.) x 5 mm length, volume 0.106 mL) - CIMac^{TM} ALD – with pore size diameter of 0.60 and 1.35 µm as well as ethylenediamine modified monolithic analytical columns - CIMac^{TM} EDA – with pore size diameter of 1.35 µm were provided by BIA Separations d.o.o. (Ajdovščina, Slovenia). TCPK treated trypsin from bovine pancreas (EC 3.4.21.4), N-α-benzoyl-L-arginine-ethyl ester hydrochloride (BAEE), N-α-benzoyl-D,L-arginine (BA), dithiothreitol (DTT), iodoacetamide, cytochrome c from equine heart (CytC), myoglobin from equine skeletal muscle (Myo), human serum albumin (HSA), bovine serum albumin (BSA), albumin from chicken egg white (OVA), IgG from human serum (IgG), Glu-1-fibrinopeptide B mass standard, analytical grade potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ammonium bicarbonate, Tris (hydroxymethil)-aminomethane (TRIS), sodium cianoborohydride ($NaCNBH_3$), ethanolamine, sodium chloride (NaCl), benzamidine hydrochloride (BAHC), glutaraldehyde grade II 25% in H_2O, 2-methylpyridine borane complex (2-PB), 2-(N-morpholino)ethanesulfonic acid (MES), sodium hydroxide, sulphuric(VI) acid, sodium periodate, formic acid (FA), trifluoroacetic acid (TFA), ammonium chloride, acetic acid, HPLC grade acetonitrile (AcCN) and HPLC grade methanol were from Sigma-Aldrich (Milan, Italy). Recombinant porcine trypsin was purchased from Shanghai Yaxin Biotechnology Co. (Shanghai, China). Sodium dihydrogenum phosphate, sodium azide, calcium chloride and ethylenediamine

(EDA) were from Merck (Darmstadt, Germany). Poroszyme^{TM} immobilized trypsin cartridge was from Applied Biosystems (Foster City, CA, USA). AmiconUltra-0.5 mL, 10 K, Centrifugal Filters were obtained by Merck Millipore (Vimodrone, Italy).

2.2 IMER preparation

Trypsin was covalently immobilized on CIMac monolithic analytical columns using eight different coupling protocols as detailed below. Each protocol was applied in triplicate.

Protocol A: 1.0 mg of trypsin from bovine pancreas was dissolved in 1.0 mL of immobilization buffer, composed of 0.1 M phosphate, pH 7.2, 2-PB (1.2 mg/mL) and BAHC (0.4 mg/mL). CIMac ALD column with an average pore size diameter of 1.35±0.15 μm was washed with 1 mL of 0.1 M phosphate buffer pH 7.2, followed by 1 mL of immobilization buffer. Then the trypsin solution was continuously recirculated for 3h at flow rate of 0.05 mL/min. Aliquots of trypsin solution were collected at the beginning, during and at the end of the immobilization procedure for subsequent trypsin mass balance analysis. After treatment of the residual aldehyde groups with ethanolamine and 2-PB the column was further kept in the deactivation solution for 15 h at 25 °C. After this time, the column was washed with 2 mL of TRIS-HCl 20 mM pH 7.4 containing 1 M NaCl, followed by 2 mL of ethanol 20 % (v/v). The trypsin-IMER was stored at 4°C in TRIS-HCl 20 mM pH 7.4 containing sodium azide 0.02%. For long-term storage (more than one month) aqueous solution of acetic acid 20 mM (or 50 mM) pH 3.5 containing, $CaCl_2$ 1.0 mM was used.

Protocol B: The procedure was similar to protocol A, apart that MES 0.1 M pH 5.6 was used as immobilization buffer.

Protocol C: The procedure was similar to protocol A, apart that $NaCNBH_3$ was used as reducing agent.

Protocol D: The procedure was similar to protocol B, apart that $NaCNBH_3$ was used as reducing agent.

Protocol E: The procedure was similar to protocol B, with the difference that a 0.5 mL a solution with trypsin concentration equals to 0.38 mg/mL was used as immobilization solution.

Protocol F: A CIMac ALD columns with average pore size diameter of 0.60 μm were used. The immobilization procedure was similar to protocol A with the only exception being the starting concentration of trypsin in the immobilization buffer (1.5 mg/mL).

Protocol K: A CIMac EDA column with average pore size diameter of 1.35±0.15 μm was used. The column was treated with 1 mL of glutaraldehyde solution (2.5 M in AcCN:H_2O, 25:75) and thermostated at 30 °C for 18 h. Then the column was washed with 5 mL of bidistilled water, followed by 2 mL of MES 0.1 M pH 5.6 containing BAHC and $NaCNBH_3$. 1.0 mL of trypsin from bovine pancreas (1.0 mg/mL) in the same buffer was recirculated in the CIMac column at 25 °C for 18 h at flow rate of 0.05 mL/min.

Protocol P: The procedure was identical to procedure A, but the immobilization was performed using recombinant porcine trypsin.

2.3 Determination of trypsin density

The amount (mg) of immobilized trypsin was calculated according to Eq. 1.

$$m_{immobilized}(trypsin) = \gamma_{load}(trypsin) \times V_{load} - \sum \gamma_n(trypsin) \times V_n \quad (1)$$

where γ_{load}(trypsin) is the concentration of trypsin in the loading solution, γ_n(trypsin) are concentrations (mg/mL) of trypsin in washing and deactivation fractions after the immobilization, V_{load} is the volume of trypsin solution applied to the column and V_n is the sum of the washing and deactivation fraction volumes, in mL.

The determination of trypsin concentration was performed by a chromatography workstation, which consisted of two pumps, an autosampler and a UV detector (Smartline, Knauer, Berlin, Germany) equipped with EuroChrom 2000 software (Knauer) for data acquisition, using a Nucleosil 100-10 C18 10 µm 150x4.6 mm column (Machery Nagel, Duren, Germany). Mobile phase A [water:TFA (100:0.1) (v/v)] and B [AcCN/TFA (100:0.1) (v/v)] were used to develop a gradient: 0%, 0-2 min, 0-90% in 2-6.5 min and 90-100% in 6.5-8.5 min. Flow rate was set at 1.0 mL/min, detection wavelength at 280 nm and injection volume was 60 µL. The column was equilibrated with initial conditions for 5 min before the next injection. Each fraction was diluted 20 times in mobile phase A before injection. A calibration curve was built injecting standard solutions of trypsin in immobilization buffer with concentrations ranging from 0.05 to 1.5 mg/mL. The area of the chromatographic peak related to trypsin was plotted against the injected concentration of trypsin (mg/mL) using Microcal Origin 6.0 Software (OriginLab, Northampton, MA, USA).

2.4 Determination of active units of immobilized trypsin

For the on-line determination of column activity, expressed as number of enzymatic active units in the column the non chromogenic substrate N-α-benzoyl-DL-arginine ethyl ester (BAEE) was used. The apparent international enzyme units (*IU), defined as the immobilized enzyme that converts 1.0 µmol of BAEE per minute at pH 8.0 and 25 °C, were estimated by inserting the trypsin-IMERs in a HPLC system consisting of a Jasco PU-1580 solvent delivery system connected to a Jasco auto sampler (model AS-2055) and a UV-vis detector (model UV-2070 plus) (Jasco Europe, Cremella, Italy). Data were processed with ChromNAV software (Jasco Europe, Cremella, Italy).

2.4.1 Zonal approach

Trypsin-IMERs were equilibrated for at least 20 min with the mobile phase consisting of 20 mM Tris HCl, 19 mM $CaCl_2$ pH 8.0 (TRIS/$CaCl_2$ buffer). Operating flow rate was set at 1.2 mL/min. For the construction of the Michaelis-Menten curves, 20 µL of BAEE solutions at increasing concentrations (from 1.56 to 100 mM) were injected. Analysis solutions were prepared by dilution of a 100 mM BAEE stock solution in TRIS/$CaCl_2$ buffer/EtOH, 90/10. For the quantification of the product, the eluates were collected in 2.0 mL volumetric flasks and analysed by the RP-HPLC method reported in *section 2.5*. If not immediately analysed, samples were kept at -20°C. Samples were diluted 1:50 in the mobile phase used for the chromatographic analysis before being analysed. Each sample was analysed in duplicate or triplicate.

2.4.2 Continuous flow approach

TRIS/$CaCl_2$ buffer containing 6.0 mg/mL of BAEE (17.51 mM) was used as mobile phase. To assess the maximum immobilized enzyme activity different flow-rates, ranging from 0.1 to 3.5 mL/min, were considered. Between each tested condition the IMER was equilibrated for at least 30 dead volumes to ensure the steady-state status. After proper equilibration, for each tested flow rate, a 200-300 µL aliquot of eluate was collected and analysed by the RP-HPLC method reported in *section 2.5*. If not immediately analysed, samples were kept at -20°C. Samples were diluted 1:50 in the mobile phase before being analysed. Each sample was analysed in duplicate or triplicate.

2.5 Chromatographic separation and quantitation of *N-α-benzoyl-DL-arginine*

The separation and quantification of the formed product (BA) from the undigested substrate (BAEE) was performed on a HPLC system using a Chromolith Performance RP-18e column (100 x 4.6 mm, Merck, Germany) and H_2O/A_CCN/TFA, 83/17/0.1, as mobile phase. Flow rate was set at 1.2 mL/min and detection weavelength was set at 223 nm. Injection volume was 20 µL.

To quantitate BA a calibration curve was built using standard BA solutions. A stock solution of BA (5.0 mM, in H_2O/AcCN/TFA. 83/17/0.1) was diluted in the mobile phase to achieve test solutions with concentrations ranging from 7.8 to 500 µM. 20-µL aliquots of each test solution were analysed in duplicate and the area corresponding to BA was plotted against the injected concentration of BA (µM) using GraphPad Prism (GraphPad Prism 4.0, GraphPad Software, San Diego, USA).

2.6 Evaluation of the spontaneous hydrolysis of the BAEE in the assay conditions.

To account for spontaneous hydrolysis in the conditions used for the activity determination a 1.46 mM solution of BAEE in TRIS/$CaCl_2$ buffer was prepared. The time-dependent formation of the hydrolysis product (BA) was monitored over 6 h by collecting an aliquot each 30 min. BA was quantitate using the RP-HPLC method detailed in *section 2.5* and the percentage of BA at a determined time t (% BA_t) was calculated using the following formula:

$$\% BA_t = 100\, x\left({Area\ BA}/{Area\ BAEE + Area\ BA}\right)$$

The rate of the spontaneous hydrolysis was determined by plotting the % BA_t versus time (min), using GraphPad Prism (GraphPad Prism 4.0, GraphPad Software, San Diego, USA).

2.7 Determination of the BAEE-units of trypsin activity: in solution studies

In-solution, the determination of BAEE-units (S&T units) of both trypsin from bovine pancreas and recombinant porcine trypsin was performed using UV-Vis differential spectroscopy and following the protocol given by the manufacturer to certify the effective active units and exclude any degradation after long-term storage.

2.8 In solution tryptic digestion of protein substrates

1.0 mg/mL trypsin stock solution was prepared by dissolving trypsin in HCl 2 mM. 5 µL of DTT (100 mM in ammonium bicarbonate 20 mM, pH 8) were added to 80 µL of each protein substrate (1.25 mg/mL ammonium bicarbonate 20 mM, pH 8) and incubated at 56 °C for 30 min under agitation (400 rpm). Afterwards 10 µL of a 10 mg/mL solution of iodoacetamide in ammonium bicarbonate 20 mM, pH 8 were added. The samples were incubated for 40 min in the dark at room temperature. Finally, a 5 µL aliquot of a 0.2 mg/mL trypsin solution (prepared by diluting trypsin stock solution with ammonium bicarbonate 20 mM, pH 8.0) was added and the sample was incubated at 37 °C, under gentle agitation (400 rpm) overnight (18 h). Digestion was stopped by adding 2 µL of a 10% aqueous solution of formic acid. Final protein concentration was 1.0 mg/mL; final concentration of DTT was 5.0 mM; final concentration of iodoacetamide was 1.0 mg/mL, final trypsin concentration was 0.001 mg/mL. Protein/trypsin ratio was 100/1.

5 µL of tryptic digest were analysed by LC-ESI-MS/MS as described in *section 2.9* and obtained data were processed as described in *section 2.10*.

2.9 LC-ESI-MSMS analysis

Chromatographic separation of tryptic digests was carried out by using a Agilent 1200 Series (Walbronn, Germany) equipped with an autosampler. Analyses were performed on a C4 (Phenomenex Jupiter; 150 x 2.0 mm i.d., 5 µm, 300 Å) column. Mobile phases A (water/AcCN/FA, 99/1/0.1) and B (AcCN/water/FA, 98/2/0.1) were used to develop a gradient. The solvent gradient was set as follows: 0-5% B, 1 min; 5-40% B, 59 min; 40-70% B, 5 min; 75-80%, 10 min. The column was equilibrated with initial conditions for 10 min before the next injection.

Mass spectrometry analyses were performed on a Q-ToF spectrometer (Micromass, Manchester, UK) with Z-spray ion source. The ESI-QToF source temperature was set at 120°C, the desolvatation temperature at 300 °C, the capillary voltage at 3.3 kV, and the cone voltage at 35 V.

Peptide ions within a m/z 400-1700 survey scan mass range were analyzed for subsequent fragmentation. 2^+, 3^+ and 4^+ charged ions exceeding a threshold abundance (TIC value 10 counts/sec), were selected for MS/MS analyses. From a single survey scan 8 ions were selected for subsequent fragmentation. Scan returned to mass survey mode when the ion intensity fell below 3 counts/sec or after 8 sec. Scan time was 1 sec for the parent ion and 1 sec for the MS/MS ions.

Collision energy was selected using charge state recognition.

2.10 Data processing

The fragment ion spectra obtained from LC-ESI-MS/MS analyses were processed using Mascot Distiller 2.5.1.0 (Matrix Science, London, UK), a software program that reduces MS raw data to high-quality peak lists for database searching. LC-MS/MS data were analyzed by searching the human SWISSPROT database (2015_12; 550116 sequences; 196219159 residues http://www.uniprot.org) allowing only three missed cleavages. The precursor and fragment ion tolerance were 1.2 and 0.6 Da, respectively. Cysteine carbamidomethylation was selected as fixed modification while methionine oxidation, cysteine sulfonylation, cysteine trioxidation, Thr phosphorylation, lysine glycation and N-term acetylation were selected as variable modifications. Values of score, matches and coverage were retrieved from the obtained report and compared.

2.11 Trypsin-IMERs: off-line and on-line digestion of proteins

2.11.1 Protein denaturation and alkylation

A 85 µL aliquot of a protein solution (1.176 mg/mL) in reaction buffer was added with 5 µL of an aqueous solution of DTT 100 mM and incubated at 56 °C for 30 min under agitation (400 rpm). Afterwards a 10 µL aliquot of a 10 mg/mL iodoacetamide solution in ammonium bicarbonate 20 mM, pH 8.0 was added and the samples were incubated for 40 min in the dark at room temperature. Final

protein concentration: 1.0 mg/mL; final concentration of DTT: 5 mM; final concentration of iodoacetamide: 1.0 mg/mL.

If not otherwise mentioned protein samples were digested without any further pre-treatment.

2.11.2 Sample pretreatment: reagent removal by ultracentrifugation

A 0.5 mL aliquot of HSA (1.0 mg/mL) previously denatured and alkylated, was ultra-filtered using AmiconUltra-0.5 mL, 10 K, Centrifugal Filters (Millipore). Sample centrifugation was performed on a Centrifuge 5417R (Eppendorf Italia); the HSA sample was first concentrated to about 100 µL by centrifugation for 30 min at 15 °C and 5000 g. To completely remove reagents, the concentrated sample was subjected to three washing cycles (30 min, 15 °C, 5000 g). Before each cycle, 400 µL of ammonium bicarbonate, 20 mM, pH 8.0 were added. Finally the concentrated protein (100 µL) was diluted with ammonium bicarbonate 20 mM, pH 8.0 to 500 µL to reach a final concentration of 1.0 mg/mL.

2.11.3 Off-line studies

Trypsin-IMERs were inserted in a chromatographic system consisting of a Jasco PU-1580 solvent delivery system connected to a Jasco auto sampler (model AS-2055) and a UV-vis detector (model UV-2070 plus) (Jasco Europe, Cremella, Italy). Data were processed with ChromNAV software. Each IMER was equilibrated for 20 min at 0.8 mL/min with the digestion buffer (ammonium bicarbonate 20 mM, pH 8.0) before use. Previously denaturated proteins (CytC, OVA, BSA, HSA and Myo) were injected in the trypsin-IMER and the elution profile of the digested protein was monitored at 223 nm. The eluates were collected in eppendorf tubes (400-500 µL) and adjusted to a final volume of 500 µL. 100 µL aliquot (13 µg) of each evaluate was analyzed by LC-ESI-MSMS.

2.11.4 Optimization of operational parameters

Injection volume. Different volumes (64, 50, 40, 30 and 20 µL) of a CytC solution (1.0 mg/mL) were injected into the trypsin-IMER and digested. The mobile phase consisted of ammonium bicarbonate 20 mM, pH 8.0 and the flow-rate was set at 0.3 mL/min. Aliquots of eluates corresponding to digested and undigested protein were collected, analyzed by LC-ESI-MSMS and the digestion efficiency was compared.

Flow-rate. 64 µL of a 1.0 mg/mL solution of either HSA or CytC (64 µg) were injected in the trypsin-IMER equilibrated with the mobile phase (ammonium bicarbonate 20 mM, pH 8.0). IMER performances were compared at different operative flow-rates: 0.3, 0.5, 0.75, 1.0 and 1.5 mL/min. Each selected condition was analyzed in duplicate by the LC-ESI-MSMS.

Concentration and type of buffer. Ammonium bicarbonate buffer (pH 8.0) at increasing concentration (20, 50 and 100 mM) was used as mobile phase and three different flow rates (0.3, 0.5 and 1.0 mL/min) were evaluated. The study was carried out on two representative IMERs, i.e., K and F. The IMERs were equilibrated for 20 min, afterwards 64 µL of 1.0 mg/mL solutions of CytC, OVA, BSA and HSA were injected and elution profiles were monitored.

To evaluate the influence of the buffer type on the digestion efficiency of large protein substrates, 64 µL of HSA were digested by IMER F previously equilibrated either with ammonium bicarbonate 20 mM pH 8.0 or with TRIS-$CaCl_2$ buffer. The flow rate was set at 0.3 mL/min and the digestion efficiencies were compared.

2.11.5 Evaluation of analysis reproducibility

64 µL of BSA 1.0 mg/mL were injected in triplicate in the trypsin-IMER F equilibrated with ammonium bicarbonate 20 mM, pH 8.0. The operating flow rate was 0.3 mL/min. The eluates were analyzed in triplicate by the LC-ESI-MSMS approach.

2.11.6 On-line protein digestion

The first dimension of the on-line set-up consisted of an isocratic pump, an autosampler, the IMER, a C18 trapping column (Chromolith Guard Cartridge RP-18e, 10 x 4.6 mm, Merck) and an UV–Vis detector. The C18 trapping column was inserted to retain and concentrate the digested peptides. The protein sample (50 µL) was loaded onto the trypsin column using 20 mM ammonium bicarbonate, pH 8.0 as mobile phase and 0.5 mL/min as flow rate. The second dimension consisted of a binary gradient pump coupled to a diode-array detector (DAD) and to a ESI-Q-TOF mass spectrometer. The analytical column was a Jupiter; 150 x 2.0 mm i.d., 5 µm, 300 Å (Phenomenex). Retained compounds were flushed from the trapping column in the gradient mode (solvent A: water/AcCN/FA, 99/1/0.1 and B: AcCN/water/FA, 98/2/0.1) and peptides were analyzed using the LC-ESI-MSMS methodology described below. Both the IMER and the trap column were equilibrated with the working buffer for 20 min at 0.8 mL/min before the first analysis and for 10 min between two analyses.

Protein stock solutions were diluted with ammonium bicarbonate 20 mM, pH 8.0 to reach the final concentration of 0.26 mg/mL and 50 µL-aliquots were injected. The trapping step was 5 min for all the IMERs except for tryp-IMER K, which required longer trapping time (30 min). Peptides were transferred on the LC-ESI-MS system for the proteins identification.

On-line protein digestion by Poroszyme™ immobilized trypsin cartridge (Applied Biosystems) was performed using the same experimental setup described above.

2.11.7 Method validation

The parameters addressed to assess the performance of the tryp-IMERs included intra-day and inter-day reproducibility, IMER to IMER repeatability, ruggedness and sensitivity. All parameters were

determined using pretreated HSA at 0.26 mg/mL as test solution and the on-line set-up described in section *2.11.6*. To assess the intra-day repeatability 13 µg of pretreated HSA were digested and analyzed in triplicate. Inter-day repeatability was evaluated over three consecutive days (two replicates for each day). IMER-to-IMER (batch-to-batch) repeatability was estimated by digesting the test solution of HSA with three tryp-IMERs resulting from the same immobilization protocol (three replicates for each IMER). The ruggedness was estimated by comparing the results of the analysis carried out using the same tryp-IMER over four weeks (n =8). Finally, sensitivity was assessed by digesting and analyzing HSA solutions at decreasing concentrations (from 260 to 0.5 µg/mL, two replicates for each tested concentration).

2.11.8 On-line IgG analysis

A 5.0 mg/mL stock solution of IgG from human serum in NaCl 100 mM was prepared, denatured, alkylated and diluted to 0.26 mg/mL with ammonium bicarbonate 20 mM, pH 8.0. 50 µL aliquots were on-line digested using the on-line set-up described in section *2.11.6*. A control sample was prepared by digesting IgG sample in solution with trypsin from bovine pancreas at the ratio of 100/1 (w/w), overnight at 37°C.

2.11.9 High molecular weight plasma protein fraction on-line analysis

A 9 mL sample of peripheral blood sample from a single donor from our laboratory was withdrawn from the brachial vein, collected in a sterile tube containing ethylenediaminetetraacetic acid (EDTA) and centrifuged at 3000 rpm for 10 min to obtain plasma. The high molecular weight (HMW) protein fraction was obtained by ultrafiltration. In details, plasma (100 µL) was ultra-filtered using AmiconUltra-0.5 mL, 100 K, Centrifugal Filters (Millipore). Five centrifugation cycles (30 min each, at 5,000 g and 4 °C) were performed in a 5417R Centrifuge (Eppendorf Italia, Italy) adding 300 µL

of ammonium bicarbonate buffer (20 mM, pH 8.0) at the end of each cycle. The HMW fraction was collected and the protein content was spectrophotometrically quantified using an Eppendorf BioPhotometer (Eppendorf, Italy). An aliquot of HMW protein fraction (167 mg/mL) was diluted to 1.176 mg/mL with ammonium bicarbonate buffer (20 mM, pH 8.0) and 85 µL of this solution were reduced and alkylated as described in section 2.11.1. Finally the denaturated and alkylated sample was diluted to the final concentration of 0.26 mg/mL and on-line digested at 37°C. Due to the higher complexity of the analyzed sample, the chromatographic analysis of the digested peptides was performed using the following gradient: 0-5% B, 1 min; 5-60% B, 120 min; 60-80% B, 5 min; 80-0%, 5 min. A control sample was prepared by digesting overnight an aliquot of the same HMW plasma fraction with the classical in solution assay. Trypsin from bovine pancreas ratio (100/1 w/w substrate to enzyme) and 37°C were used as optimal conditions for in solution digestion. Analyses were performed in duplicate using the same analytical set up.

3. Result and discussion

Short methacrylate monolithic columns commercialized under the trademark Convective Interaction Media® (CIM) [26, 27] have been previously shown to be suitable supports for IMERs [28-31] including trypsin-IMERs [32-34]. CIMac™ analytical columns are short bed, high performance monolithic columns based on CIM® technology that have been primarily developed for the separation of large biomolecules. All columns employed in this work, apart that used for the preparation of tryp-IMER F, were characterized by an average pore size of 1.35±0.15 µm and small dimensions [5.2 mm in diameter (I.D.) x 5 mm in length], a volume of 0.106 mL and porosity of 62±2 %. The column used for the preparation of tryp-IMER F held the same dimensions but the average pore size was 0.600 µm (Table 1).

The coupling chemistry may affect enzyme stability and activity. Furthermore, the immobilization protocol also defines the final surface chemistry and, as a result, the so-called nonspecific interactions, i.e., all interactions not occurring with the immobilized enzyme, but involving the column surface. Such interactions may lead to peak broadening and carry over effects.

Since previous studies by Nicoli *et al.* [32] showed the optimal coupling procedure on CIM disks was achieved using ethylendiamine (EDA)-CIM disks derivatized with glutaraldehyde, as 8-carbon spacer moiety, CIMac^{TM} EDA columns were considered as good option and trypsin immobilization through EDA-glutharaldehyde (EDA-GLA) chemistry was performed (tryp-IMER K). After the coupling reaction, EDA-GLA exhibits secondary amino groups which, at the optimal pH for trypsin activity (7-9), are positively charged. In the light of limiting nonspecific ionic interactions between charged peptides and monolith surface, aldehyde (ALD) activated CIMac^{TM} columns were also used as chromatographic support for IMER preparation. ALD columns do not show any spacer linker and surface is preferentially neutral. IMERs A-D were prepared using trypsin from bovine pancreas and different immobilization conditions based on aldehyde coupling chemistry as detailed in Table 1.

Walmstey *et al.* [25] highlighted significant differences for tryptic digests generated by trypsin from bovine and porcine source. Thus trypsin origin cannot be considered a negligible factor. To assess best enzyme source, an IMER based on recombinant porcine trypsin (tryp-IMER P) was prepared and its performance was compared to that of an identical IMER based on trypsin from bovine pancreas.

Finally, when dealing with high molecular weight proteins, pore size may be an issue. On the other hand by enlarging the average pore diameter the surface available for immobilization reduces and the amount of immobilized trypsin should decrease. Thus, columns with the average diameter of the pores of 1.4 µm (commercially available) were compared with a prototype column with approximately twice lower pore size diameter (0.6 µm) and consequently twice higher Brunauer-Emmett-Teller

(BET) surface area (IMER F). The main differences and similarities between differently prepared trypsin IMERs are gathered in Table 1.

Table 1. Characteristic of CIMac columns and conditions used for the preparation of CIMac-based trypsin IMERs

Immobilization protocol	Pore size diameter (µm)	Immobilazation buffer pH	Reducing agent	Starting amount of trypsin	Type of trypsin	Coupling chemistry	Immobilized trypsin per column (mg)[a]	Density of immobilized trypsin[a,b]
A	1.35±0.15	7.2	2-PB	1.0	From bovine pancreas	ALD	0.24	2.3
B	1.35±0.15	5.6	2-PB	1.0	From bovine pancreas	ALD	0.34	3.2
C	1.35±0.15	7.2	$NaCNBH_3$	1.0	From bovine pancreas	ALD	0.39	3.7
D	1.35±0.15	5.6	$NaCNBH_3$	1.0	From bovine pancreas	ALD	0.48	4.5
E	1.35±0.15	5.6	2-PB	0.2	From bovine pancreas	ALD	0.19	1.8
F	0.60±0.05	7.2	2-PB	1.5	From bovine pancreas	ALD	0.74	7.0
K	1.35±0.15	5.6	$NaCNBH_3$	1.0	From bovine pancreas	EDA-GLA	0.18	1.7
P	1.35±0.15	7.2	2-PB	1.0	Recombinant porcine	ALD	0.24	2.3

[a] RSD < 10% (n=3), [b] amount of trypsin (mg) per mL of chromatographic material.

3.1 IMER characterization – trypsin ligand density

When preparing an analytical tool based on immobilized enzymes, two key aspects need to be taken into consideration: the yield of immobilization in terms of protein bound to the matrix and how many units remain active or are accessible to the substrate after immobilization. These two parameters both contribute to the evaluation of the optimal immobilization conditions. To define the immobilization yield a RP-LC method, able to baseline separate trypsin from the immobilization reagents, was optimized enabling the determination of trypsin in less than 10 min (t_r 2-PB = 5.8 min, t_r BAHC = 6.2 min, t_r trypsin = 7.4 min). To quantitate the amount of trypsin in the immobilization buffer a calibration curve [y(mg/mL) = 2.03x10e5 x - 0.01; R^2 = 0.999] was built. By following the decrease

of trypsin concentration in the re-circulating solution, the optimal immobilization time was set at 3 h and was kept constant in all protocols. Such extent was considered enough to have the maximum immobilization yield. In fact, after 60 min, no significant reduction of the residual amount of trypsin in the immobilization solution was detected.

A 1.0 mg/mL solution of trypsin (volume 1.0 mL, total amount = 1.0 mg) was considered suitable to avoid it being a limiting factor in the immobilization (tryp-IMERs A-D). In agreement with such hypothesis, independently from the immobilization protocol, a significant residual amount of unbound trypsin (ranging from 52% to 76% of the initial amount) was detected in the immobilization buffer after the completion of the immobilization procedure.

The yield of immobilization was influenced by the type of reducing agent and buffer pH. In particular, the immobilization efficiency increased by decreasing the buffer pH from 7.2 to 5.6 and by using a stronger reducing agent ($NaCNBH_3$ instead of 2-PB), i.e., compare immobilization yield of tryp-IMER B *vs* A, tryp-IMER C *vs* A and tryp-IMER D *vs* B, Table 1. $NaCNBH_3$ as reducing agent and MES buffer with a pH value of 5.6 (protocol D) resulted in an immobilization yield of 48% and the highest possible immobilized trypsin density (4.5 mg per mL of support). Based on these considerations, a higher immobilization yield should be achieved by reducing the initial amount of trypsin. As expected, when the starting amount of trypsin was reduced to 0.19 mg (protocol E) no unbound trypsin was detected after the completion of the immobilization procedure (immobilization yield = 100%).

Considering that the molar mass of trypsin from bovine pancreas is 23.8 kDa and the BET surface area of monoliths with average pore size diameter of 1.35 µm is 3.2 ± 0.1 m^2 per mL of support [35], each trypsin molecule in tryp-IMER D (the one with the highest immobilized trypsin density) occupies approximately 30 nm^2 of surface. Based on Saha *et al.* [36] the molecular dimension of native trypsin from porcine pancreas is 4.8 nm x 3.7 nm x 3.2 nm. Assuming that the spatial

dimensions of bovine-derived trypsin are similar and that enzyme molecules are surrounded by a hydration layer of approximately 0.2 nm, one trypsin molecule would occupy between 13 and 20 nm^2 surface, leading to approximately 50% surface coverage. Therefore, even at the highest immobilized ligand density the trypsin molecules are not overcrowded on the surface and tryptic activity should not be negatively affected by intermolecular steric hindrance.

As a further consideration, by enlarging surface area the amount of immobilized trypsin should increase. According to expectation, approximately twice larger amount of trypsin could be bound per mL of monolith when a prototype column with pore size diameter of 0.60 µm and, consequently, about twice higher BET surface area was used. Indeed, resulting enzyme density was 7.0 mg per mL of monolith support (trypsin-IMER F), a value which is about three times higher than in that achieved by using CIMac ALD column with larger pore diameter (protocol A).

The effect of the immobilization chemistry on the immobilization yield was evaluated comparing ALD and EDA-GLA supports. Even if in both cases covalent attachment of trypsin occurs via a reductive amination, the immobilization yield on the EDA-GLA column was only 18%, about three times lower than that obtained with ALD column D. This yield obtained for the EDA-GLA column is in agreement with that obtained by Nicoli et al. (22%) using a similar column (CIM column) and the same immobilization chemistry [32].

As final remark, identical yield in terms of mg of immobilized enzyme was achieved when recombinant porcine trypsin and trypsin from bovine pancreas were immobilized using the same protocol (tryp-IMER P *vs* A).

3.2 *Determination of immobilized active units*

Enzyme immobilization is the confinement of the enzyme to a phase (chromatographic support) different from that surrounding the enzyme in solution. Both reaction conditions and confinement

may seriously affect enzyme activity. Thus, the amount of immobilized enzyme reflects the binding capacity of the support in the chosen immobilization conditions and does not necessarily correspond to that of amount of active enzyme. Evaluation of the enzyme activity after immobilization is therefore a key step in IMER characterization.

The Enzyme Commission of the International Union of Biochemistry recommends trypsin activity to be expressed in terms of μmoles of substrate transformed per minute at 25 °C and optimum pH. One international unit (IU) of trypsin catalyzes the hydrolysis of one μmole of N-α-benzoyl-L-arginine-ethyl ester hydrochloride (BAEE) per minute at 25 °C, pH 8.0 [37]. Since BAEE and the hydrolysis product N-α-benzoyl-D,L-arginine (BA) share similar spectroscopic properties and they co-elute from the tryp-IMERs, to estimate product formation a RP-HPLC method was developed to separate the residual amount of substrate from the formed product. In the optimized conditions the retention times (t_r) of substrate and product were 3.85 min (k '= 1.75) and 1.92 min (k' = 0.37), respectively. The method showed good selectivity (α = 4.76), good resolution (Rs = 4.23) and short analysis time (6 min) (Figure S1). The μmoles of BA were calculated by building a calibration curve (y = 94140x – 38560) which showed good linearity (R^2 = 0.9983) in the selected BAEE concentration range (7.8-500 μM).

Because tryptic digestion of BAEE is performed at slightly alkaline pH (8.0), the ester bond in BAEE might undergo spontaneous hydrolysis, thus affecting the accuracy of trypsin activity measurement. For a correct determination of trypsin activity the amount of BA formed by spontaneous hydrolysis should be subtracted, if significant. Thus, the spontaneous hydrolysis of the substrate was evaluated and resulted to follow a linear trend (*y(% BA formed)* = 0.0039 *x* + 0.4936; R^2 = 0.995). The rate of hydrolysis is given by the slope of the linear regression and it was equal to 0.0039% per min (0.23% per hour). It is worth to note that the commercial BAEE also contains a small percentage of BA (about

0.47%) as impurity. This amount of BA was subtracted when calculating the trypsin-IMER activity. Worth to mention, the spontaneous hydrolysis of BAEE in the acid conditions used for the RP-HPLC analysis resulted negligible.

To have a correct estimation of the yield of immobilization in terms of active units retained in each IMER, the BAEE-units (S&T units) of the trypsin batches used for the preparation of the IMERs were determined following the manufacturer's protocol. The obtained values (13973 BAEE-units/mg of trypsin from bovine pancreas and 7237 BAEE-units/mg of recombinant porcine trypsin, respectively) were in agreement with the value reported by the manufacturers for the same batch of protein, indicating that no degradation occurred in the commercial samples during storage before the immobilization.

Based on previously reported studies on tryp-IMERs [32, 33, 38-40] retained activity was initially determined by a classical zonal approach: 20 µL-aliquots of increasing concentrations of BAEE (1.56-100 mM) were injected in each trypsin-IMER. The eluates corresponding to each substrate concentration were collected in 2 mL-volumetric flasks and the amount of product formed was determined by RP-HPLC analysis. The Michelis-Menten graphs were obtained and the apparent Vmax (Vmax*) values were calculated. To estimate the effective contact time the peak width of eluting product was considered. Results are shown in table S1 and the observed trend quite nicely matched the amount of immobilized protein on each IMER.

For high-rate conversion enzymes, in a zonal approach the injection volume may influence the observed enzyme activity because it defines the portion of the IMER that is in contact with the substrate at a given moment, thus the portion of the IMER that can exert the hydrolyzing activity.

In agreement with this consideration, preliminary results with increasing injection volumes (from 20 to 64 µL) of a 20 mM BAEE solution showed a volume-dependent IMER activity. Thus, to verify this issue, the Michelis-Menten plot was built using injection volumes of 20 µL and 64 µL,

corresponding to 0.31 and 1 bed volumes (BV), and resulting plots were compared (Figure S2). Provided that the plateau is reached, the Vmax value calculated from the Michaelis-Menten plot should be independent from the range of substrate concentration, thus the significant increase of the Vmax value when a 64 μL-injection volume was used (2.44 μmol/min *versus* 3.00 μmol/min for an injection volume equals to 20 and 64 μL, respectively, using tryp-IMER K, Figure S2) indicated a clear influence of the injection volume on maximum trypsin activity. Thus, when using a zonal approach, an underestimation of the apparent active units of trypsin in each IMER seems to occur.

Based on these results, the tryp-IMER activity was investigated using a continuous flow approach, in which the substrate is solubilized in the mobile phase and the IMER is continuously supplemented with a constant amount of substrate. Thus, the whole IMER (and not just a part of it) should work in steady-state conditions. In such approach, provided that a saturating concentration of substrate is dissolved in the mobile phase and that the flow-rate is set to ensure that the substrate is supplemented to the IMER enough quickly to guarantee that the concentration of substrate throughout the IMER remains constant (i.e., the portion of substrate converted to product is negligible), the IMER works in steady-state conditions at its maximum catalytic rate, thus the amount of active units can be estimated. Thus, for a given concentration of substrate in the mobile phase, increasing the flow rate the velocity of substrate conversion should increase up to a plateau.

17.5 mM BAEE was found to be a saturating concentration for all IMERs. For each IMER, the μmoles of BA/min were plotted as a function of the flow-rate/dead volume ratio (BV = 0.064 mL) (Figure 1). This ratio indicates the number of dead volumes of mobile phase passing through the IMER per unit of time.

A clear dependence of the trypsin activity from the velocity at which the substrate is supplemented, was observed (Figure 1). At the lowest flow-rates the amount of substrate supplemented to each

IMER per minute represents a rate-limiting factor. Indeed increasing the flow-rate the amount of hydrolyzed substrate per minute increases up to a plateau. This is in agreement with the high turnover number (k_{cat}, number of chemical conversions of substrate molecules per second at a single catalytic site) for BAEE hydrolysis by trypsin (16.5 ± 1.8 s^{-1}, bovine trypsin, at pH 8.0) [41].

Higher the amount of active units in an IMER, higher the flow-rate required to reach the plateau, i.e. the maximum conversion rate. Accordingly, for the IMERs with the highest activity (namely F and D) the maximum catalysis rate is not reached even at the highest flow-rate, i.e., 3.5 mL/min, corresponding to 54.7 BV/min. This trend confirms that the IMER activity is significantly underestimated when the zonal approach is used. Indeed, injections of 20 and 64 µL correspond to BVs of 0.31 and 1.0, respectively.

The immobilized apparent active units of trypsin in each IMER were calculated from the plateau of the corresponding curve. Results are reported in Table 2.

Table 2. Apparent trypsin units retained in each IMER expressed as apparent international units (IU*) and as apparent S&T units. S&T units were calculated considering that 1IU = 270 S&T [42].

	IU* of trypsin	**S&T units* of trypsin**	**IU* per mg of immobilized trypsin**	**% active units over theoretical units[a]**
A	7.42	2004	30.9	59.6
B	11.51	3109	33.9	65.3
C	11.96	3228	30.7	59.1
D	16.76	4525	34.9	67.3
E	4.51	1219	23.7	45.8
F	27.67	7471	37.4	72.1
K	7.40	1998	41.1	79.3
P	7.19	1941	30.0	57.8

[a]The value was calculated dividing the apparent S&T units of trypsin in each tryp-IMER for the S&T units calculated using the S&T units/mg determined in solution.

The conversion factor determined by Bergmeyer [42], i.e., 270 BAEE units correspond to 1 international unit (U) at 25°C, was used to convert IU into most commonly used BAEE-units.

Immobilization procedures were satisfactory reproducible. Indeed, for all procedures, RSD% on the final active units retained on each tryp-IMER was less than 10% (n = 3).

Based on the active units per immobilized mg of trypsin for IMERs A-D (Table 2), some considerations can be drawn on the effects of the immobilization pH and reducing agents on the enzyme activity. Indeed no difference in enzyme activity was detected when either 2-PB or $NaCNBH_3$ was used (compare IU*/mg of trypsin for tryp-IMER A *vs* C, and tryp-IMER B *vs* D), while immobilization at pH 5.6 gave a higher residual activity per mg of immobilized trypsin (compare IU*/mg of trypsin of tryp-IMER B *vs* A, and tryp-IMER D vs C), meaning that at acidic pH the enzyme is less denaturating condition, in agreement with data from literature. Protocol D and B, which were prepared using $NaCNBH_3$ and 2-PB, respectively, gave the best results in terms of yield in active units (67.3% and 65.3%). Additionally, according to our prediction from calculation of percentage of surface area occupied by trypsin molecule no negative effect of intermolecular steric hindrance due to higher trypsin density was observed (compare tryp-IMER C with A or tryp-IMER D with B). To note, while pH affected both the yield of protein immobilization and % of residual active units, the selection of the reducing agent was a key factor only for the immobilization yield. Interestingly, notwithstanding the quite low amount of immobilized trypsin in tryp-IMER K, the yield in terms of retained active units per mg of immobilized trypsin was the highest (79.3%), confirming the beneficial effect of the introduction of a spacer arm, as previously reported for both trypsin [32] and other immobilized enzymes [28].

3.3 *Protein digestion by tryp-IMER*

To estimate the digestion efficiency three parameters were considered: the protein score, the number of matches and the sequence coverage (SQ%). Five standard proteins, namely cytochrome C (CytC, MW ~ 11.7 kDa, pI = 10.0-10.5), myoglobin (Myo, MW ~16.9 kDa, pI = 7.3 - major component, and 6.8 - minor component), albumin from chicken egg white (OVA, MW ~42.7 kDa, pI = 4.54), bovine serum albumin (BSA, MW ~66,0 kDa, pI = 5.3) and human serum albumin (HSA, 66,4 kDa, pI = 4.7) were selected as substrates in order to cover a wide range of protein size and isoelectic point, and different accessibility to cleavage sites. Most of these proteins have been employed as substrate to test the efficacy of previously developed trypsin-based IMERs [43-45], thus allowing comparison.

3.3.1 Optimization of operational parameters

Notwithstanding trypsin shows good catalytic activity with several buffer, TRIS/$CaCl_2$ buffer with a pH ranging from 7 to 9 is the buffer most commonly suggested in the technical bulletins of various companies for in solution studies. However, this buffer is not directly compatible with MS analyses. On the other hand ammonium bicarbonate (20 mM, pH 8.0) is fully compatible with direct MS coupling and it has previously been used for on-line trypsin digestion with excellent results [32, 46]. Thus, protein digestion of HSA as representative substrate was performed in TRIS/$CaCl_2$ buffer and in ammonium bicarbonate (20 mM, pH 8.0). Optimal working parameters for tryp-IMER activity were set up using an off-line approach. Quite interestingly (figure S3A) the digestion efficiency resulted higher when ammonium bicarbonate (20 mM, pH 8.0) was used as mobile phase instead of TRIS/$CaCl_2$ buffer (score: +28.2%; matched queries: +41.1%; coverage: +18.4%). This trend is opposite to that observed with small substrates such as BAEE (data not shown). Considering the final application to protein digestion, ammonium bicarbonate (20 mM pH 8.0) was selected as mobile

phase for further studies. The optimal injection volume was also investigated. Digestion efficiency increased by increasing the injection volume up to a plateau which was reached at volumes equal or higher than 50 µL (Figure S3B). On the basis of these results the injection volume selected for the on-line studies was 50 µL.

Finally, since the flow-rate value defines the contact time, i.e. the time during which the substrate is in contact with the immobilized enzyme, the flow rate should influence the digestion efficiency. To address this issue, the digestion efficiency at flow-rates ranging from 0.3 to 1.5 mL/min (corresponding to theoretical contact times from 12.6 s to 2.6 s) were investigated using CytC and HSA as representative low and high molecular weight substrates. Notably, the theoretical contact time is extremely short, particularly when compared with the incubation time used for in solution digestion (from 4 to 18 h). As expected, the digestion efficacy (Figure S3C,D) worsened by increasing the flow rate and this effect was more marked when HSA, a high molecular weight protein, was used as substrate. A flow rate of 0.5 mL/min was selected for on-line studies, to obtain good efficiency and short digestion time. In these conditions theoretical contact time (or residence time) is 7.6 s.

It should, however, be considered that, working with proteins resistant to digestion, a decrease in the flow-rate may be suggested in order to allow a longer contact time and facilitate digestion.

3.3.2 Investigation of tryp-IMER performances by off-line approach

Tryp-IMERs were challenged with five standard proteins with different molecular weight and shape and protein score, the number of matches and the SQ% were compared to assess the best performing IMER. As shown in Figure 2A, all tryp-IMERs showed very good performances independently from the amount of immobilized trypsin or pore size. The similar good efficiency is likely related to the fact that all prepared IMERs have a suitable high amount of active enzyme and are able to generate a sufficient amount of tryptic digest for a proper database search and protein identification. Indeed, as

reported by Hustoft *et al.* [6], a peptide is correctly identified if it exceeds a threshold value, independently from its absolute intensity. Consequently, above a given concentration value, tryptic peptides are correctly identified in the database, thus search score, number of matches and coverage are no longer key discriminating parameters. This result also highlights that the chosen immobilization protocols are suitable for highly efficient tryp-IMERs.

On the other hand IMER performance was influenced by the type of immobilized trypsin. Indeed, tryp-IMER P, which contains recombinant porcine trypsin, showed significant lower digestion efficiency when compared to the corresponding bovine trypsin-based IMER A (score: -61%; matches: -70%; SQ%: -48%) (Figure 2A). It must be noticed that this lower efficiency cannot be ascribed either to support porosity, either to surface chemistry or to amount of active units of immobilized trypsin, which do not significantly differ between IMER A and P (7.19 vs 7.42 *IU). A lower intrinsic catalytic activity of the porcine recombinant enzyme can also be excluded since in solution studies showed good performances for the recombinant porcine trypsin, similar or even better than those showed by trypsin from bovine pancreas, in agreement with supplier's specification. It can be hypothesized that the lower efficiency of the tryp-IMER P may be attributed to a reduced accessibility to the trypsin active site or P sites to large substrates, as consequence of the immobilization process. Indeed, notwithstanding the high sequence homology, porcine trypsin lacks four lysine residues [45]. Hence, since binding chemistry involves the side chains of the lysine residues, a different orientation of the immobilized porcine trypsin on the IMER surface may occur.

The stationary phase nature may greatly affect the activity and the stability of enzyme bioreactors. The support material in fact could either stabilize the immobilized enzyme or cause its denaturation [18]. Furthermore, strong interactions between the tryptic digest and the chromatographic support may lead to significant protein or peptide adsorption (with consequent sample loss) and/or determine peak tailing (with consequent longer analysis time) [13, 22]. In agreement with the fact that they are

endowed with different surface chemistry, EDA-based IMER and ALD-based IMERs showed a different chromatographic behavior. In particular, working with EDA-based IMER peak width was about 10 times larger than that observed for protein digests generated by ALD-based IMERs (Figure S4). Furthermore, since these interactions influence the residence time, i.e., the time the substrate flows through the column, the effective contact time for the EDA-based IMER was much higher. The significant peak broadening observed for the EDA-based tryp-IMER K may be ascribed to electrostatic interactions between substrates/peptides digest and the residual amino groups on the surface of the chromatographic support, that are partially protonated at the working pH (pH = 8.0). To confirm this hypothesis, since the operating pH cannot be varied to maintain optimal tryptic digestion, variations of the retention time and peak width at increasing ionic strength values were evaluated. Three different concentrations (20, 50 and 100 mM) of ammonium bicarbonate buffer pH 8.0 were used as mobile phase and the elution profiles of digested protein were monitored. Retention time and peak width gradually reduced by increasing the ionic strength of the mobile phase (Table S2). Indeed, at 1.0 mL/min, when ammonium bicarbonate 20 mM is used as mobile phase the peak width is about 11-12 min for all substrates (independently from their molecular weight or complexity), while peak width is 7.4-8.1 min and 4.1-4.5 min when ammonium bicarbonate 50 mM and 100 mM is used, respectively. Because of the significant peak broadening, tryp-IMER K could not be compared with other IMERs in the off-line studies but was characterized only by the on-line approach.

3.3.3 On-line protein digestion

In order to obtain a complete automation and a more straightforward workflow of the analytical process, the on-line platform was optimized. The trypsin-IMER was connected to a C18 trapping column and to the LC-MS/MS system through a switching valve. Proteins were on-line digested,

tryptic digests were concentrated on a C18 trapping column using optimized operational conditions. Retained peptides (and eventually the residual protein) were flushed from the trapping column in the gradient mode to the analytical C4 column and peptides were analyzed by a MS/MS method. On the basis of the elution profiles, the trapping time was set at 5 min for all ALD-based IMERs while for the tryp-IMER K the trapping time was set at 30 min. The effective residence time of proteins within ALD-based IMERs was very short, about 77 ± 9 s (total time from injection to baseline peak 90 ± 9 s), a value which is in the same time scale of most of the previously developed monolith-based capillary reactors (from 20 to 240 s) [13, 18]. Impressive faster digestion (within 4 s) of CytC was accomplished by Bischoff *et al.* [47] using a 10 mm × 1 mm i.d. cartridge IMER containing an acetylated form of trypsin. However, as a general consideration, protein digestions within dozens of seconds, as in the case of the CIMac-based tryp-IMER, can be considered more than satisfactory in terms of high-throughput analysis. Indeed even if, analyses within few seconds would enable higher throughput in terms of number of digested samples per hour, in a typical shotgun approach involving LC-MS analysis, the chromatographic separation becomes the key limiting step, being usually achieved in tens of minutes (use of slow gradients) to increase separation and number of identified proteins. Indeed, as an example, in the work by Bischoff *et al.* [47] a (about) 50 min chromatographic run was performed for the analysis of the tryptic digest resulting from the 4 s on-line digestion.

The carry over effect may be a serious issue in on-line studies performed with trypsin reactors. Hence, a carry-over effect between consecutive analyses was evaluated and excluded by analyzing a blank sample immediately after protein analysis, using HSA as model protein. Indeed, based on Mascot search no protein identification was obtained, confirming that no carry-over effect takes place. On-line digestion efficiencies of all trypsin-IMERs were initially compared using in solution digestion (Ctrl) and the commercial available trypsin-column Poroszyme™ from Applied Biosystem as references for the digestion of HSA (Figure 2B). Data obtained with the on-line set up were in

agreement with those obtained with the off-line approach. Indeed, accordingly with off-line studies, tryp-IMER P showed a significantly lower digestion efficiency. For all IMERs containing trypsin from bovine pancreas, IMER efficiency closely matched the immobilized active units, i.e., efficiency increasing with increasing immobilized active units. More interestingly, apart IMER P, for all IMERs including tryp-IMER E, which contains the lowest amount of immobilized trypsin, the digestion efficiency was comparable or even higher than that obtained by overnight in solution digestion (Figure 2B), underlining the applicability of such IMERs as alternative choice to in solution assays. As mentioned for the off-line studies, such good efficiency is likely related to the fact that they are all able to generate a sufficient amount of tryptic digest for a proper database search and protein identification. Indeed the most accurate approach for the determination of trypsin efficiency in protein digestion is a challenging issue. The efficiency of protein digestion is often related to the sequence coverage value; nevertheless, as also reported by Hustoft [9], this could be a misleading parameter since the sequence coverage value also depends on the type of mass spectrometer as well as on the parameters used for database search. Furthermore, since the sequence coverage value is related to the number of missed cleavages, an incomplete digestion could result in the formation of longer peptides which, in turn, will give a higher coverage value, as for the CytC digestion. Finally, a high sequence coverage is not achievable with large and hardly hydrolysable proteins. Thus, the evaluation of the residual amount of undigested protein was suggested be a better approach to compare trypsin activity in different IMERs. Based on these considerations, the amount of the residual undigested protein was evaluated by recording TIC chromatograms. Notably, residual protein was scarcely detectable in all IMERs including tryp-IMER E which contains the lower amount of active enzyme immobilized.

Among ALD-based tryp-IMERs characterized by 1.35 µm pore diameter the best performance was obtained with tryp-IMER D: score 2450±18 (+27% *vs* Ctrl and +11% *vs* Poroszyme^{TM}), matched queries 88±0 (+26.7% *vs* Ctrl and +17.0% vs Poroszyme^{TM}) and sequence coverage 69±1 (+1% vs

Ctrl and +1% vs Poroszyme^{TM}). Tryp-IMER F, which is characterized by a lower pore diameter (0.6 µm), showed a digestion performance similar or better than tryp-IMER D, indicating that a pore size of 0.6 µm is also suitable for the digestion of proteins with molecular weight up to 66 kDa.

To note, the high score, matched queries and %SQ values recorded for tryp-IMER K cannot be ascribed to a higher digestion efficiency but to the considerably higher contact time (peak width 19 min rather than 2 min) which was caused by strong nonspecific interactions (mainly ionic) with the column surface. As confirmation, when buffer concentration was increased from 20 mM to 100 mM a significant peak sharpening (peak width from 19 to 11 min) and reduction of digestion efficiency were observed. This confirmed that the longer contact time was responsible for the high score and sequence coverage values.

As best performing IMERs, D and F were further compared for the digestion of all selected protein substrates. Tryp-IMER K was also evaluated to exclude that what observed was related only to a specific substrate. As shown in Figure 3, all tryp-IMERs gave higher score values and similar or higher %SQ values, when compared to the in solution protein digestion (18h), for all selected substrate with the exception of CytC. Noteworthy, considering the short residence time (less than 2 min), the obtained results are particularly satisfactory. Furthermore, despite the short residence time, the results in terms of efficiency are fully comparable to those obtained with microscale IMERs, which are characterized by similar contact time [20, 21, 44].

Considering the significant aspecific interactions observed for tryp-IMER K, that lead to a significantly lower analysis throughput and may cause sample loss, tryp-IMER K was not used for further studies.

3.3.4 Digestion of proteins mixture

Since protein digestion efficiency can be less effective when standard proteins are digested in mixture instead of alone [9], a mixture of CytC, Myo, OVA, BSA and HSA (final protein concentration for each substrate of 52 µg/mL) was digested by Tryp-IMER F as the best performing IMER. All five proteins were identified with excellent Mascot scores (Table 3).

Table 3. Protein digestion efficacy of tryp-IMER F (in terms of SQ%, coverage and matches) as a function of injected amount (µg) of protein.

S**:** Score; M**:** Matches. The analysis were performed in duplicate and the RSD% were ≤ 26% for the score, ≤28% for the matches and ≤15% for the SQ% values.

Injected amount of protein	CytC			Myo			OVA			BSA			HSA		
	S	M	SQ %	S	M	SQ %	S	M	SQ %	S	M	SQ %	S	M	SQ %
2.6 µg	110.5	2.5	23.5	475.5	7.0	51.5	641.0	12.0	38.0	1302.0	26.5	42.0	1235.5	30.0	43.5
0.200 µg	131.0	3.50	37.5	\	\	\	398.5	7.0	32.5	659.5	12.0	24.0	583.0	13.0	31.5

Aiming at comparing the digestion efficiency of the new tryp-IMERs with microscale tryp-IMERs previously developed by others, the same analysis was performed on samples containing 200 ng of each protein (3-17 pmol; depending on the protein MW), which is a threshold concentration value used in previous studies to assess performances of microscale systems [20, 21, 44, 48]. Notably, with the exception of Myo, all proteins were well identified in terms of both score and SQ%. More importantly, results in terms of SQ% were comparable to those obtained with microscale reactors coupled to micro- or nano-separative systems such as the microchip-based IMERs proposed by Liang *et al.* [21] and the capillary tryp-IMER developed by Ma *et al.* [48]. In details, in the work by Liang *et al.* a mixture of BSA, Myo and Cytc, 200 ng for each protein, was analyzed by a nanoRPLC–ESI-

MS/MS achieving %SQ values of 51.29%, 60.78%, and 59.61%, respectively. Similarly, Ma *et al.* achieved protein %SQ ranging from 17% to 52% when a mixture of four proteins, i.e., CytC, Myo, BSA and carbonic anhydrase, was digested and analyzed by a a microflow reversed-phase liquid chromatography/tandem mass spectrometry (μRPLC-MS/MS system) [48].

Finally, having close activity, performance of tryp-IMER D was also evaluated. Based on results listed in Table S3, also tryp-IMER D offered good performance even if with score and SQ% values slightly lower than those obtained with Tryp-IMER F.

3.3.5 Method validation

Method validation for on-line protein digestion included the assessment of intra-day and inter-day reproducibility of protein digestion, IMER to IMER (batch-to-batch, n=3) reproducibility, ruggedness and sensitivity. All parameters were determined using pretreated HSA at 0.26 mg/mL as test solution. %SQ and score values were considered as most representative parameters to describe tryp-IMER performance. Therefore, for each tested parameter, %SQ and score values were compared and the relative RSD% values were calculated. A very good reproducibility was obtained in terms of %SQ for the intra-day, inter-day and IMER-to-IMER analysis with %RSD values equal to or lower than 1.2 (Table S4). Also reproducibility of score values was satisfactory showing %RSD values of 2.4, 3.0 and 5.2 for intra-day, inter-day and IMER-to-IMER analyses, respectively.

More interestingly, the evaluation of method ruggedness over a five month-period showed that, notwithstanding a slight decrement of the score values likely caused by a slight decrement of IMER activity during intensive use, the general performance of the tryp-IMER remained satisfactory high (n = 8, %RSD = 3.9 for score values; %RSD = 1.4 for %SQ values). Indeed, the study was conducted comparing analyses of HSA on the same IMER over four different weeks along a five-month period.

This period was considered enough long to cover common daily variability in terms of buffer pH, different operators, laboratory temperature, instrumental response, sample preparation.

Analysis sensitivity, at a given amount of injected protein, strongly depends on fraction of protein that is digested by immobilized trypsin. Good sensitivity is required because biological samples often contain very low amount of analytes of interest. Microscale immobilized bioreactor have been shown to be advantageous due to the low sample consumption and high sensitivity [18]. In order to test the performance of the IMER-based analytical system in terms of sensitivity, decreasing concentrations (from 13.0 to 0.020 µg) of HSA were processed. HSA was selected as representative substrate being, among the others, the largest selected protein and, thus, the most difficult to digest. Till the injected quantity of protein exceeded 5.0 µg (76 pmol, 1.5µM) digestion efficiency did not decreased significantly (Figure 4), in agreement with the concept of the threshold value for good protein identification [6]. When an amount of protein lower than 5.0 µg was injected, score, %SQ and matches progressively decreased. Nevertheless, protein amount as low as 50 ng yielded signals that were sufficient for a correct identification (Figure 4). Considering HSA molecular weight, this corresponds to correct protein identification with only 0.76 pmol of protein. As further consideration, even when the sequence coverage was only 3-4% (0.05-0.10 µg of protein), unique peptides were correctly identified, allowing a trustable protein identification. These results are very satisfactory , allowing the correct use of such analytical platform even with low amount of available protein sample. Nonetheless, it must be noted that higher sensitivity can be achieved by off-line coupling with micro- or nano-LC-MS/MS systems. Such approach has been already proposed by several authors. As few examples, two-order of magnitude higher sensitivity for albumin digestion (7 fmol of protein) could be achieved by Wu S. *et al.* in 2011 using matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) as detection system [20], Similarly, Spross J. *et al.* used a capillary tryp-IMER off-line coupled with nano-HPLC/MALDI-TOF/TOF-MS/MS for the

digestion of a 10 fmol protein mixture [46] while Ma J. *et al.* analyzed a 10 fmol sample of Myo off-line coupling a capillary-tryp-IMER with a μRPLC-MS/MS system [48].

3.2.6 Digestion of human IgG.

IgGs are large molecules of about 150 kDa composed by four peptide chains. Human polyclonal IgG is a combination of four slightly different subclasses characterized by different plasma abundance: IgG1 (66%), IgG2 (23%), IgG3 (7%) and IgG4 (4%) [49]. Identifying changes in IgG sequence and post transcriptional changes (PTM) is important to monitor possible changes in IgG activity and/or immunogenicity [50]. However, when dealing with IgGs, sample preparation for bottom-up analysis may be difficult due to protein glycosylation [51], tendency to form large aggregates, resistance to denaturation process and formation of both too large and/or too small peptides when trypsin digested [52]. Tryp-IMER D and F (with different pore diameter, to account for eventual substrate accessibility limitations) were employed for the on-line digestion of a pool of human IgG (hIgG) and results were compared with those obtained with the in solution digestion (Ctrl) and with the commercial column Poroszyme™ (Table 4).

Table 4. Protein digestion efficacy for the identification of IgG heavy (*γ*) and light chains (*k* and *ʎ*).

	Ig*γ*1		**Ig*γ*2**		**Ig*γ*3**		**Ig*γ*4**		**Ig*k***		**Ig*ʎ***	
	SQ%	**U**	**SQ%**	**U**	**SQ%**	**U**	**SQ%**	**U**	**SQ%**	**U**	**SQ%**	**U**
Ctrl, 37°C	27.0	3.0	15.5	2.0	18.0	5.0	15.5	0.0	53.5	3.5	21.0	2.0
Poroszyme, 37°C	27.0	3.5	21.5	2.5	12.0	0.5	11.5	0.0	83.0	7.5	23.5	2.5
Tryp-IMER F, 37°C	35.0	4.5	29.0	2.5	19.0	1.0	14.0	0.0	83.0	6.0	35.0	3.0
Tryp-IMER D, 37°C	32.5	5.0	27.5	4.5	13.5	1.0	12.5	0.0	83.0	4.5	41.0	3.5
Tryp-IMER F, r.t.	27.0	3.0	15.5	2.0	18.0	5.0	15.5	0.0	53.5	3.5	21.0	2.0

U**:** unique peptides. r.t: room temperature. The analyses were performed in duplicate and the RSD% values were ≤20% for the SQ%, and ≤28% for the matches and ≤20% for the U values.

In tryp-IMERs D and F each trypsin molecule occupies approximately 30 nm^2 (D) and 42 nm^2 (F) of surface which is in both cases less than half of of IgG hydrodynamic size [53]. It means that theoretically each flowing IgG molecule inside the monolith pore is in contact with more than one trypsin molecule simultaneously regardless the tryp-IMER D or F.

To enhance the protein digestion efficiency studies were performed at 37°C. SQ% was select as parameter to compare the digestion efficiency while the number of unique peptides was listed to evaluate the identification validity.

The low SQ% values obtained are in agreement with the resistance to digestion which lead to formation of low amount of suitable peptides for a proper MS analysis. However, tryp-IMER F and D gave SQ% values significantly higher than those obtained by overnight in solution digestion, with the exception of Igγ4. Moreover, all IgG heavy and light chains were correctly identified by Mascot and the digestion efficiency of tryp-IMER F was significantly better than that of the commercially available Poroszyme™ in the same experimental conditions (Table 4).

As further consideration, digestion efficiency of selected tryp-IMERs can also be compared to that of nanoscale trypsin-bioreactor by Krenkova *et al.* [22] considering that the higher SQ% values achieved in that work were related to hyphenation with a highly sensitive nano-LC-MS, and not to a better digestion rate of the bioreactors, as demonstrated by comparison with the in solution digestion.

As further challenge, IMER F was also tested at room temperature and showed SQ% values slightly better than those by in solution digestion. As further outcome, pore diameters equal of larger than 0.6 μm seem to be suitable for the digestion of high molecular weight proteins such as immunoglubilins G. In fact, reducing pore diameter from 1.35 to 0.6 μm did not negatively affect efficiency in digesting large substrates.

3.2.7 Analysis of high molecular weight (HMW) plasma proteins

On-line digestion and analyses were performed in triplicate at 37°C injecting a 13 µg aliquot of the HMW protein fraction from plasma obtained by ultracentrifugation of plasma sample from a single donor. Mascot search on database led to the successful identification of 281 plasma proteins with 979 unique peptides. For comparison, the same sample was digested by the classical in solution protocol (overnight digestion, 37°C) leading to the identification of 312 proteins with 1346 unique peptides by a conventional HPLC-MS system. These results confirm the use of CIMac–based tryp-IMERs as promising alternative to classical assays even for processing complex samples. Indeed, considering the high number of proteins the result can be considered satisfactory (10% difference in terms of identified plasma protein). Similar trends were obtained by Wu *et al.* using a poly(acrylamide-co-methylenebisacrylamide)-based microTryp-IMER coupled to nanoRPLC–ESI-MS/MS (333 *vs* 411 identified proteins from a protein mixture extracted from *Escherichia coli*)[20]. Better performances were achieved by *Ma et al.* [48] in the analysis of 20 µg of proteins extracted from *E. coli* (208 proteins identified by a 10 cm capillary tryp-IMER vs 176 proteins recognized by the in-solution digestion). To note, integration of on-line digestion with a two-dimensional nano-HPLC-MS/MS system would significantly increase the analytical efficiency, enhancing the complexity of samples to be processed, as shown by Yuan H. *et al.* [13].

3.2.8. Operational and storage stability

The reagents used for the reductive alkylation of protein substrates (DTT and iodoacetamide) may significantly affect tryp-IMER stability. The IMER stability was evaluated by determining the residual active IU* of immobilized trypsin using BAEE as substrate after determined period of use. In the conditions used for the on-line studies, final DTT concentration was equal to 1.3 mM and final iodoacetamide concentration was 1.43 mM. Samples were injected without any further pretreatment. In such conditions, no activity loss was detected over six days of continuous use.

However, further studies highlighted that DTT and iodoacetamide concentration are key parameters for IMER stability. Indeed, when undiluted 1.0 mg/mL protein sample were injected a significant decrease of IMER activity was observed. Each injection of a protein sample containing DTT 5 mM and iodoacetamide 1.0 mg/mL (typical concentration of DTT and iodoacetamide for 1.0 mg/mL denatured protein without dilution) reduced the IMER activity by 0.85 ± 0.5%, thus significantly shortening the tryp-IMER lifetime (n= 80 injections).

Interestingly to note, for IMERs with the highest content of immobilized trypsin, such decrease of IMER activity was not paralleled by a reduction of the IMER performances in the digestion of standard protein samples such as HSA. This is in agreement with the fact that, provided that the amount of peptides generated during digestion exceeds a given threshold value, the peptides are correctly identified in the database and protein coverage is not affected [6]. However, this observation highlights that monitoring efficiency in the digestion of a sample protein, as used in some works (e.g. BSA in [22], myoglobin in [32], HSA in [40]) is not a suitable approach to assess trypsin stability but BAEE (or other chromogenic substrates such as D,L-BAPNA) needs to be used.

As operative information, the removal of reagents used for the reductive alkylation by centrifugal filters (AmiconUltra-0.5 mL, 10 K) prevented the alkylation of primary amines (such as lysine residues) and greatly enhanced IMER stability. Indeed no difference in the enzyme activity was observed when, after alkylation, the 1.0 mg/mL protein samples containing high concentration of DTT and iodoacetamide were pretreated with centrifugal filters, over 25 injections of denatured protein substrate.

Finally, tryp-IMERs were highly stable when stored for over one year not in use. Indeed no significant reduction of IMER activity, in terms of BAEE units, was detected when IMERs were store at 4°C in acetic acid 50 mM pH 3.5 containing $CaCl_2$ 1.0 mM.

4. Conclusions

This work aimed at providing new tryp-IMERs based on short-bed monolithic columns, which could be inserted in common HPLC systems. These IMERs were designed to provide performances which could match those provided by microscale bioreactors, with evident advantages related to an easier-to-use analytical platform. Results obtained have demonstrated the aldehyde-CIMac columns were suitable supports for the development of highly efficient tryp-IMERs. Besides activation chemistry also the type of trypsin, being immobilized on the support, and the amount of immobilized trypsin have been shown to be crucial parameters. In particular, the best performing IMERs were tryp-IMER D and F, with high density immobilized trypsin from bovine pancreas, containing 16.8 and 26.7 apparent international units of trypsin, respectively. Interestingly IMERs D and F were characterized by different pore diameter (1.35 and 0.60 μm, respectively). The lower pore size of tryp-IMER F allowed a larger BET surface and, consequently, a higher amount of immobilized enzyme without preventing the IMER to be used for the digestion of large and complex substrates such as a pool of human IgG, with molecular weights exceeding 150 kDa.

The optimized IMER-based analytical platform allowed the on-line digestion of protein substrates, the separation of peptides and the acquisition of the MS/MS spectra in an automated manner for the subsequent search in the data bank (SwissProt). All five selected protein substrates of different MWs and isoelectric points were correctly identified with excellent sequence coverage, generally higher that those obtained by in solution digestion (overnight) or with the commercially available trypsin-IMER Poroszyme^{TM}. Importantly, in the optimized working conditions no memory effects, nor significant nonspecific interactions were detected and very short digestion time (about 90 s), good intra–day and inter-day analysis reproducibility (%RSD in terms of %SQ ≤ 1.2) and good sensitivity, which permitted good protein identification down to picomol level, were achieved.

The high efficiency protein digestion was confirmed by the analysis of the HMW fraction of plasma proteins, as challenging real sample. Indeed, efficient protein digestion could be achieved within 90 s albeit sample complexity, allowing the identification of 281 plasma proteins.

Although the proposed on-line set up offers advantages in terms of automation and limited sample handling, CIMac-based tryp-IMERs can also be used in an off-line approach, i.e., collecting the peptide digests downstream the IMER and analyzing them separately. The latter approach, even if lacking automation, offers advantages over the *in solution* assay in terms of time saving and lower artifacts, and allows off-line coupling with micro- or nano-LC-MS systems as well as MALDI-TOF/TOF instruments, which cannot be directly hyphenated with on-line digestion. This approach has been used in previous works giving good results in terms of sensitivity and %SQ.[20, 46, 48]. However it must be considered that the sample collected downstream the tryp-IMER encounters dilution, thus an analytical approach of appropriate higher sensitivity should be employed for the analysis of the collected tryptic digest.

Finally, the protocol for sample denaturation ensured tryp-IMER stability without requiring any sample pretreatment. The latter translated in time saving on the overall process. The best performing tryp-IMER, the ALD-based try-IMER F which contains trypsin from bovine pancreas, held suitable performances for large scale applications in the proteomic field as alternative to in solution assays or to more complex and skill-requiring analytical systems.

Ancillary but definitively similarly important outcomes of this work are those related to operational issues. Indeed, even if several papers have been published in last decades on trypsin-bioreactors, in most cases a deep investigation for optimal procedures was not undertaken, leading to results that are not fully comparable or have been obtained in non optimal conditions. Investigations carried out within this work have highlighted the importance of using a continuous flow approach to determine the active units of enzyme after immobilization and during usage. The quite often used zonal approach

would lead to an underestimation of the immobilized active units. Furthermore, the importance of using small non proteic substrates, such as BAEE, to determine and monitor tryp-IMER stability was also highlighted. Indeed, monitoring protein substrate digestion by MS/MS detection would lead to an overestimation of enzyme stability, due to the intrinsic ability of the MS/MS-database search system to correctly identify proteins when the amount of digested peptides is higher than a threshold level, without any further discrimination when such level is exceeded. A proper characterization is a key issue in order to have a robust and trustable tool and to obtain a system which could effectively find applications in the field of proteomics. As a more general consideration, the procedures and considerations related to operational issues may have broaden applications either in the characterization or for in process control of trypsin-based analytical systems, with important implications for scientists working in the field.

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FIGURE CAPTIONS

Figure 1. Overlaid activity profiles for the tryp-IMERs as determined by continuous-flow approach. Tryptic activity is expressed as µmoles of BAEE hydrolyzed by the immobilized trypsin per minute. Dependence of activity from flow-rate has been investigated using flow-rates from 0.1 to 3.5 mL/min. BV stands for bed volume.

Figure 2. Digestion efficiency of tryp-IMERs by off-line and on-line approaches. In the off-line analyses 64 µL of HSA 1.0 mg/mL was used as substrate; ammonium bicarbonate (20 mM, pH 8.0) was used as mobile phase and flow-rate was set at 0.3 mL/min. The on-line analyses were performed

injecting 50 µL of HSA 0.26 mg/mL; ammonium bicarbonate (20 mM, pH 8.0) was used as mobile phase and flow-rate was set at 0.5 mL/min. White bars (Crtl) refer to the overnight in solution digestion by trypsin from bovine pancreas, while grey bars refer to on-line digestion by Poroszyme™ in the same operational conditions.

Figure 3. Digestion efficiency, expressed as score (A) and SQ% (B) values, for the on-line digestion of CytC, Myo, OVA, BSA and HSA (0.26 mg/mL) by tryp-IMERs D, F and K. Ammonium bicarbonate (20 mM, pH 8.0) was used as mobile phase and flow-rate was set at 0.5 mL/min. White bars (Crtl) refer to the overnight in solution digestion by trypsin from bovine pancreas.

Figure 4. SQ%, matches and score values as a function of the injected amount (µg) of HSA. Data refers to digestion by tryp-IMER F. Ammonium bicarbonate (20 mM, pH 8.0) was used as mobile phase and flow-rate was set at 0.5 mL/min.