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Trends in Analytical Chemistry 144 (2021) 116419

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Immobilized-enzyme reactors integrated into analytical platforms: Recent advances and challenges

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ARTICLE INFO

Article history:
Available online 19 August 2021

Keywords:
Hyphenation
Enzymatic reaction
Immobilization
Liquid chromatography
IMER
IMER-LC

ABSTRACT

Immobilized-enzyme reactors (IMERs) are flow-through devices containing enzymes that are physically confined or localized with retention of their catalytic activities. IMERs can be used repeatedly and continuously and have been applied for (bio)polymer degradation, proteomics, biomarker discovery, inhibitor screening, and detection. Online integration of IMERs with analytical instrumentation, such as high-performance liquid chromatography (HPLC) systems, reduces the time needed for multi-step workflows, reduces the need for sample handling, and enables automation. However, online integration can also be challenging, as reaching its full potential requires complex instrumental setups and experienced users. This review aims to provide an assessment of recent advances and challenges in online IMER-based (analytical) LC platforms, covering publications from 2014–2021. A critical discussion of challenges often encountered in IMER fabrication, sample preparation, integration into the analytical workflow, long-term usage, and of potential ways to overcome these is provided. Finally, the obstacles preventing the proliferation of IMERs as efficient tools for high-throughput pharmacological, industrial, and biological studies are discussed.

1. Introduction

Enzymes are crucial in the analytical scientists' toolbox, where they play a vital role as biological catalysts in chemical, biochemical, and biological applications. Enzyme-induced conversion is used in many applications, such as (bio)polymer degradation, proteomics, biomarker discovery, inhibitor screening, and detection. However, classical protocols rely on in-solution approaches, often leading to enzyme autolysis, single-use assays, and long reaction times (up to 24 h) with difficult automation.

Immobilized-enzyme reactors (IMERs; infrequently called immobilized capillary enzyme reactors, ICERs) can be defined as flow-through devices, which contain enzymes physically confined or localized with retention of their catalytic activities, and which can be used repeatedly and continuously [1]. The digestion reaction can take place under a flow of liquid, (dynamic) or when the flow has been paused (static). IMER supports include particles [2–18], monolithic supports [19–35], open-tubular [36–43], and porous-layer open-tubular [44,45] columns, and each format can influence the efficacy of the IMER. Reactor formats can be classified as either conventional or microfluidic, depending on the internal diameter of the reactor. Microfluidic immobilized-enzyme reactors have been applied mostly for analytical purposes, and several excellent reviews on this topic are available [46–48]. IMERs have drawn the attention of researchers thanks to several advantages when compared with in-solution digestion. Primarily, shorter digestion times (minutes to seconds instead of many hours) and increased sample throughput can be achieved. This is thanks to improved mass transfer, resulting from reduced diffusion distances, convection (in the case of monoliths), and the higher local concentrations of enzymes. The latter is possible as immobilization allows autodigestion to be avoided. Additionally, the product stream does not contain auto-digestion by-products. Enzyme stability often increases upon immobilization and the IMERs can be reused. In the field of proteomics, the first mention of an IMER can be traced back to a 1989 publication by Cobb and Novotny [49]. They immobilized trypsin on agarose gel and packed this into Pyrex tubing (300 mm × 1 mm ID) for the digestion of β-casein. Since

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https://doi.org/10.1016/j.trac.2021.116419

Abbreviations

AChE	Acetylcholinesterase
ACN	Acetonitrile
AhPNP	Purine Nucleoside Phosphorylase
BA	Nα-benzoyl-L-arginine
BAEE	Nα-benzoyl-L-arginine ethyl ester
BChE	Butyrylcholinesterase
BuMA	Butylmethacrylate
CE	Capillary Electrophoresis
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
FAC	Frontal Affinity Chromatography
HEMA	Hydroxyethylmethacrylate
HILIC	Hydrophilic Interaction Chromatography
HPLC	High-Performance Liquid Chromatography
ICER	Immobilized Capillary Enzyme Reactor
ID	Internal Diameter
IEC or IEX	Ion-Exchange Chromatography
IMER	Immobilized-Enzyme Reactor
IPC	Ion-Pair Chromatography
LC	Liquid Chromatography
Lys	Lysine
MS	Mass Spectrometry
NTPDase	Nucleoside Triphosphate Diphosphohydrolase
OT	Open Tubular
PBS	Phosphate-Buffered Saline
PEGMA	Poly(ethylene glycol) Methacrylate
PLGA	poly(lactic acid-co-glycolic acid)
PEG	Poly(ethylene glycol)
PLOT	Porous Layer Open Tubular
PNGase F	Peptide:N-glycosidase F
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
TPCK	l-(tosylamido-2-phenyl) Ethyl Chloromethyl Ketone
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultra Violet
α-GLU	α-Glucosidase

then, IMERs have taken on numerous formats, allowing their integration with analytical instrumentation, such as capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) systems, to aid the detection of fragmented or product molecules. IMERs can be essential parts of the analytical workflow, but need to be integrated with upstream or downstream (offline or online) sample-processing steps. Coupling the IMER online to an analytical system reduces the time needed for multi-step workflows, reduces the need for sample handling in-between steps (avoiding sample loss and contamination), and enables automation.

The aim of this contribution is to provide an overview of recent advances in online IMER-based (analytical) LC platforms, covering publications from 2014–2021. The review includes a critical discussion of challenges often encountered in online LC applications and of potential ways to overcome these. IMER fabrication, sample preparation, integration into the analytical workflow, and long-term usage will be discussed. Recent advances in enzymatic synthesis (e.g. biocatalysis) and digestion (e.g. bottom-up proteomics) will be described. Outside the scope of this review are capillary electrophoresis-based methods, and offline coupling.

2. Online IMER-based LC analytical platforms

2.1. IMER design and fabrication

2.1.1. General challenges

A multi-step approach to IMER fabrication is most common, using an optimized method to create a support structure (Fig. 1) such as particle-packed beds, open-tubular (OT) or porous-layer open-tubular (PLOT) columns, or monolithic media. Usually, the enzyme is immobilized to suitable surface groups through chemical coupling, which may be achieved in several steps. Generally, the attachment of the enzyme to a support structure can be achieved using one of three types of interactions, *viz*. hydrophobic, electrostatic, or covalent reactions [50]. Covalent reactions are most commonly employed to enhance the stability of the IMER, but this can be at the expense of IMER activity [50]. When creating an IMER, the choice of which support type to use can be challenging, with many factors to consider, such as enzyme loading, porosity, pH stability, etc.

A major challenge for the application of IMERs is non-specific adsorption of reaction substrates or products on the support surface, causing sample loss and carryover. Fabrication techniques vary, and factors such as hydrophilicity of the surface and secondary interactions due to charged groups are considered for each application. Non-specific adsorption can be addressed by hydrophilization of the support (using hydrophilic building blocks [25] or grafting [51]).

The choice of support, the residence time, and the flow dynamics supporting the digestion (continuous or paused flow) impact the efficacy of the digestion. This is particularly relevant for large substrate molecules with multiple reactive (e.g. lysing) sites. Different IMER supports and substrates present different challenges. For example, when large diffusion distances are encountered (porous media with pores >5 μm or single-conduit open-tubular IMERs) reduced efficacy is reported [50]. The sizes of protein substrates may also cause issues. For example, digestion residence time is critical in the biocatalysis of large molecules, which feature a number of cleaving sites [52]. Specific challenges associated with the support types are detailed in Fig. 2.

2.1.2. Open-tubular and porous-layer columns

Open-tubular columns/open channels (OT), either with a porous layer (PLOT) or with chemical modification (i.e. nano-architectures [42]), provide low-pressure candidates for online integration and automation of IMERs [36,38,43,44]. OT-IMERs can be housed in microfluidic chips [53], capillaries [14,20,34,36,37,39–41,43], or parallel-channel capillaries (photonic crystal fibres) [42,44]. The end of an IMER capillary can be pulled to form an electrospray (ESI) needle to facilitate the coupling with mass-spectrometric (MS) detection [54]. Generally, OT columns exhibit a limited surface-to-volume ratio, resulting in a low loadability. The latter can be partly overcome with long OT columns [44], or by enhancing the surface area through additional channels [42,44] and/or surface architectures (e.g. PLOT [44], nanoparticles [9]). Even in an OT format, IMERs outperform in-solution digestion in most circumstances [39], with improved repeatability when compared to monolithic IMERs [41]. However, in OT columns mass transport is limited by diffusion [39] and the contact time between enzyme and substrate is critical [38]. To facilitate enzyme-substrate interactions, longer reaction (residence) times are often required. The residence time should be increased when a reduced efficacy is observed in IMERs compared to free enzyme [43], or when a reduced sequence coverage is found with MS [54], especially when no change in substrate affinity is observed [38].

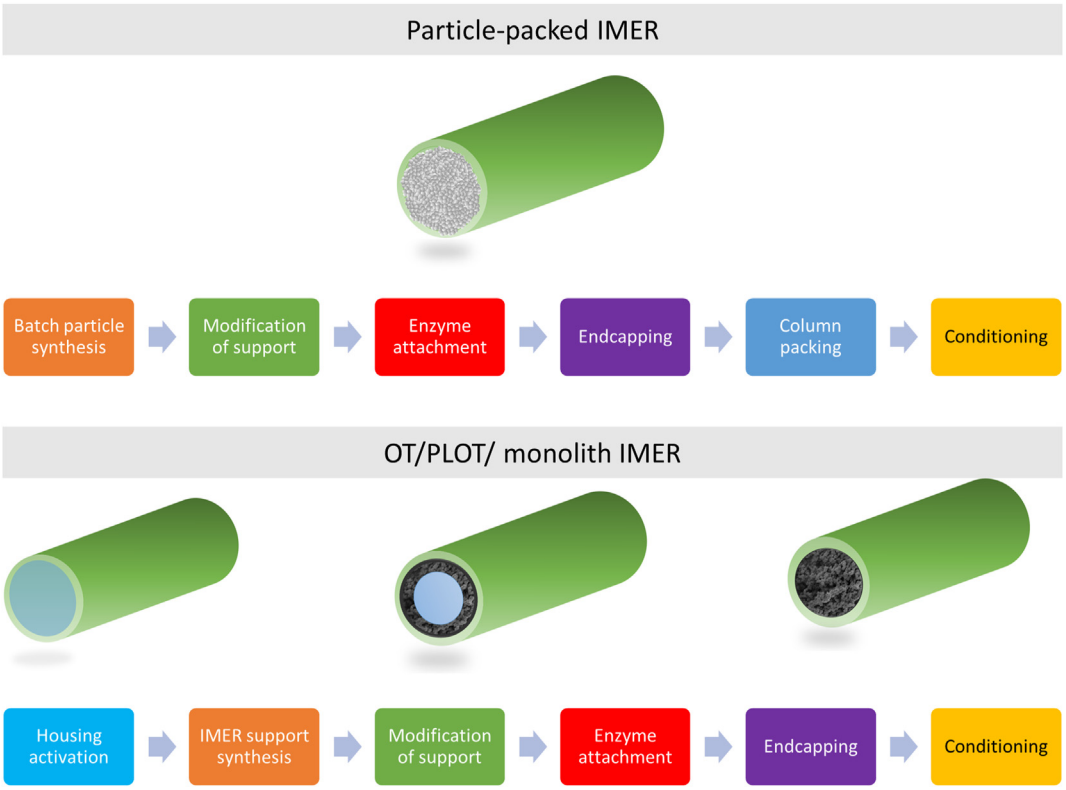

Fig. 1. Overview of the fabrication steps taken in the preparation of IMERs for particle-packed IMERs, and for either open-tubular (OT), porous-layer open-tubular (PLOT), or monolithic-media IMERs.

2.1.3. Monolithic columns

Compared to OT-IMERs, monolithic materials can provide higher efficacy, thanks to increased surface areas and convective mass transport [30]. Monolithic beds contain a single piece of porous material, within the confines of a housing material. They are prepared from organic or inorganic constituents, or a mixture of both [55]. Due to the interconnected structure of monoliths, they demonstrate high permeability, with fast mass-transfer thanks to convection, unlike the diffusion-restricted mass-transfer encountered within the pores of packed columns. Monoliths exhibit a number of benefits in IMER production, ranging from the available surface chemistries, the spacing of the immobilized enzymes [24,27,56], enzyme loading [27], accessibility/pore size [25,27,52], and convective flow. Ease of modification and porosity are the main factors to consider when optimizing the IMER scaffold. The main challenges with monolith fabrication are summarised in Fig. 2.

Monolith fabrication and surface modifications are usually performed *in-situ*. This makes for a technically straightforward approach to the introduction of surface-active groups. Organic-polymer monoliths are prepared from monomer precursors with porogenic solvents. They are macroporous in nature and lack the high population of micro- and mesopores encountered in silica monoliths [57]. Surface-specific groups for IMER attachment can be introduced using co-polymerization or post-fabrication modification reactions. Co-polymerization of a functional monomer, such as glycidyl methacrylate or vinyl azlactone, can provide a low surface expression of pendant reactive groups, ready for modification. Other approaches include the introduction of a spacer arm, such as glutaraldehyde, which may improve the efficacy of the proteolysis. This is thanks to a combination of factors, including a reduction in steric hindrance (reduced enzyme-population of the surface) [27], maintaining the conformational structure of the enzyme (reduction of surface-enzyme side interactions), and a resulting increase in the potential interactions between the enzyme and the substrate [24]. This also relates to controlling the enzyme load, as a high load may result in a lower efficacy and performance, likely due to a combination of distortion of protein structure, and steric/diffusional limitations of accessibility between the enzyme and incoming substrate [27,52,56]. Some studies suggest that specific limitations, such as a reduced efficacy, are observed with large macromolecules, such as large enzymes [27,56], or with large and complex substrates [25]. These limitations may possibly be overcome by manipulating the porous nature of the support [52] or by increased residence times.

Silica monoliths are hydrophilic in nature [19] and well suited to reducing non-specific binding. However, pH stability may be an issue during fabrication/immobilization and application [57]. Passivation of the surface may be required to minimize the effects of free silanols [55], which may interfere with the digestion [41,55]. Hybrid monoliths, a combination of silica and polymer monoliths, have been reported to yield higher sequence coverages of IMER-digested proteins [55], thanks to a higher binding-capacity, improved permeability, and enhanced mechanical strength [57]. However, organic-polymer monoliths, with low back pressures, high permeability, high pH stability (large working pH range) [57], and high solvent resistance, are generally more suited to applications involving macromolecules [25]. For polymer monoliths, the hydrophobicity is controlled by the functional monomers. The inclusion of a hydrophilic monomer, such as HEMA [52] or PEGMA, can reduce the surface hydrophobicity during fabrication. Alternatively, hydrophilization can be achieved via surface modification.

Organic monoliths allow quick fabrication [13] and control of porous properties [52], without an adverse change in surface chemistry. Wide-pore silica monoliths and polymer monoliths are

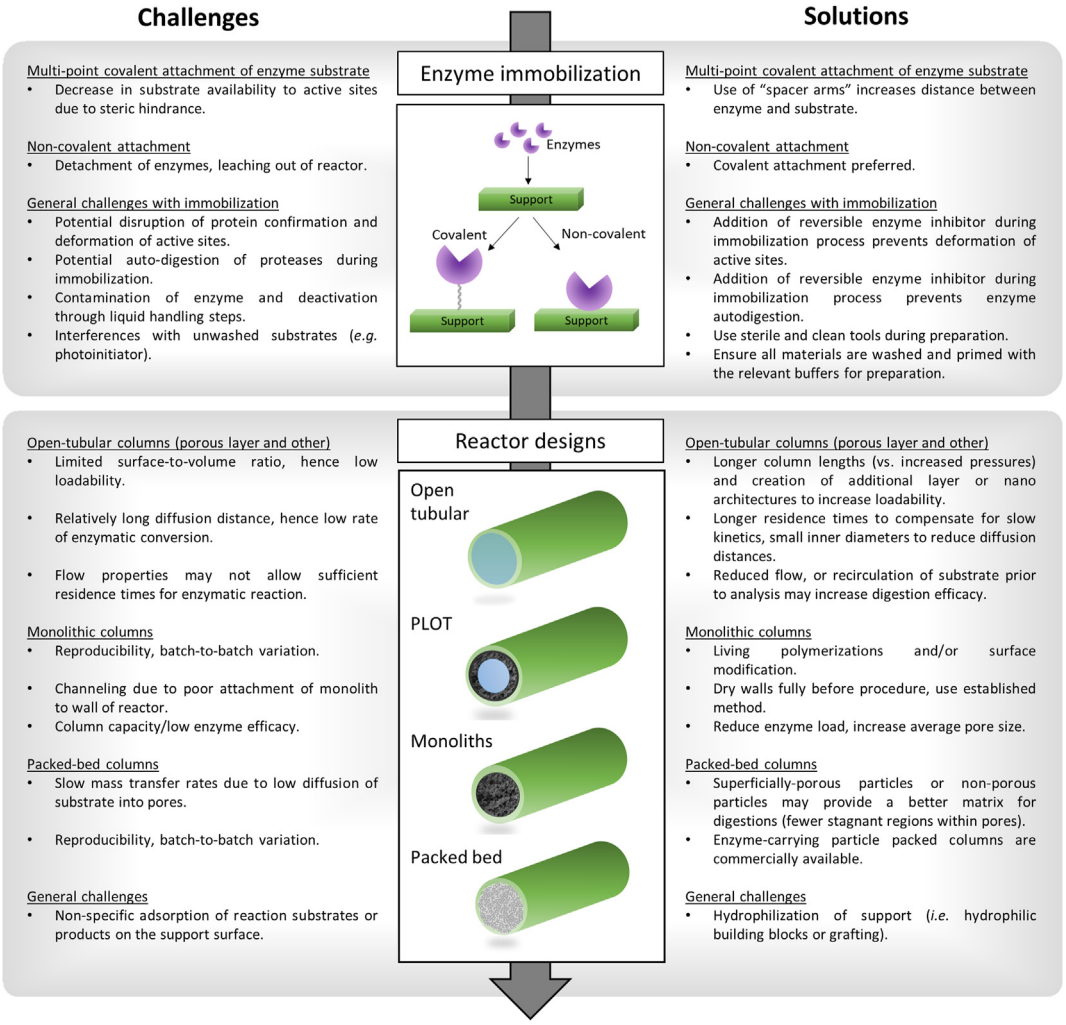

Fig. 2. Challenges and solutions for various aspects of enzyme immobilization and reactor design.

ideal for IMER fabrication, thanks to the absence of stagnant regions within the materials [19] and a limited diffusion-path length [13], leading to better accessibility of the immobilized enzyme for substrates.

Residence time in IMERs is inversely proportional to flow rate. Digestion with high residence times [28] and short residence times [26] have both been reported with monolithic media. However, the impact of residence time (when reported) has not been consistent [56], which may indicate an application-specific or technical issue, likely due to factors such as enzyme size and load, substrate size, bed length and/or volume, structure of IMER support, etc. [52].

2.1.4. Packed-bed columns

A packed-bed flow-through IMER consists of a column packed with (often porous) particles, on which enzymes are immobilized. Substrate molecules percolating through the reactor diffuse through a stagnant solvent layer present between the mobile phase and the particle surface, and partition into the particle pores, where an enzymatic reaction takes place. Subsequently, reaction products leave the enzyme active site, partition into the stagnant solvent layer, diffuse into the bulk mobile phase, and are swept out of the IMER [18]. Compared to monolithic columns, mass transfer rates are slow, due to diffusion in and out of the pores. This can be mitigated by using superficially porous or non-porous particles, albeit at the expense of loadability. Enzyme-carrying particle-packed columns can be purchased commercially, including manufacturers such as OraChrom (StyroZyme columns containing TPCK-trypsin [4,17,58], pepsin, or papain), Perfinity (columns containing trypsin [5,18] or lys-C), and Genovis (FabRICATOR-HPLC columns containing a cysteine protease for digestion of monoclonal antibodies). Alternatively, researchers have purchased enzyme-carrying particles (e.g. Poroszyme by Thermo Scientific) and packed these into columns [9,10]. However, especially for applications outside of proteomics, most of the recent publications report various in-house enzyme-immobilization protocols for particles, which are then manually packed into columns. This may affect the reproducibility and repeatability of results.

2.2. IMER integration into the analytical workflow

This section discusses challenges related to the integration of the IMER in an online LC analytical platform. An overview of various common IMER-integrated configurations found in literature is shown in Fig. 3. The section is divided into three parts, *viz.* challenges in sample preparation, integration, and storage, with an overview of these challenges summarised in Fig. 4.

2.2.1. Challenges related to sample preparation

Two steps are common in sample preparation prior to protein digestion, i.e. denaturation and alkylation. For in-solution

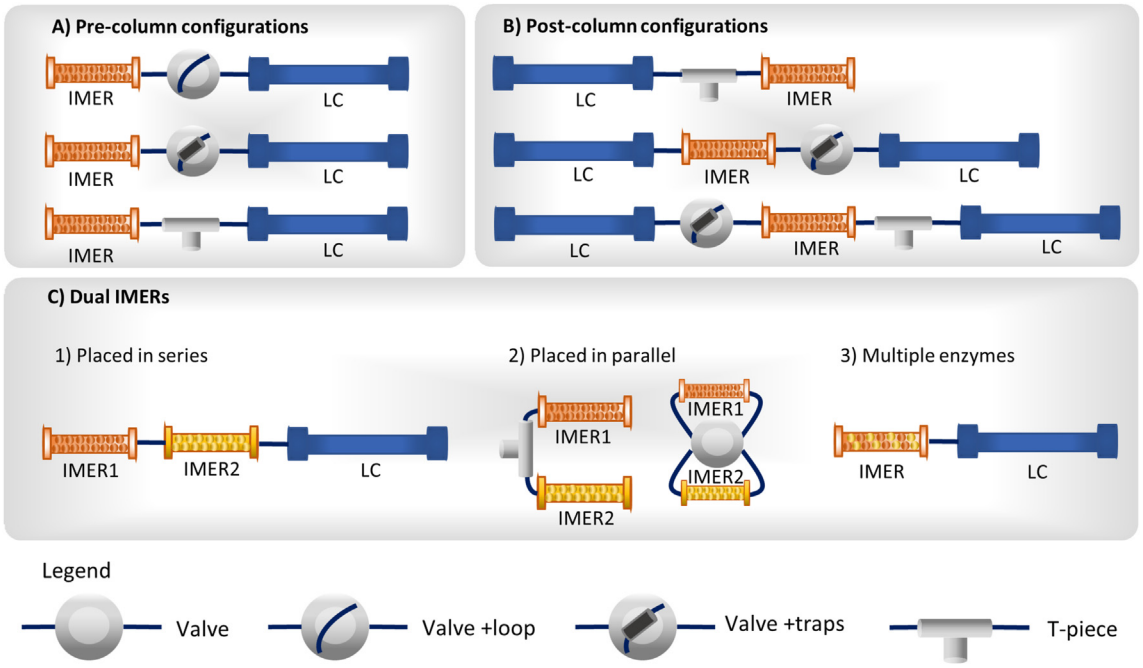

Fig. 3. Overview of IMER configuration in analytical LC workflow. a) pre-column configurations, b) post-column configurations, and C) dual-enzyme reactors.

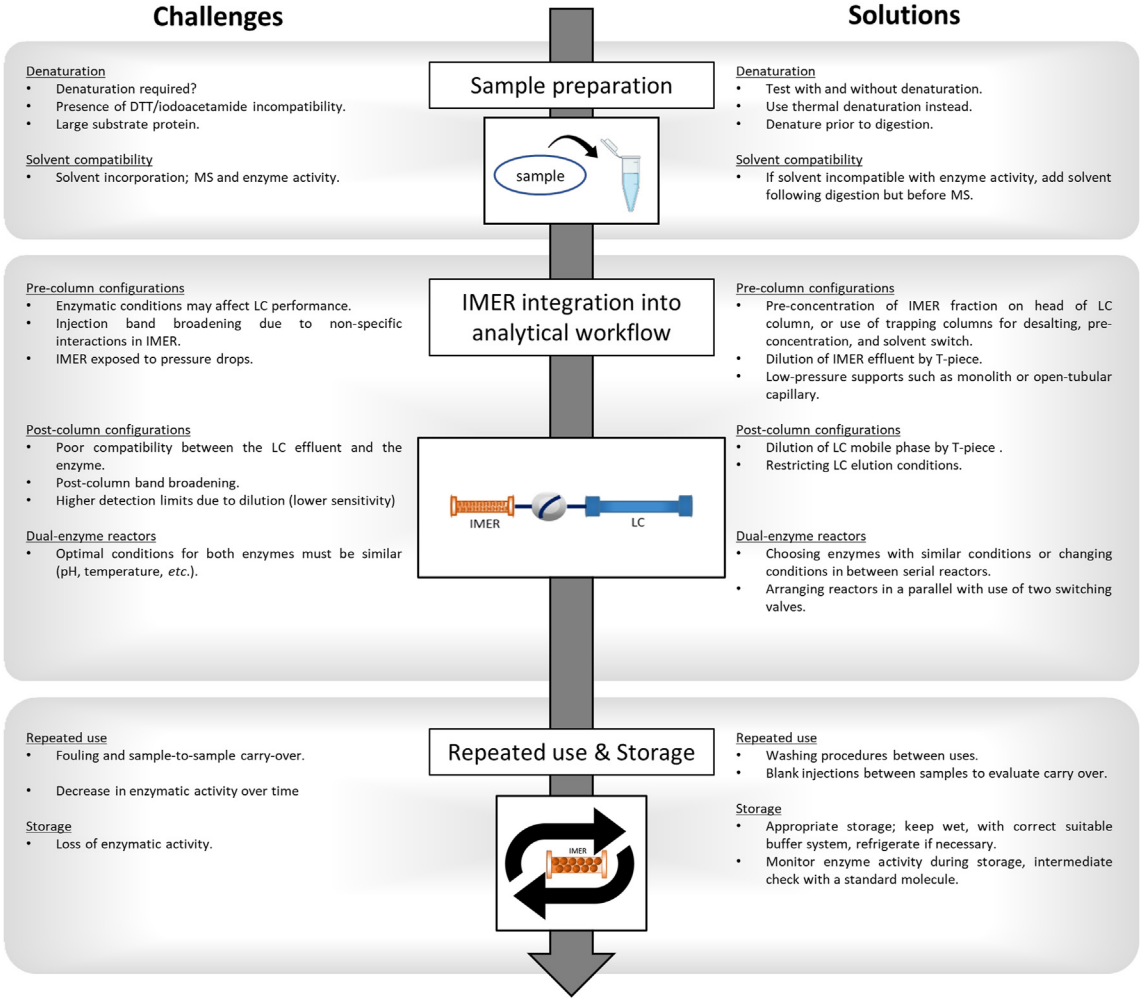

Fig. 4. Challenges and solutions for various aspects of sample preparation, IMER integration into the analytical workflow, repeated use, and storage.

approaches, proteins are often denatured and solubilized with chemical agents (e.g. SDS) to improve enzymatic efficiency, particularly for target proteins with multiple disulphide bridges. The decision whether to use these sample preparation steps is not clear when using IMERs. The latter are reported to have a higher digestion efficacy compared to in-solution approaches [30]. Yet, some reports with trypsin use denaturation [55], while some do not [24]. The size of the substrate or large complex proteins has been shown to have an effect on the efficacy of digestion in IMERs [52]; in such cases the use of denaturation would be recommended.

In addition to the digestion, the immobilized-enzyme's environment must also be considered, as it will be exposed to the sample solution as a whole (i.e., DTT/iodoacetamide included). It has been reported that the use of alkylating and denaturing agents can be detrimental to IMERs, and that these may need to be removed prior to digestion to lengthen IMER lifetime [23]. The size and structure (e.g. disulphide bridges) of the analyte protein should be assessed before digestion. If reduced efficacy is acceptable for the study, then intact native proteins may be digested. If denaturation and alkylation are to be used, an assessment of IMER lifetime [23] should be performed. For online IMER digestion, the presence of denaturants could prove incompatible with MS. Thermal denaturation prior to digestion may be a useful alternative. Additionally, Liu et al. studied the effect of the pre-screening solvent on enzyme activity [59]. The effect of different extraction solvents (ethanol, methanol, and DMSO) and the different proportions of organic solvent to PBS (v/v) on the enzymatic activity of α-glucosidase was investigated [59] and it was found that 20% (v/v) DMSO-PBS still retained more than 60% relative activity of α-glucosidase, striking the best balance between solubility of the original plant extract and tolerance of the enzyme.

2.2.2. Challenges related to integration

2.2.2.1. Pre-column configurations. Hyphenation of LC and IMERs in an online analytical platform can be achieved in either pre-column (IMER-LC) or post-column (LC-IMER) configurations (see Fig. 3). In pre-column configurations, samples are injected into the IMER for enzymatic reaction, and the IMER effluent is transferred online to an LC column (Fig. 3A). The IMER-LC configuration avoids exposure of the enzymes to LC mobile phases and allows optimization of the enzymatic conditions (e.g. buffer, pH, residence time), independently from the LC separation. Three main challenges can be discerned for IMER-LC configurations. Firstly, the enzymatic conditions may affect the performance of the LC separation due to solvent incompatibility. Secondly, as the sample passes through the IMER prior to the LC separation, injection band broadening may occur due to non-specific interactions of the analytes with the enzyme and the support material. Finally, in the case of a microfluidic device, the IMER may be exposed to high pressures. Gstottner et al., for instance, reported that the trap behind the IMER was kept rather short to achieve a back pressure below the limit of the trypsin column [17].

To address the above challenges, IMER effluent can be transferred via a modulation interface, consisting of a switching valve with either empty loops or with trapping columns, that allow for pre-concentration and a solvent switch. Sample transfer is typically done for at least four IMER volumes to ensure full transfer [11]. As an alternative to switching valves, Lynen et al. demonstrated the use of a T-piece to mix the IMER effluent with LC mobile phase prior to the separation column [11]. While the simplicity of this approach is attractive, the IMER effluent was continuously percolated through the LC column. As a consequence, the IMER reaction buffer had to be compatible with the ion-pair chromatography (IPC) and ion-exchange chromatography (IEC) mobile and stationary phases.

Pre-concentration on the head of the analytical column has mostly been used for inhibitor screening, online sample preparation, and biocatalysis, with UV detection rather than MS. Hyphenation with LC allows for online enzymatic studies including kinetic analyses and the identification of ligands. Examples include screening for ligands with nucleoside triphosphate diphosphohydrolase (NTPDase-1) [39] and Cathepsin D [37], online-screening of acetylcholinesterase inhibitors [20] or α-glucosidase (α-GLU) inhibitors [59] in natural products, with characterization and affinity-screening studies of new inhibitors by frontal affinity chromatography coupled to MS (FAC-MS) [30]. Another popular application field for integrated LC-IMER platforms is to investigate biocatalytic properties of immobilized enzymes, by coupling the bioconversion step with the product separation in an integrated platform. Recent examples include immobilization of ω-transaminases for the synthesis of chiral amines [19], and purine nucleoside phosphorylase (AhPNP) for the evaluation of the substrate specificity on nucleosidelibraries [30] or to study the phosphorolysis reaction [16] in flow-synthesis of nucleosides. Finally, Wouters et al. reported a lipase-containing microfluidic reactor hyphenated with a size-exclusion-chromatography system for online degradation of PLGA-PEG-PLGA nanoparticles and subsequent analysis of the polymer-degradation products [60]. Rather than using an organic solvent to dissolve the polymeric samples, polymeric nanoparticles in an aqueous environment yielded sample-solvent compatibility with the IMER. This platform introduced a novel tool for online characterization of synthetic polymers, reducing the necessary polymer degradation time from more than 24 h to 2 min.

Alternative to empty loops on the switching valve, traps can serve to clean up the sample from salts and other interfering compounds, allow for pre-concentration of the sample, and enable a solvent switch prior to LC-MS analysis. Several factors need to be taken into account when traps are used, such as digestion buffers (pH) that may damage the trap [22], carry-over [22] or breakthrough effects [61]. Chen et al. investigated the effects of digestion buffer and found that a pH of 8.5 could cause deterioration of a C18 trap [22]. This problem was solved with the addition of a T-piece and a make-up flow from another pump after the IMER to mix the digested peptides with low-pH solvent prior to the trap. Barr et al. developed an integrated IMER × LC-MS/MS platform for the simultaneous quantitative analysis of eight apolipoproteins [5]. Their setup consisted of a ten-port valve with two traps that allowed simultaneous digestion and LC-MS/MS analysis. Each consecutive sample was digested and trapped on one trap, while the previously digested and trapped sample was eluted from the other trap and analyzed by LC-MS/MS, effectively doubling the LC-MS/MS sample throughput to one sample each 12.5 min. The group of Massolini et al. have demonstrated an example of the use of traps for selective enrichment [35]. An integrated LC-MS method was developed with online digestion of (phospho-) proteins, coupled with the selective enrichment on a TiO_2 trap and separation by LC-MS for both normal and phosphorylated product peptides. Their method significantly enhanced the ability to digest and enrich the phosphorylated peptides in a complex protein mixture in a high-throughput platform.

2.2.2.2. Post-column configurations. A limited number of recent publications have opted for the post-column configuration, either in one- or two-dimensional LC platforms (LC-IMER, LC-IMER/ × / LC, or LC × IMER × LC, shown in Fig. 3B). When placing the IMER downstream from the LC separation, researchers should consider compatibility between the LC effluent and the enzyme, post-column band broadening, and higher detection limits due to dilution (lower sensitivity). Overall, two strategies can be

distinguished to deal with solvent-compatibility issues, i.e. dilution using a T-piece and/or restricting the elution conditions to accommodate the enzyme. An example of the first approach was shown in a publication by Gstottner et al., describing an online "four-dimensional" HPLC/MS approach (IEX × trap × IMER × RP-MS) for the characterization of unknown monoclonal-antibody variants [17]. The second dimension consisted of RP trapping and reduction, and the third dimension was an online tryptic digestion. A T-piece was used to introduce digestion buffer (50 mM TRIS, 10 mM $CaCl_2$) at pH 8.0 on the trap, leading to a final solvent composition of 11.6% ACN diluted in digestion buffer. No effect of reducing agents (i.e. DTT) on enzymatic activity was reported. As an example of the second approach, Liang et al. used hydrophilic monoliths with immobilized gold nanoparticles for highly selective enrichment and online deglycosylation of glycopeptides [33]. The enrichment by hydrophilic-interaction chromatography (HILIC) was achieved in weak alkaline environment, enabling online deglycosylation by the IMER without the requirement of buffer exchange and pH adjustment. One completely different approach is the integrated sample pre-treatment platform for quantitative *N*-glycoproteome analysis described by Weng et al. [15]. They developed a nitrogen-assisted interface for sample exchange between a HILIC-IMER interface to eliminate residual ACN from the HILIC column.

2.2.2.3. Dual-enzyme reactors. A limited number of publications report on the use of multiple enzymes, for instance by co-immobilizing multiple enzymes in one reactor [24,26,40] or by placing two reactors in series or parallel in the workflow [27,34,36,41,44,62] (shown in Fig. 3C). Trypsin is the most commonly-used proteolytic enzyme immobilized in reactors, followed by chymotrypsin, Lys-C or Lys-N. Combining trypsin and Lys-C provides more complete digestion of proteins by reducing the number of missed cleavage sites [40]. Such an approach can result in a higher sequence coverage, which is particularly important when the reliable identification of one target protein is needed. Jiang et al. immobilized a trypsin/Lys-C mixture and Lys-N in a monolithic column [26]. A proof-of-concept experiment demonstrated a significant improvement in sample-preparation efficiency and a reduction of experimental time compared to in-solution digestion, with similar protein-sequence coverage and protein identification. Several groups have used multiple enzymes by coupling two IMERs either in series or parallel. During integration, the main challenge is to maintain optimal conditions for both IMERs (pH, temperature), either by choosing enzymes with similar conditions or by allowing a change in conditions in between the two IMERs. Foo et al. successfully coupled a deglycosylation reactor with a trypsin reactor in series to detect both the non-glycosylated, and glycosylated peptides in an online LC-MS/MS configuration [41]. There was a noticeable effect of the position of the trypsin reactor and deglycosylation reactor in the flowpath on the detection of the target peptides. Another way to overcome the challenge of maintaining optimal conditions for both IMERs is to couple two IMERs with the use of switching valves to arrange the two reactors in a parallel manner [36,62]. In a recent paper Seidl et al. described an on-flow method based on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [36]. The IMERs were arranged in parallel between LC and MS instruments utilizing two 10-port/two-position switching valves, with each IMER independently connected to each of the switching valves. The novelty of this work was that two different enzymes could be screened at the same time, with only one single sample injection and in less than 6 min. The group of Seidl introduced a 'bridge' concept to allow both enzyme reactors to work simultaneously.

2.2.3. Challenges related to long-term usage

When a reactor is used repeatedly in an analytical workflow the risks of fouling, carryover, and reduced activity increase over time. To overcome carryover and fouling, washing procedures between uses have been suggested, while blank injections between samples are used to evaluate carryover. IMER-stability studies have rarely been described, with no standard approach for reporting. In the few studies described, time points range from 60 uses [43], 7 days of consecutive use [55], to longer term studies of 3 months, 6 months, 10 months, and to 1 year [8,16,23,63].

If an IMER is stored under poor conditions, a loss of enzymatic activity can occur over time [37,63,64]. Usually, a buffer suitable to the enzyme (often the digestion or running buffer), a metal salt to preserve the enzyme's conformation, and storage at 4–5°C are recommended [8,23,29,30,42]. Many storage conditions have been reported, including both extremes of room temperature and refrigeration, with varying enzymes. Some authors reported an activity retention of 20% after 5 months [37], while others report no adverse effects from up to 6 months of storage [8]. The exact cause of activity loss (e.g. autolysis, loss of structure) has not been alluded to. In one study the activity of the enzyme dropped to 64% after 3 months of storage, which still outperformed a frozen in-solution aliquot (14% activity retained [63]). Some reports also show no change in efficacy following 6 months of use and storage [8].

To monitor enzymatic activity following storage and multiple uses, a validation digestion of a standard molecule prior to and following any study would be pertinent. Intermediate checks have been recommended with a standard molecule, such as BAEE, similar to quality assurance in the pharmaceutical industry [29]. However, when using BAEE and generating BA as a system-check, spontaneous hydrolysis may occur in solution (alkaline conditions), which may need to be corrected for [23].

3. Conclusions and future perspectives

The challenge of analyzing large numbers of volume-limited samples drives the need for automated, sensitive, and robust analytical tools, such as online IMER-based LC platforms. In this review, the advantages and disadvantages of IMER-based LC platforms were discussed, and potential solutions were suggested for challenges encountered. The online integration of different steps in the analytical workflow, including enzymatic reactions and separation of mixtures, offers clear advantages to the user. Reduced sample loss, improved repeatability and reproducibility, and increased sample throughput can be achieved, thanks to reduced sample handling, automation, and a greatly reduced total analysis time. However, online integration can also be challenging, as complex instrumental setups and experienced users are needed to reach its full potential. The main application areas of online IMER-based LC platforms include inhibitor screening, online sample preparation, and biocatalysis. A majority of the recent publications involved proteolytic enzymes, such as trypsin, pepsin, PNGase F, Lys-C, and Lys-N, underlining the popularity of proteomics as a development and application area. However, IMERs are not yet widely accepted in the analytical and industrial communities. Hurdles preventing their acceptance include the reproducibility of IMER production, their robust integration in analytical platforms,

and sample-to-sample carryover. Comparing IMERs is still difficult and consensus is needed on parameters and methods. Standardized methods would also allow assessing the effect of storage and repeated use on the efficiency of the IMER, which may instill confidence in potential users. Addressing these shortcomings will stimulate the acceptance of IMERs as efficient tools for high-throughput pharmacological, industrial, and biological studies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

N. Abdulhussain acknowledges The STAMP project, which is funded under Horizon 2020-Excellent Science-European Research Council (ERC), Project 694151.

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