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Review

Immobilized enzyme-based analytical tools in the -omics era: Recent advances

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ABSTRACT

Protein analysis is a field under rapid development mainly thanks to technological advances which have granted miniaturization of analytical devices, automation and higher detection sensitivity. The interest in the field has paralleled the expansion of the -omics era, laying down the bases for the current applications in proteomics and glycomics. Advances in protein sample transformation prior to analysis have led to reduction of sample consumption and contamination, enhancing throughput. Within this context, and thanks to the availability of new high performing materials and technologies, increasingly more efficient and miniaturized enzyme-based analytical tools have been proposed to overcome shortcomings encountered in the in-solution enzymatic reactions (protein digestion and protein deglycosylation, for proteomics and glycomics, respectively). In this context, immobilized enzyme reactors (IMERs) and IMER-based platforms have been developed as promising approaches toward automation and higher analysis throughput. The scenario is in continuous development as underlined by thirty-four papers published in the last five years. This review encompasses recent advances in the design and operational set-ups of IMERs purposely developed for the analysis of proteins and glycoproteins. Recently developed dual IMERs, which integrate more than one processing step into a single IMER, and analytical platforms exploiting tandem IMERs are also reviewed and commented.

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1. Introduction

Protein analysis is an area of continuous growing importance with implications in several research fields spanning from basic molecular biology to diagnosis, drug discovery and clinics. The growing number of biotechnological drug candidates entering clinical trials each year [1] has also generated new analytical challenges to be fulfilled. The large and increasing interest for a detailed analysis of complex protein mixtures has definitively paralleled, and at the same time paved, the proteomic era. Indeed, rapid technology-driven progress has allowed processing more complex samples with progressively higher informative outputs. The

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rush toward proteome identification was prompted by the tremendous scientific impact coming from advances in the field. Proteins play different major roles in living organisms, hence, correlation between structural motives and their biological function as well as between structural modifications and pathological *stati* are at the basis of the understanding of physiological and pathological processes, respectively.

Liquid chromatography (LC) coupled to mass spectrometry (MS) is currently dominating the field of protein identification and macromolecule analysis. Currently available high resolution LC-MS systems allow the analysis of low abundant and highly heterogeneous samples with a suitable selectivity and resolution. Fueled by similar recent analytical and technological advances, the past decade has also seen the rapid growth of other younger "-omics" including glycomics, which addresses the comprehensive analysis of the glycans in glycoproteins or glycolipids within a tissue, organ or biological fluid. As for proteomics, the growing interest in glycomic studies has stimulated the investigation on new enzymatic and analytical tools to enable processing large sample sets.

Indeed, -omics are a land of challenges from an analytical point of view. Notwithstanding the higher throughput in analytical workflows, in both proteomics and glycomics, protein sample transformation prior to analysis still represents a limiting step. When performed in a classical way, sample transformation, either protein digestion or glycan release, is often tedious and time consuming.

In this scenario, the use of low-volume immobilized enzyme reactors (IMERs) has gained increasing popularity as attractive alternative approach to classical in-solution methods being trypsin the most exploited enzyme. To be appealing for -omics applications, the reactor should be a device of small dimensions that could be interfaced with separation and MS systems and able to process a small amount of sample with high efficiency. With respect to the latter point, it has been shown that IMER miniaturization allows processing smaller sample volumes with improved efficiency [2]. Hence, an anlysis of recent literature clearly shows a run toward miniaturization, as underlined by recent reviews by Jänis and coworkers [3] Yixin Li *et al.* [4] and Hajba1 and Guttman [5].

IMERs can be prepared in various formats using different support materials and re-used for multiple cycles. From a theoretical standpoint, all the pre-MS analysis procedures, both in proteomics and in glycomics, could be performed by multiple reactors properly interfaced with desalting and/or trapping columns.

The interest for IMERs is not recent. The first report on a IMER for proteomic studies dates back to 1989 when Kelly A. Cobb and Milos Novotny [6] published on the preparation of a trypsin reactor by packing trypsin-agarose into a 30 cm × 1 mm thick-walled Pyrex tube. The IMER was used for the off-line peptide mapping of low amounts of standard proteins (down to 40 nM). Since then, numerous immobilized enzymes have been developed for applications in proteomics and have shown great advantages, allowing to overcome the most common shortcomings of in-solution assays, as discussed in some of the major former review articles on this subject [7–11]. Among these advantages, the high enzyme-to-substrate ratio achievable with immobilization has shown to significantly improve efficiency and shorten reaction time even when low-abundance proteins are processed. In addition, the high repeatability of the catalyzed reaction, i.e. digestion or deglycosylation, could provide reliable outputs.

In parallel to the advances in immobilization techniques these last years have foreseen advances in supporting materials, with particular focus on monoliths [12,13], that have been used to prepare IMERs of different formats such as beads, membranes, fused-silica capillaries, and on-chip reactors [14], which have been used off-line or on-line upon integration with high-performance separation methods, mostly liquid chromatography but also capillary electrophoresis [15].

Hence, herein, we discuss recent advances in the design and operational set-ups of immobilized enzyme reactors of different formats for application in sample preparation for proteomic- and glycomic-related studies. Although a variety of works is published each year addressing similar issues, e.g. high efficient tryptic digestion, their specific applications may not overlap because of differences in the IMER format or stability. Thus, particular attention will be given to parameters that are important for the selection of a proper approach such as (i) IMER validation by complex biological samples, other than isolated probe substrates, (ii) stability over multiple cycles of use and (iii) format since this defines, to some extent, the separation and detection system. Finally, since notwithstanding the good number of publications in this field, only a small number of them effectively proposes analytical platforms for on-line sample processing and analysis, this aspect will be underlined along the whole review.

2. IMERs in proteomics and protein analysis

2.1. Trypsin IMERs

MS-based protein analysis relies on efficient and unbiased protein digestion protocols. Reduced experimental variability, limited contamination and increased automation are essential goals to achieve fast and reliable results [16]. Trypsin is the gold standard enzyme used in shotgun proteomics for the conversion of proteins into MS-friendly peptides [17]. Notwithstanding the in-solution trypsin digestion tends to be the simplest approach in terms of sample handling, it is endowed with some common drawbacks, among which long incubation time, trypsin autolysis and low stability at higher temperatures or in the presence of organic solvents [18]. Because of the extensive use of trypsin in protein digestion, its commercial availability and its low cost, several research groups have attempted to enhance the proteolysis efficiency by designing a variety trypsin-based IMERs [3,19–24]. Furthermore several different formats of trypsin-based IMERs are commercially available, among which magnetic (Mag-Trypsin™ from Takara Bio Europe) and non-magnetic beads (Thermo Scientific Pierce), buffered aqueous suspension of trypsin immobilized on agarose (Sigma-Aldrich), micro spin columns containing highly purified, TPCK-treated porcine trypsin (Sigma-Aldrich) as well as quite expensive trypsin-based columns, namely Poroszyme™ Immobilized Trypsin Cartridge (2.1 mm × 30 mm) from Applied Biosystems (Poroszyme™ immobilized trypsin also available as bulk media) and Perfinity™ Trypsin Column and NoRA™ Trypsin Column of identical dimensions (2.1 × 33 mm) from Perfinity Biosciences. The commercially available IMERs or beads ensure fast protein digestion within minutes and can be on-line or off-line coupled with LC–MS systems.

Notwithstanding the commercial availability, the constant interest in achieving trypsin-based analytical tools of improved performance is underlined by the number or works published each year.

For reviewing purposes recently proposed trypsin-IMERs were classified, based on their format, in i) flow-through IMERs and ii) micro- nano-particle IMERs, including nano-flowers, magnetic beads, core-shell particle and graphene-oxide. Table 1 summarized the main characteristics of the trypsin-based IMERs developed in the time-frame considered for this review.

2.1.1. Flow-through IMERs

This paragraph refers to those IMERs whose format allows their insertion in a flow-through system. On the basis of their physical