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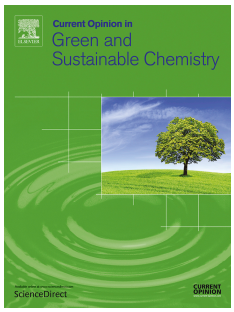

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Characterization and evaluation of immobilized enzymes for applications in flow-reactors

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Highlights

- Enzyme immobilization is a key enabling technology for flow-biocatalysis.
- Materials and immobilization protocols need to be adapted for continuous applications.
- Enzyme loading, recovered activity, space-time yield, and turnover numbers must be reported when applying immobilized enzymes in flow.
- Productivity and operational stability of immobilized enzymes are maximized through rationally engineering the protein-surface interface, the material and the reactor configuration
- There is a positive correlation between the space-time yields and turnover numbers of immobilized enzymes applied in flow

Abstract

Flow-biocatalysis mainly exploit immobilized enzymes for continuous chemical manufacturing. In the last decade, the use of immobilized biocatalysts in flow is growing rapidly, yet the immobilization protocols need to be optimized. In this review, we have discussed the most fundamental aspects to be considered when designing immobilized enzymes for productive and stable operations in flow. Furthermore, we analyze the protein loading, the activity recovery, the space-time yield (*STY*), the specific productivity and the turnover number (*TN*), as complementary metrics to assess the efficiency and longevity of immobilized enzymes integrated into flow-reactors. The science of the immobilization engineers the surface-protein interface, the material features and the reactor design to achieve highly active and robust heterogeneous biocatalysts under continuous operations. Hence, we encourage the flow-biocatalysis community to accompany those engineering efforts with accurate assessments of both activity and stability.

Keywords; biocatalysis, protein immobilization, advanced materials, packed-bed reactors, flow-chemistry

INTRODUCTION

Modern chemistry demands more sustainable, efficient and environmentally friendly manufacturing schemes that contribute to mitigate the harmful impact of our economic development on climate. Biocatalysis and flow-synthesis are two fundamental areas to meet that goal. In the last decade, there is an exciting wave of utilizing new enzymatic reactions in flow tubular reactors for the continuous synthesis of fine chemicals at the bench scale [1,2]. Nevertheless, the transition to industrial implementation will require facing new challenges at the level of both biocatalyst and reactor development.

Enzyme immobilization is certainly the key enabling technology to push biocatalysis into flow chemistry. Immobilization methods aim at either fabricating heterogeneous biocatalysts suitable to be integrated into packed-bed reactors (PBRs) or functionalizing the walls of microreactors to make chemistry under microfluidics environments [3–5]. Besides the reactor format, the catalytic activity and operational stability of the immobilized enzymes must be maximized (**Figure 1**). Ultimately, the implementation of the immobilized biocatalysts in flow needs to transcend the trial-and-error approaches and advance towards more rational designs that fulfill the process requirements [2].

The immobilization itself enables the use of enzymes for continuous processes but does not guarantee high conversions during long operational times, since not all immobilization protocols render highly active and stable enzymes [6,7]. High volumetric activity and operational stability can only be attained through controlling the immobilization process both at molecular level (interface between the enzyme and the

solid material), and system level (interface between the biocatalytic material and the reactor configuration) (**Figure 2)**. Recently, Paradisi, Weiss and Turner groups have revised the state of the art of flow biocatalysis using immobilized enzymes, listing a great number of examples including both single-step and step-wise biotransformations [1,2,5,8]. Complementary to these reviews, our aim is to discuss the requirements for fabricating heterogeneous biocatalysts to be applied in flow and accordingly analyze the data reported in the most relevant and recent works in the field.

In this review, we dissect the innovative elementary aspects of the enzyme immobilization science for flow applications and address them through standard quantitative metrics. To evaluate the impact of the immobilization on flow-biocatalysis, we need to quantify the enzyme load in the solid phase, recovered activity upon the immobilization, the space-yield time (*STY*), specific productivity (*SP*) and the turnover number (*TN*) along the operation time (**Figure 1)**. Having these numbers in hand, we will assess the potential of most recent proof of concepts to meet the industrial demands (up to 10^5 $kg_{product}$ x $kg^{-1}_{immobilzed\ enzymes}$) [9].

Engineering activity at the solid interface.

The first aim of any immobilization methodology is incorporating enough active enzymes into the reactor volume. The maximum *STY* scales directly to the enzyme activity and, hence, the reaction time decreases reciprocally. For comparative purposes, the STY values must be accompanied by the flow rate, the substrate concentration and the reactor volume (enzyme packed-bed) to assess both catalytic activity and net yield (conversion) of flow-reactors operated by immobilized enzymes. Hence, the STY itself informs neither about the degree of conversion, the isolated yield nor the specific

enzyme activity packed in the reactor. The latter is a consequence of both the quantity of protein and the immobilized specific activity. Thus, assessing the enzyme effectiveness in flow-reactors demands to calculate the protein loading (mg x $g_{carrier}^{-1}$) and the relative recovered activity of the immobilized enzyme (**Figure 1**). Protein load relates mainly to carrier materials, whereas the specific activity depends on the protein-material surface interface.

Among the different strategies to engineer *surface-protein binding* (**Figure 2A**), reversible interactions (i.e. physical adsorptions) often preserve the activity of the immobilized enzymes [10,11], yet the protein orientation is hardly controlled and the enzyme leaching is plausible. To strengthen the binding and direct the immobilization, enzymes have been genetically fused to both binding modules and peptide tags. Through this strategy, both purification and immobilization are accomplished in just one-step [12–15], easing the reactor preparation by directly using protein crude extracts. Using protein-based cationic modules (Z-basic) and solid materials functionalized with anionic groups, uniform surface coverage and high immobilized specific activity have been achieved for phosphorylation, glycosylations and transamination reactions operated in both wall-coated reactors and PBRs [11, 12,16,17]. Likewise, immobilization of His-tagged enzymes on porous particles functionalized with metal chelates yield both high loads (>100 mg/g) and catalytic effectiveness (>50%), resulting in highly productive PBRs (335 g^{-1} x L x h^{-1}) [18]. However, implementing this immobilization chemistry in wall-coated reactors faces difficulties during the material functionalization [13]. Contrarily, irreversible immobilization can guarantee stable binding without enzyme leaching, although recyclability of the reactor/material might be problematic. Orthogonal or self-immobilizing techniques using Spy, Halo and streptavidin protein motifs**,** and formylglycine-generating enzymes have been used in

microfluidic bed reactors or in wall-coated reactors [19–23]. HaloTag™ technology based on halogenases domains promotes a quick irreversible immobilization on porous beads that can be readily integrated into PBRs, with 40-65 % recovered activity and *STY*s of 1.58 g^{-1} x L^{-1} x h^{-1}[14,15].

Material engineering (**Figure 2B**) aims at increasing the available surface for immobilization, which depends on both materials properties and reactor configuration. Hence, the internal surface of the packed material, the surface area generated during the monoliths manufacturing, and the inner area of microfluidic tubular reactors are decisive parameters for the enzyme loading. Most recent examples rest in the translation from batch reactors to PBRs using medium mesoporous or macroporous particles of diverse nature, such as cross-linked agarose, cross-linked polyacrylic polymers, and silica [18,24–28]. The combination of medium-high protein loadings (10-100 mg/g), and dense packing into PBRs leads to a high catalyst concentration, and hence high *STY*. For example, the immobilization of an acyl transferase into hydrophilic agarose carriers containing 1-10 mg x $g_{carrier}^{-1}$ displays 73 % of immobilization yield and 30 % of recovered activity leading to an unprecedented 5 min reaction time at high conversion and *STY* (106.25 g^{-1} x L^{-1} x h^{-1}) [24]. Similarly, a transaminase is also immobilized on agarose carrier through covalent attachments (5 mg x $g_{carrier}^{-1}$) with a recovered activity of 30 %, enabling the continuous oxidation of amines to aldehydes with *STY* of 0.91 g^{-1} x L^{-1} x h^{-1}. The high local enzyme concentration within PBRs and the adequate mass transfer of the substrates explain the short residence times. [25]. Xylanase was immobilized on methacrylic polymer-based supports activated with glyoxyl groups, achieving high protein loads (10-40 mg x $g_{carrier}^{-1}$). The high activity into PBR (*STY*: 3453 g^{-1} x L^{-1} x h^{-1}) decreased the reaction time for the

oligosaccharides production, although the enzyme activity depended on protein loading due to mass transfer resistances [26]. High *STY* enables to use short residence times (high flow rates), which might originate practical problems (e.g. high pressure drops). In this regard, fabrication of new materials, as hollow silica microspheres, allows achieving high protein loads and high flow rates without suffering pressure drops [29]. Advances in exploiting the architecture of porous materials can harness the application and boost the flow implementation of complex biosystems (e.g. cofactor dependent multi-enzyme cascades). For example, the co-immobilization of phosphorylated cofactors (PLP, FAD^+, and NAD^+) and enzymes onto the same solid material allows operating without exogenous addition of cofactors during continuous flow [22,30,31].

Enhancing the practical use of intensified enzymatic reactors is also being assisted by *reactor engineering* that includes new reactor concepts and fabrication technologies. 3D printed reactors [32] and groove-typed channel microreactors have been tailored to increase the loading capacity [33]. In wall-coated reactors the *STY* scales reciprocally with the diameter of the channel. Consequently high volumetric activities can be reached with high surface-to-volume ratio at the microfluidic scale, enabling the operation under a kinetic control regime in the absence of diffusion limitations (**Figure 2C**). These features can be exploited as a tool to determine the intrinsic kinetic parameters of the immobilized enzymes [34] or to optimize both conversion and *STY*s aided by timescale analysis [35] and mathematical modeling [16]. Studies comparing the effect of reactor format on *STY* are scarce but useful [36]. In an illustrative example, a comparison between different laccase/reactor formats revealed that the catechol oxidation was more efficient when the enzyme was immobilized on the surface of microchannels [37]. The immobilization into the inner surface of wall-coated reactors

usually aims at forming uniform monolayers by directed immobilization [12,16,17] or by the controlled formation of thin films [38,39]. However, when the inner reactor area is insufficient, surface coating with nanomaterials (i.e nanoparticles, nanosprings, nanotubes…)[40–43] and polymers [44] increases the enzyme loadings by 10-15 fold.

Finally, the monolithic reactors can combine the goodness of PBRs and wall-coated microreactors since inner porous surface is created for enzyme immobilization but structured channels are developed to order the flow. Silica monoliths enable high loading (92 % yield, up to 80 mg x $g_{carrier}^{-1}$) and are suitable to work under high flow rate at low backpressure, obtaining high *STYs* (up to 1229 g h^{-1} L^{-1}) [45,46]. For instance, macrocellular silica monoliths prepared by a sol-gel method based on emulsion templating [47,48] have been used for the adsorption and covalent grafting of transaminases (16 % activity recovered). Besides silica, monoliths can be also formed with biopolymers [49] like agarose [50,51]**.** Through this approach, several thermostable enzymes have been entrapped, recovering 80-90 % activity upon the immobilization process. Alternatively, carrier-free immobilization has been proven very effective to achieve high enzyme loadings. Herein, the protein aggregation can be genetically programmed by protein domains fused to the enzymes, in order to trigger the self-assembly of a 3D gel network within microreactors [19–21]. Such packed-bed retains several dehydrogenases and NADH to continuously asymmetrically reduce prochiral ketones [20].

Engineering stability at the solid interface.

Enzymes normally fail to fulfill the robustness requirements demanded by the implementation of intensified continuous processes. Immobilization has been the

classical solution to stabilize native enzymes, and lastly have been synergistically combined with protein engineering for adapting enzymes to work under non-physiological conditions. For example, Merck & Co and Codexis Inc. successfully immobilized several engineered enzymes (galactose oxidase and kinases) for the synthesis of the antiviral Islatravir, gaining control over the synthetic process and dramatically reducing the number of synthetic steps in comparison with the chemical synthesis [52]. Furthermore, immobilization techniques allow us stabilizing both native and engineered enzymes, and enable their integration into flow-reactors to intensify these valuable processes. As occurred for the activity, several aspects like the immobilization chemistry, the material properties and the reactor configuration need to be optimized to make robust heterogeneous biocatalysts (**Figure 2**). As a working metrics to illustrate the contribution of these aspects, we have calculated i) the specific enzyme productivity, which informs of the time-averaged efficient use of the enzyme, and ii) the turnover number (*TN*), that means the enzyme operational stability under operation conditions. It should be emphasized that *TN*s does not mean total turnover numbers, since the reported data rarely explore the operational limit given by enzyme inactivations (e.g. short times and low substrate concentration are used).

Engineering the *material-protein interface* is critical to stabilize immobilized enzymes (**Figure 2D**). The enzyme stability relies both on the reversibility and valency of the enzyme-carrier interaction. It is widely accepted [6,7] and lastly supported by single-molecule experiments [53], that irreversible multivalent attachments contribute to the structural stabilization of immobilized enzymes. Semproli et al demonstrated that one transaminase from *Vibrio fluvialis* immobilized through glyoxyl chemistry on agarose porous microbeads works 22 fold more efficiently in flow (5.35 $mg_{product}$ x mg_{enzyme}^{-1} x

h^{-1}) than in batch [54]. After operating the reactor at 0.2 mL x min-1 for 500 minutes, the enzyme *TN* was 19000, which demonstrates the high operational stability of the resulting heterogeneous biocatalyst. Using both the same carrier and immobilization chemistry, Contente et al reported an outstanding specific productivity of 960 $mg_{product}$ x mg_{enzyme} x h^{-1} for the synthesis of melatonin using irreversibly bound acyl transferase from *Mycobacterum smegmatis*. After 24 h of operation, the immobilized biocatalyst gave rise to a *TN* of 3.36 x 10^6[24]. This extremely high *TN* has even been overtaken by the xylanase from *Streptomyces halstedii* JM8 immobilized on metacrylate porous carriers activated with glyoxyl groups applied for the continuous synthesis of xylooligosaccharides (XOS). The stability of this heterogeneous biocatalyst was challenged in a PBR operating at extremely high flow rates (10 mL x min^{-1}), reporting a specific productivity of 3277 mg_{XOS} $^{-1}$ x mg_{enzyme} x h^{-1} for 120 h of operation, which meant an *TN* of 2.6 x 10^8 [26]. This latter value falls within that one reported for the glucose isomerase immobilized on carriers activated with glutaraldehyde, that are utilized for the fructose syrup industrial production [55]. Therefore, aldehyde chemistry reveals enormously efficient to achieve highly productive and stable heterogeneous biocatalysts for flow processes.

On the other hand, carriers activated with epoxides have been exploited to immobilize and apply an engineered transaminase from *Chromobacterium violaceum* (W60C) for the continuous kinetic resolution of amines. After testing different epoxide groups linkers, Abaházi *et al* discovered that the most hydrophilic bi-epoxides resulted in the most efficient heterogeneous biocatalysts with a specific productivity of 4.97 $mg_{product}$ x mg_{enzyme} $^{-1}$ x h^{-1} and an enzyme *TN* of 4265 after 60 minutes of operation [56]. Epoxy and aldehyde chemistries have also been exploited to graft monoliths for the

immobilization of transaminases and lyases, although their volumetric activity and operational stability were lower than those reported for the PBRs [45,48].

Enzyme stabilization through aldehydes and epoxides proceeds establish irreversible bonds that anchor the enzymes through the abundant ε-NH_2 of their surface Lys. These chemistry result in random immobilizations where predicting and controlling protein orientation is extremely difficult. Without renouncing to the stabilization, protein orientation can be controlled through a combination of directed immobilization and covalent multivalent attachment on heterofunctional carriers. These solid materials are functionalized with two types of reactive groups; affinity groups (i.e metal chelates) that drive the selective binding through the tags (i.e His-tag), and reactive groups (i.e epoxide, aldehydes) that establish irreversible covalent bonds mainly with the Lys residues surrounding the tag. The group of Paradisi has immobilized and stabilized His-tagged transaminases and alcohol dehydrogenases through this approach [28]. These heterogeneous biocatalysts were further integrated in PBRs for the continuous manufacturing of primary and secondary alcohols, obtaining more than 80% yield after up to 5 operation days ($TN_{transaminase}$ = 3960) [28].

Besides engineering the protein-material interface, the hydrophic/hydrophilic balance (*material engineering*) of the material surfaces have revealed fundamental to render highly stable immobilized enzymes when operating in anhydrous (or quasi-anhydrous) media, (**Figure 2E**). Bohmer et al., have immobilized different His-tagged transaminases on controlled pore silica functionalized with polymers of different polarity and conjugated with Fe^{3+} complexes for the site-selective enzyme binding [57]. The more hydrophilic surfaces rendered more stable enzymes when submitted to low

water contents using apolar solvent like toluene. Under continuous operation using neat toluene, this heterogeneous biocatalyst operates at 0.034 $mg_{product}$ x mg_{enzyme}^{-1} x h^{-1} during 5 days which corresponds to a *TN* of 1000, a significantly lower value than the ones observed for transaminases operating under aqueous/buffer conditions [18].

Macroscopically, the stability of the enzymes involved in continuous processes can also be maximized through *reactor engineering* (**Figure 2F**). This approach is frequently used when synthetic cascades, often chemo-enzymatic cascades, meet stability incompatibilities between the different catalysts required for each reaction step [58]. In this context, the spatial segregation of (bio)catalysts is mandatory. This challenge is normally faced by connecting two reactors in-line but operating them at different reaction conditions. As example, Grabner et al. connected a PBR containing a phenolic acid decarboxylase from *Bacillus megaterium* entrapped on alginate beads to perform the decarboxylation reaction at 30º C with a second PBR loading a Pd-heterogeneous catalyst to sequentially catalyze the Heck reaction at 85º C [59]. Besides increasing the temperature of the second reactor, the reaction media needed to be reformulated between the two PBRs to sequentially perform these steps in flow. Under this configuration, the immobilized decarboxylases presented an specific productivity of 0.12 $mg_{product}$ x mg_{enzyme}^{-1} x h^{-1} during 23 hours, which meant a *TN* for the enzyme of 103, much lower than the overserved for other enzymes immobilized through irreversible bonds on pre-existing carriers. Compartmentalizing the two catalysts in two PBRs, the incompatible chemo-enzymatic cascade was feasible.

Conclusion

The use of immobilized enzymes in flow requires engineering the protein-carrier interface, the material and the reactor configuration to maximize the productivity and the robustness of the heterogeneous biocatalysts under continuous operations. In this review, we identify the protein loading, the recovered activity, *STY* and enzyme turnover number as the relevant metrics to assess the operational specific productivity and stability of immobilized enzymes. We remark that each value by itself is unable to inform about both effectiveness and robustness of one immobilized enzyme packed into a plug-flow reactor.

From the most recent advances in flow-biocatalysis using immobilized enzymes, we have extracted, and even calculated for the most encrypted cases, the abovementioned metrics. These data were mapped in an industrial landscape using *STY* of 100 g x L^{-1} x h^{-1} and *TN* of 10^5 as borders. In this 2D-map, we find a positive correlation between the natural logarithms of *STY* and *TN* turnover, regardless the type of enzyme, immobilization and reactor design (**Figure 3**). Based on these numbers, we conclude that engineering activity and stability tends to follow a parallel path, giving rise to cases that mainly occupy low/low and high/high activity/stability windows. Contrarily, we also find some few cases where the enzyme immobilization improves the reactor SY (activity) to higher extent than the enzyme TN (stability) - example ***k*** [45] - or *vice versa* - example ***o*** [27]. Nevertheless, the realistic evaluation of the operational stability (total turnover number) will require further studies that bring enzymes to their inactivation limits. So far, this metric analysis must be fed with much more experimental data involving all the enzymes types to consolidate the trend suggested by **Figure 3**. Therefore, more rational and predictable designs to fabricate more

reproducible heterogeneous biocatalyst must come along with an accurate parameterization following the metrics herein proposed. We envision that new biocatalytic reactions under non-physiological conditions will pose new challenges to develop efficient and robust heterogeneous biocatalyst.

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Annotations

• 15 Covalent directed immobilization by using the HaloTag™ technology promotes a quick irreversible one step purification-immobilization on porous beads that can be readily integrated into PBRs, with 40-65 % recovered activity. The approach allows reaching high *STYs*.

• 20. Biopolymers-based monoliths are formed by genetically programmed protein aggregation. A 3D gel network is developed where the *STY* is maximized due to the high enzyme loading achieved

• 24. Immobilization of a versatile acylase on agarose beads activated with glyoxyl groups. This heterogeneous biocatalyst integrated into a PBR produces melatonin at multi-gram scale in 5 minutes residence time.

•• 26. A xylanase immobilized on methacrylate beads activated with glyxoyl groups and integrated into PBRs operates the flow-synthesis of xylooligosacharides at 10 mL x min-1 flow rate with the highest productivity ever reported to the best of our knowledge.

•• 45. Silica monoliths enable high loading (92 % yield, up to 80 mg x $g_{carrier}^{-1}$) and are suitable to work under high flow at low backpressure at high *STY* (1229 g x h^{-1} x L^{-1}). Protein-surface chemistry can be adapted to improve the recovered activity.

• 58. His-tagged transaminases immobilized on hydrophilic porous glass particles activated with metal chelates operate under quasi-anhydrous conditions for 5 days at low productivities though.

Figures

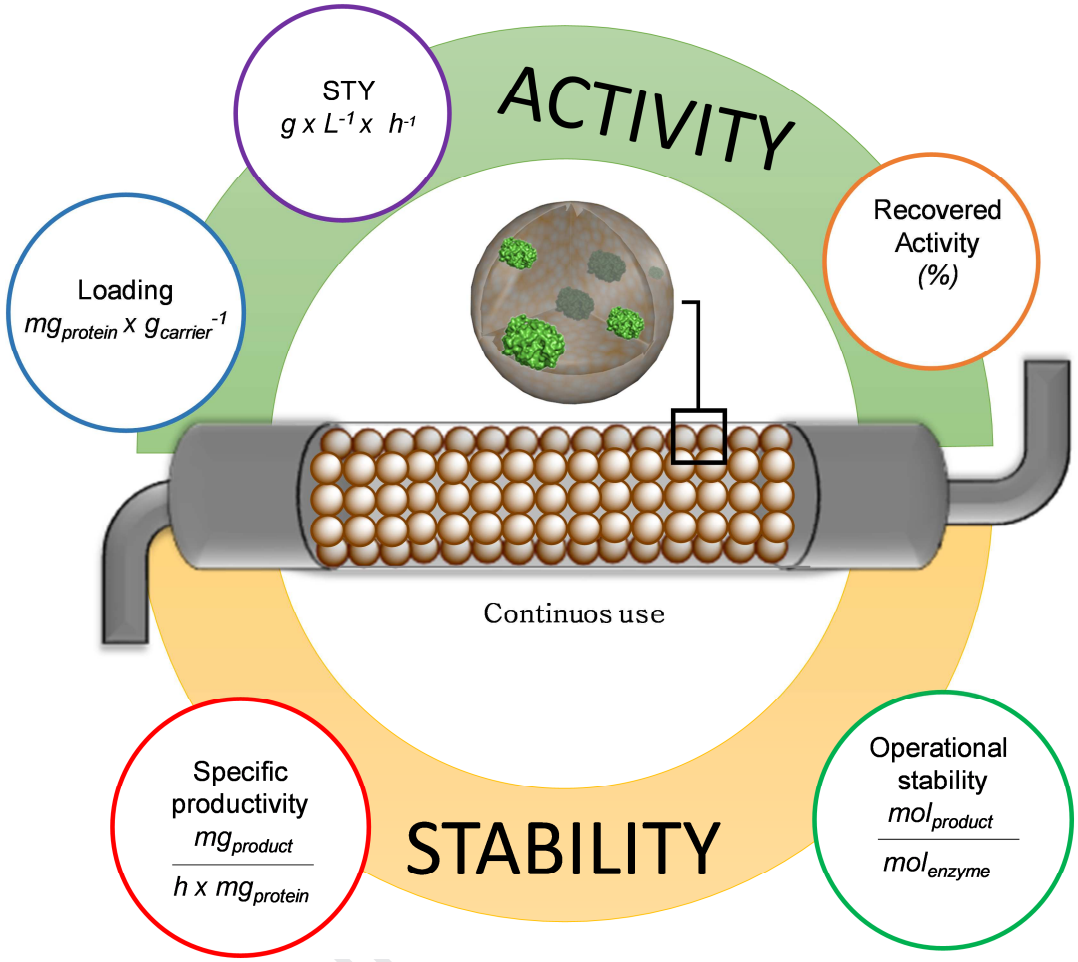

Figure 1: Most relevant metrics to assess the activity and the stability of immobilized enzymes under continuous operation. The recovered activity is defined as the percent value of the immobilized specific activity (U x $mg_{protein}^{-1}$) regarding its soluble counterpart.

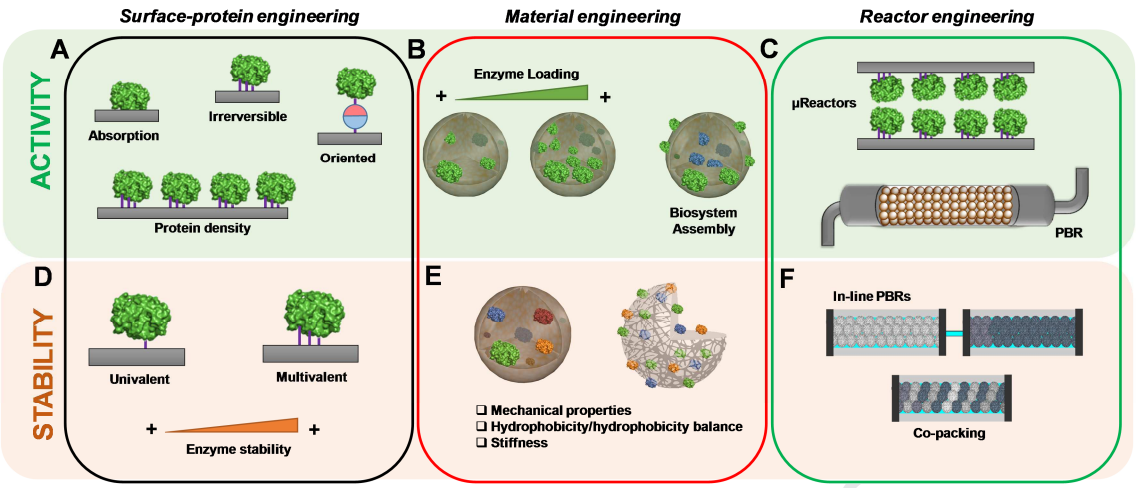

Figure 2: Fundamental aspects to be considered when engineering an immobilized enzyme for flow applications. Properties and strategies to engineer the activity (top green panels) and the stability (bottom orange panels) of the immobilized enzymes, related to the following aspects; protein-carrier interface (A and D), material properties (B and E), and reactor configuration (C and F)

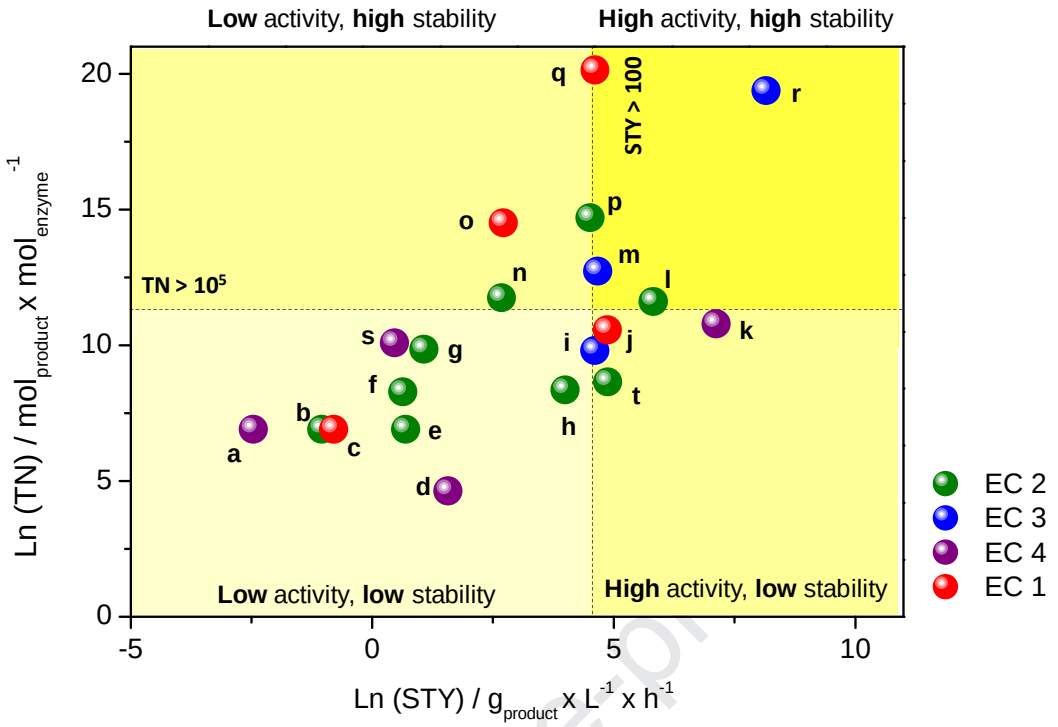

Figure 3. 2D-Map of metrics that define the productivity and operational stability of immobilized enzymes in continuous operations. This graph plots the natural logarithms of the corresponding *STY* and *TN* values calculated for the most recent and relevant papers that describe the use of immobilized enzymes in flow. Different enzyme families are represented with different colors. Data occupy different windows depending on the productivity and operational stability of each immobilized enzyme. 100 g x L^{-1} x h^{-1} and *TN* of 10^5 were set as windows borders. Each letter corresponds to the literature references as follows; a (22); b (22); c (22); d(59); e (57); f (28); g (54); h (56); i (46) j (11); k (45); l (18); m (24); n (17); o (27); p (12); q (37); r (26); s (14); t (41).

Declaration of interests

X 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: