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***In-situ* Enzyme Immobilization by Covalent Organic Frameworks**

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Abstract: Enzyme immobilization is a widely reported manner to favor the applicability of enzyme by enhancing their stability and re-usability. Among the various existing solid-supports and immobilization strategies, the *in-situ* encapsulation of enzyme within crystalline porous matrices is a powerful tool to design biohybrid with a stable and protected catalytic activity. However, to date, only a few metal-organic frameworks (MOFs) and hydrogen bonded organic frameworks (HOFs) have been reported. Excitingly, for the first time, Y. Chen and coworkers expanded the *in-situ* bio-encapsulation to a new class of crystalline porous material, namely covalent organic frameworks (COFs). The enzyme@COF materials not only exhibited high enzyme loading with minimal leaching, high catalytic activity and selectivity, chemical and long-term stability and recyclability but could also be scaled up to a few grams. Undoubtedly, this works opens striking new opportunities for enzymatic immobilization and will stimulate new research on COF based matrix.

Enzymes are highly active natural catalysts that display high catalytic activity often coupled with chemo-, regio- or stereo-selectivity under ambient pressure and temperature. These properties have continuously placed enzymes as particularly appealing catalysts in a vast array of scientific domains ranging from synthetic chemistry, to environmental and biomedical applications. Despite their intrinsic qualities as catalyst, free enzymes are fragile entities that can be easily denatured, inducing a loss of their activity, and are difficult to handle, limiting their applications. These drawbacks can be mitigated by their immobilization onto/into solid supports.^[1] For biocatalysis purposes, the support needs to allow high catalytic activity and selectivity, improve chemical and long-term stability, and provide re-usability of the system. To maintain a high catalytic activity, it is necessary to achieve high enzyme loading with minimal leaching while allowing the diffusion of reactants and products to and from the enzyme active site. Among the immobilization strategies, encapsulation, i.e. inclusion of the biomolecular entities within the host matrix (as opposed to surface immobilization strategies), is particularly interesting for enzyme stabilization and protection as it can create a convenient micro-environment in their surroundings and/or mechanically prevent their degradation.^[2] It also enables 3D accessibility

to the enzyme active site compared to the 2D access of surface immobilization. Two main strategies exist for encapsulation. It can be performed by direct inclusion, i.e. the spontaneous diffusion of enzymes into preformed porous matrices (such as mesoporous (alumino)silica,^[3] cationic clay minerals,^[4] and more recently metal-organic frameworks (MOFs)^[5] and covalent organic frameworks (COFs)^[6]). This method offers a very simple loading process, and interesting properties have been demonstrated in terms of catalytic activity (enzyme accessibility, stability, recyclability,..) and of enzyme stabilisation under non-natural conditions, such as organic solvent.^[7] This approach requires a size matching between the pores of the host matrix and the enzyme, and afterwards to preserve voids within the matrix to permit diffusion of reactants to and from the biological entity.^[8] Another strategy is the *in-situ* encapsulation, where the matrix formation occurs simultaneously with the immobilization process. While this method overcomes the size limitation issue, it comes with a fundamental synthetic challenge, as host matrix formation needs to occur in mild enough conditions to avoid degrading the fragile enzymes (i.e. aqueous media, reasonable pH, low temperature....). This strongly limits the material that can be used, historically mainly amorphous material such as biopolymers, polyelectrolytes matrices obtained by layer-by-layer process or sol-gels processed materials.^[9] Recently, MOFs^[10] and HOFs^[11] have appeared as exciting new family of materials for *in-situ* encapsulation of bio-entities, due to the precise control over both the chemistry and the porous network structure. However, the harsh synthetic conditions required for the elaboration of these porous solids significantly hamper the number of reported hosts. Among the porous crystalline solids, COFs would also be highly attractive host matrices due to their tuneable organic structure with easy functionalization and adjustable hydrophobic/hydrophilic balance, their adjustable pore-size network in the mesoporous range and the absence of metal elements which may be desirable for some (bio)applications.^[12] Although, promising results were indeed obtained with COF based bio-immobilization matrices, only matrices for direct inclusion, surface immobilization or obtained through protective templating have been reported, due to the typically harsh synthetic conditions (pressure, high temperature, acid conditions, organic solvent).^[13]

The newly reported results by Y. Chen and coworkers are exciting news for the domain of bio-encapsulation.^[14] The researchers reported the *in-situ* encapsulation of proteins within COF networks (Figure 1). Using mild synthesis conditions (room temperature, aqueous media), a COF, named NKCOF-98, was constructed using the hydrazide-aldehyde coupling reaction between two precursors monomers, (2,5-bis(2-methoxyethoxy)terephthalohydrazide (BMTH) and 1,3,5-triformylbenzene (TB)), in slightly acidic conditions (acid acetic 1 vol%).

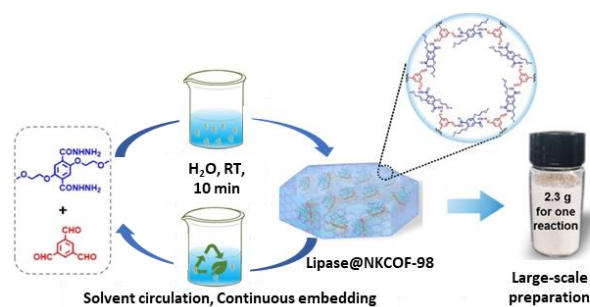


Figure 1. Schematic representation of the *in-situ* assembly of lipase@NKCOF-98 developed by Y. Zheng et al. Reproduced with permission.^[14] Copyright 2022, John Wiley and Sons.

The direct construction of an hydrazone-linked COF in such mild conditions is particularly thrilling, owing to the enhanced chemical stability conferred by these bonds to the framework.^[15]

Y. Zheng et al demonstrated that the *in-situ* formed NKCOF-98 matrix was indeed an exceptional bio-immobilization matrix, first for a lipase but also for various proteins, with the following advantages.

1) High loading and minimal leaching. The obtained materials exhibited high lipase loading (0.74 g/g), homogeneously distributed throughout NKCOF-98 particles. In agreement with the encapsulation strategy, enzyme leaching was minimal, and below values reported for materials prepared by adsorption. Interestingly, the authors showed that during the synthesis, the amino groups of lipase were binding with some aldehyde groups from the COFs network, which may result in the formation of defects within the COF framework. Such defects could contribute to the embedding of lipase within the NKCOF-98 particles and, consequently, to the stabilization of the lipase@NKCOF-98 material.

2) High catalytic activity. A key aspect when performing *in-situ* bio-immobilization is to avoid enzyme denaturation and to preserve the enzymes' conformation and catalytic activity. In this work, the immobilization process was not detrimental to the lipase conformation as the immobilized lipase exhibited a high catalytic activity, the authors showing that for the hydrolysis of 4-nitrophenyl acetate, the activity was comparable with that of the free enzyme. Also, and importantly, the enantioselective resolution activity of immobilized lipase remained unaltered and was similar to that of free lipase toward chiral resolution of 1-phenylethanol reaction and other chiral alcohols.

3) Chemical and long-term stability. The COF platform also displayed the desired protection and stabilization properties of an immobilization matrix. The storage stability as dry powder or in aqueous media of the immobilized lipase was much improved compared to free enzyme. The

matrix also provided protection against denaturing conditions (citric acid, 100 °C and surfactant).

4) Recyclability. The system allowed for a high recyclability (less than 25 % loss of activity after 15 cycles).

5) Various proteins immobilized. The system could be extended to other proteins with different size and isoelectronic points (Bovine Serum Albumin, Glucose Oxidase and Cytochrome C). The assembly process does not seem to be driven by electrostatic interactions between the proteins and the precursors, allowing encapsulating a wide range of proteins. However, the exact mechanism of formation remains to be elucidated

6) Tuneability of the chemical composition of the COF matrix. Interestingly, the possibility to tune the nature of the COF matrix was possible as the method was extended to other related COF bearing hydrophobic or hydrophilic groups and to azine COF (NKCOF-99).

7) Scale-up. For potential industrial applications, the need for synthesis easily scaled-up is mandatory. Excitingly, the lipase@NKCOF-98/99 could be scaled up to few grams in 30 min or less (2.3 g for lipase@NKCOF-98).

8) Added value compared to other matrices. The relevance of the *in-situ* COF immobilization platform was further demonstrated by comparison with lipase immobilized by adsorption on a COF, within the pores of traditional silica (MCM-41) or with a MOF formed *in-situ* (ZIF-8). All of which exhibited lower catalytic activity and protection effect. This highlights the benefit of the *in-situ* strategy and the significance of the chemical stability of the system. Compared to *in-situ* ZIF-8, it also suggests the importance of matrices with larger pores, that may favored the reactants diffusion.

The reported *in-situ* COF immobilization platform fulfills all of the compulsory requirements to act as an efficient immobilization matrix, as it allows for high catalytic activity and selectivity, provides chemical and long-term stability, and re-usability to the system. Thanks to the inherent properties of COFs, this study provides new exciting opportunities for enzymatic immobilization. The ease of functionalization allowing for a fine-tuning of the hydrophobic/hydrophilic balance of the host matrix opens perspectives for “on demand” design of enzyme/COF couples with a maximized bio-catalytic efficiency. COF matrices that could act beyond a simple host support but also contribute to the catalytic properties, as previously demonstrated with MOFs,^[16] should lead to unprecedented biocatalyst. While challenges lay ahead to encapsulate more labile bio-entities (more fragile enzyme, nucleic acids, cells...), this work will undoubtedly act as a stepping stone and stimulate a whole new field in COF based research.

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Keywords: porous solid • covalent organic frameworks • enzyme • bio-immobilization • biocatalysis

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