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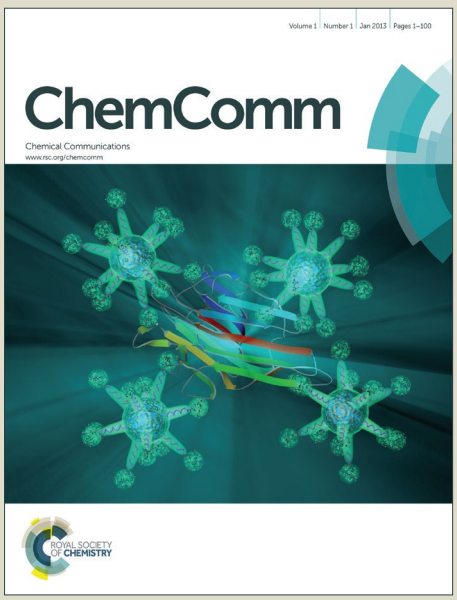





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# Enzyme encapsulation in zeolitic imidazolate frameworks: a comparison between controlled co-precipitation and biomimetic mineralisation

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**Recent studies have demonstrated that metal-organic frameworks can be employed as protective coatings for enzymes. Two efficient strategies have been reported for the synthesis of such composite materials: biomimetic mineralisation and controlled co-precipitation using polyvynilpryrrolidone. We assesed the relative efficacy of each approach by comparing the thermal stability of encapsulated urease. The resulting data shows that over a range of temperatures biomimetic mineralisation offers superior protection than the co-preciptation method.**

Enzymes are a class of proteins that are well known for catalysing a wide range of reactions in both biological and chemical systems.[1,2] Compared to synthetic catalysts, enzymes predominantly operate under mild conditions, are often highly selective (i.e. chemo-, regio-, stereo-), and afford minimal by-product generation.[3,4] As a result, enzymes are being adopted in the pharmaceutical, food, chemical, and agricultural industries.[5–8] However, a major limitation of enzymes is that their structural instability significantly restricts their potential for widespread industrial application.[9–11] One strategy that aims to address this challenge is to encapsulate the enzyme within a protective shell that shields the biomacromolecule from the reaction medium.[12–17]

Recently, metal-organic frameworks (MOFs) have been explored as protective coatings for enzymes.[18–23] MOFs are constructed from organic and inorganic components using a modular synthetic approach,[24–28] and compared to other materials such as, mesoporous silica and calcium carbonate,[29–31] offer superior thermal and chemical protection for biomacromolecule cargo.[18] Typically, MOFs possess open architectures and large pore volumes[32] that can facilitate the selective transport of enzyme substrates through the porous network[18,33] while protecting the biomacromolecule from the external environment. Among the wide variety of potential MOF based 'containers',[18–23] zeolitic imidazolate frameworks (ZIFs) have been primarily studied due to their exceptional chemical and thermal stability and negligible cytotoxicity.[34–36] In addition, ZIFs can be grown under mild biocompatible conditions that allow for preservation of enzymatic activity.[18] Here, we compare the stability of urease encapsulated in ZIF-8 by two competing 'one-pot' strategies. The first employs a co-precipitation method where enzymes stabilized by a capping agent (i.e. polyvinylpyrrolidone, PVP) are introduced into a solution containing the ZIF precursors.[19,20,37]

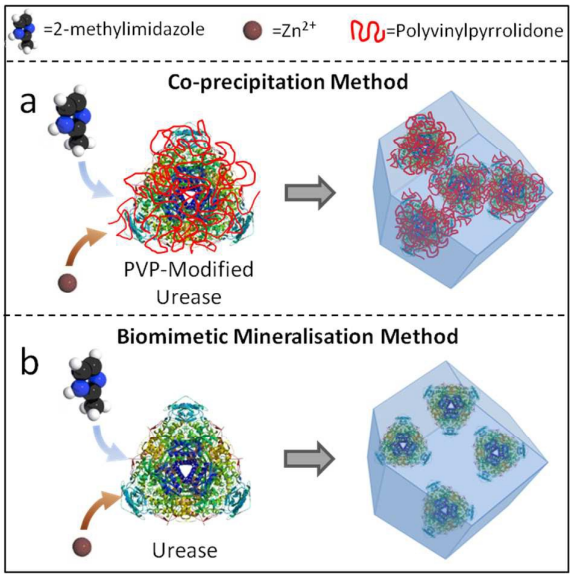


Fig. 1. Schematic showing the synthesis of urease-encapsulated ZIF-8 by a) co-precipitation in the presence of a capping agent (PVP), and b) biomimetic mineralisation method (spontaneous formation of the MOF on biomacromolecules).

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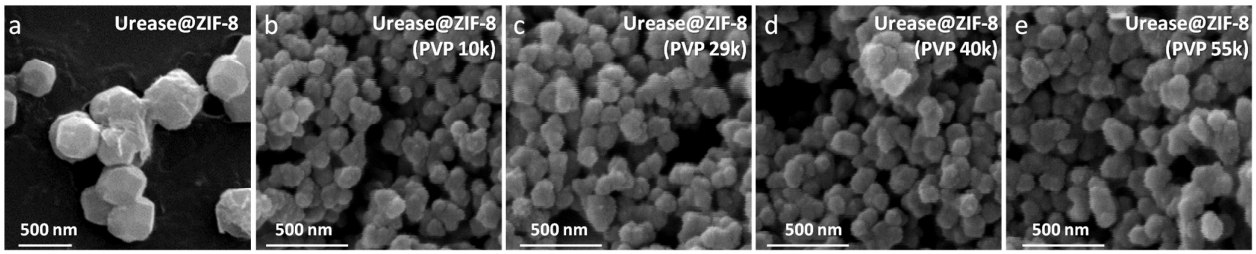


Fig. 2. a-e) SEM images of the urease@ZIF-8 crystals generated from the biomimetic mineralisation method (a) and the controlled co-precipitation method using PVP (b-e) with different molecular weight (10, 29, 40, and 55 kDa respectively).

The second approach, termed 'biomimetic mineralisation', is carried out in the absence of capping agents. In this case nucleation of the ZIF-8 precursors is triggered by biomacromolecules in an aqueous solution [18,38] resulting in a ZIF-8 protective shell (Fig. 1).[18] Given the similarity of these approaches we sought to ascertain if the capping agent plays a role in stabilizing the enzymes. Such knowledge is crucial to the development of this burgeoning area and will help accelerate the application of MOFs to a wide range of biotechnological applications.

Jack bean urease, a large, hexameric protein, with an approximate M.W. of 600 kDa, was selected as the model enzyme in this study due to its structural instability at temperatures exceeding 45 °C and its potential chemical and pharmaceutical applications.[39,40,41] The synthesis of ZIF-8 coated urease using a controlled co-precipitation method was performed by addition of $Zn(OAc)_2$(aq) to an aqueous solution containing 2-methylimidazole (HmIm), urease and the capping agent (PVP). In contrast, the biomimetic mineralisation of ZIF-8 by urease was performed by directly adding $Zn(OAc)_2$(aq) to an aqueous solution mixture of urease and HmIm. In each case the mixtures were incubated overnight at room temperature. The respective, crystalline, products were collected by centrifugation, washed three times in EtOH:$H_2O$ (1:1) and re-suspended in water. Scanning electron microscope (SEM) images were collected on each sample to assess the size and morphology of the ZIF-8 biocomposites and are shown in Fig 2. The average size of the biomimetically mineralised urease@ZIF-8 crystals is circa 500 nm (Fig. 2a) however, particles formed by the co-precipitation method are considerably smaller with an average size of 120 nm regardless of the molecular weight of the PVP capping agent that was employed (10, 29, 40 and 55 kDa, Fig. 2b-e). The reduced particle size can be attributed to the PVP capping agent which acts as an efficient MOF nucleating agent for the encapsulation of both inorganic and biological agents.[41,42] Small angle scattering (SAXS) experiments were performed to confirm the bulk structure and crystallinity of the respective biocomposites. Analysis of the data presented in Fig 3 shows a slight difference in the relative intensities of the peaks with respect to simulated ZIF-8 can be explained by the irregular morphology of the crystals (Fig. 2)

The original report of controlled co-precipitation initiated enzyme encapsulation employed 40 kDa PVP as the capping agent.[20] Accordingly, we compared this system to the biomimetic mineralisation approach. Thermal gravimetric analysis (TGA) (SI Fig. S1) performed on the urease@ZIF-8 biocomposites showed significant weight loss from 300 °C. In contrast pure ZIF-8 does not thermally decompose until above 500°C.[43] The reduced thermal stability of the urease@ZIF-8 biocomposite results from decomposition of the enzyme within the framework.[19,44] Fourier transform infrared spectroscopy (FT-IR) measurements of the urease@ZIF-8 particles further evidenced the co-presence of enzymes with ZIF-8 (SI Fig. S2). To quantify the amount of urease encapsulated within the ZIF-8 crystals we used fluorescein isothiocyanate (FITC) labelled urease as a fluorescent probe. Crystalline samples obtained via each method were completely dissolved using EDTA, and the fluorescence emission of the respective solutions were collected and compared to a standardised FITC-labelled urease emission calibration curve (SI Fig. S3 and Methods section). This protocol afforded a

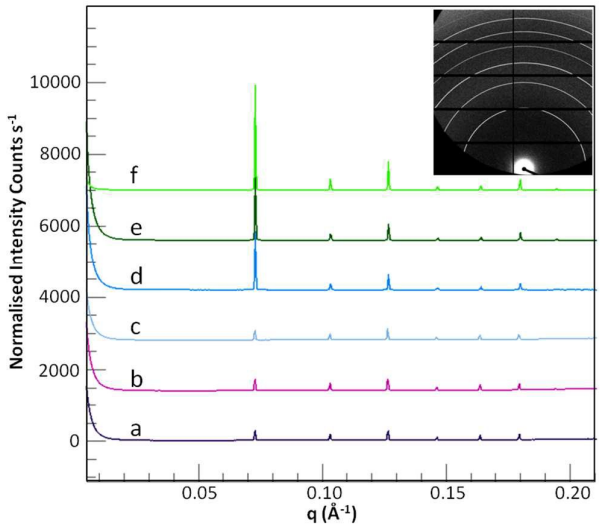


Fig. 3. Small-angle X-ray scattering (SAXS) diffraction patterns of urease@ZIF-8 prepared using controlled co-precipitation method using PVP (a) 10 , b) 29 , c) 40 , d) 55 kDa), and the biomimetic mineralisation method (e). Pure ZIF-8 pattern is also shown (f). Inset: 2D representation of SAXS patterns of the composite materials.

protein encapsulation efficiency that is independent of the amount of PVP or ZIF-8 in the biocomposites. Encapsulation of ca. 15% and 20% for particles synthesised using the PVP-capped co-precipitation and biomimetic mineralisation method, respectively. We note that the similar loadings suggest that PVP does not facilitate encapsulation.

The primary function of the ZIF coating is to protect the enzyme from inhospitable environments. Accordingly, we assessed the relative stability of the ZIF-8 encapsulated urease prepared via the two aforementioned methods. Initially, aqueous solutions containing the urease@ZIF-8 composite particles were incubated at various temperatures for 30 min to assess their thermal stability. We then performed a phenol red assay for urease[45] to evaluate the catalytic efficiency of the respective composites. In a typical assay, a solution of phenol red and urea were introduced into a sealed cuvette containing the urease@ZIF-8 composite particles. The absorbance of this mixture at 560 nm was then continuously monitored (SI Fig. S4) at room temperature. We note that the amount of biocomposite particles introduced into the assay was standardised against the enzyme encapsulation efficiency. As shown in Fig. 4, the initial reaction rate of the urease@ZIF-8 composites showed significant improvement over pristine urease. This enhancement can be attributed to the confinement of urease provided by ZIF-8 which prevents the enzyme from aggregating in solution.[44] Above 40 °C a rapid decrease in activity was seen for the biocomposite material prepared using the co-precipitation method; in contrast, the activity of the biomimetically mineralised enzymes remained relatively stable up until 60 °C before gradually decreasing. The enhanced stability of the enzymes encapsulated via the biomimetic mineralisation process is most likely due to the rigid ZIF-8 structure restricting the structural rearrangement at elevated temperatures that leads to denaturation.[45,46] Conversely PVP is known to undergo a structural rearrangement at mild temperatures (i.e. 35-65 °C)[47] that has the potential to disrupt the encased enzyme. This is further supported by data that describes unexpected physical behaviour for polymers in confined nanopores.[48] We acknowledge that there may be other contributing factors to the relative stability of the enzymes such as the disparity in crystal size. In addition, we calcined the composites at 325°C to thermally decompose the enzymes and then collected SEM images to assess the distribution of the vacant cavities throughout the crystals.[19] The distribution of cavities in Figure 5a suggests a preference of PVP-capped urease toward the surface region of the crystal. In contrast, the biomimetic mineralisation approach (Fig. 5b) gives rise to a homogeneous distribution of cavities throughout the sample. From analysis of this data we conclude that the co-precipitation method is more likely to expose the enzymes to the external environment. Indeed these hypotheses are consistent with our data that shows the activity of urease@ZIF-8 prepared by the co-precipitation method rapidly drops below that of free urease above 40 °C (Fig. 4).

We also measured the *in situ* enzymatic rate of reaction when the urease@ZIF-8 composites were added to a

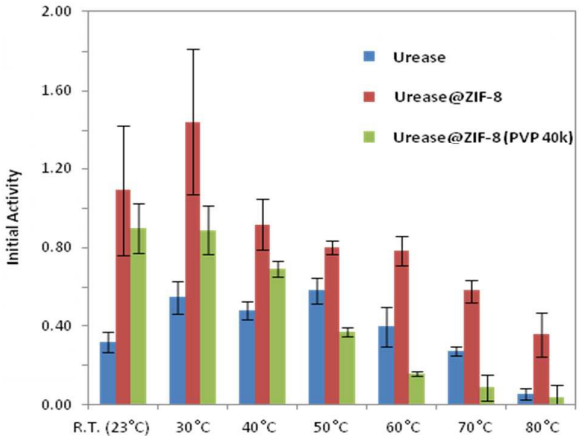


Fig. 4. Initial activity of urease and urease@ZIF-8, prepared using the biomimetic mineralisation method and the controlled co-precipitation method using PVP, after being heated for 30 min at the listed temperatures.

preheated assay at RT (23°C), 30, 40, 50, 60 and 70 °C (SI Fig. S5-6). Both the free urease and urease@ZIF-8 exhibited enhanced activity across all temperatures (SI Fig. S6). The biocomposites prepared by co-precipitation displayed slightly higher levels of activity which can be accounted for by their smaller crystal size;[49] 120 nm compared to 500 nm and their surface distribution allowing for more expeditious diffusion of the reactants and products through the structure.[19]

In summary, we have compared two strategies for the protection of biomacromolecules using metal-organic framework materials: biomimetic mineralisation and

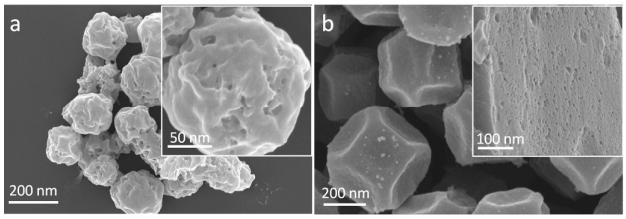


Fig. 5. SEM images of urease@ZIF-8 crystals after calcination at 325°C, revealing cavities induced by urease encapsulation. (a) Controlled co-precipitation method using PVP (inset, zoom-in at surface; (b) Biomimetic mineralisation method (inset, zoom-in at cross-section).

controlled co-precipitation using PVP as a capping agent. Both approaches exhibited comparable encapsulation efficiencies suggesting that, in aqueous solutions, PVP does not play a role in enhancing biomacromolecule loading. Additionally, we directly compared the stabilities of the urease@ZIF-8 biocomposites over a range of temperatures and our data shows that the biomimetic mineralisation technique extends the bioactive temperature range of urease compared to both the free enzyme and urease encapsulated via the controlled co-precipitation method. We envisage that this encapsulation protocol will enable the practical use of enzymes in conditions

suitable for the effective exploitation of biocatalysts for industrial applications.

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