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DOI
10.1039/c6ra19976c

Publication date
2016

Document Version
Final published version

Published in
RSC Advances

RSC Advances

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2016, **6**, 108051

Received 7th August 2016
Accepted 3rd November 2016

DOI: 10.1039/c6ra19976c

www.rsc.org/advances

Covalent immobilization of glucose oxidase on amino MOFs *via* post-synthetic modification†

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The post-synthetic modification (PSM) of two amino-MOFs with glucose oxidase is reported in this study. The multi-step approach preserved the MOFs' structure and allowed the production of enzyme-functionalized MOFs (MOFs@GOx), which retained the enzymatic activity and showed selective properties for glucose.

Enzymes are biological catalysts with applications in various industrial processes due to their high selectivity, specificity and activity under mild conditions.[1] However, immobilization of enzymes on a surface is still among the most challenging aspects hampering further use of these biomolecules in industrial biocatalysis,[2] biosensing,[3] and biomedical devices.[4]

With the purpose of enhancing enzyme stability as well as to facilitate separation and recovery while maintaining activity and selectivity, enzymes have been immobilized on different supports.[9] Conventional solid supports include ionic liquid microparticles,[5] silica gel,[6] hydrogels,[7] and nanoporous inorganic materials.[8] In this perspective, Metal Organic Frameworks (MOFs) which are self-assembled from organic ligands and metal ions,[10] are very interesting materials due to their large and accessible specific surface areas, uniform and tunable pore sizes, and the great potential to incorporate a wide range of chemical functionalities.[11] Indeed, MOFs appear as promising host matrix candidates for biomacromolecules.[12] The incorporation of some micro-enzymes such as MP-11 [13] inside the cavities of mesoporous MOFs was reported and this strategy was enlarged to enzymes,[14] whose molecular dimension exceeds the accessible pore size; however, this method may be limited to a few enzymes and other immobilization strategies are needed.

In order to covalently attach an enzyme on the surface of different MOFs, we employed a post-synthetic modification (PSM) approach.[15] In this study, we report a PSM on two different amino dicarboxylate MOFs (NH_2-MOF) (NH_2-MIL53(Al) and NH_2-MIL101(Cr)). This universal method allows the attachment of enzymes to the surface of the MOFs while maintaining the MOFs structure and the enzyme's activity. As a model enzyme, we employed glucose oxidase (GOx) due to its low cost, stability, and practical use.[16] GOx immobilization on ZIFs (zeolitic imidazolate frameworks) and other MOFs, has been recently reported in the development of glucose biosensor.[17] Although, the non-covalent (including adsorption and entrapment) methods provide many advantages, since it prevents any leaching of the enzyme and greatly improves its stability, this method is limited by the pore size of MOFs. The covalent binding can provide an efficient way to attach not only enzyme but in general biomolecules on MOFs surface.

The developed multi-step synthetic route to obtain GOx functionalized NH_2-MOFs (MOF@GOx) is illustrated in Fig. 1. In order to covalently immobilize the enzyme on the MOF surface through the NH_2 groups of the enzyme, free carboxylic acids have to be introduced into the MOF structure.[18]

Therefore, both considered MOFs bearing the NH_2 moiety (Fig. 1a) reacted with glutar anhydride after synthesis to produce pendant carboxylic acids (COOH–MOF) (Fig. 1b). The activation of the carboxylic acid of COOH–MOFs *via* the formation of NHS ester (NHS–MOF) (Fig. 1c) was used to covalently couple the GOx (MOF@GOx) (Fig. 1d) through the formation of an amide bond. The detailed experimental procedure is described in the ESI.†

As revealed in the scanning electron microscope (SEM) images (Fig. S1, ESI†) the original particle size (~500 nm and ~100 nm for NH_2-MIL101(Cr) and NH_2-MIL53(Al)) does not change upon enzyme immobilization (Fig S2, ESI†). XRD

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† Electronic supplementary information (ESI) available: Synthesis and functionalization of MOFs, SEM, DLS, TGA, UV-Vis additional characterizations. See DOI: 10.1039/c6ra19976c

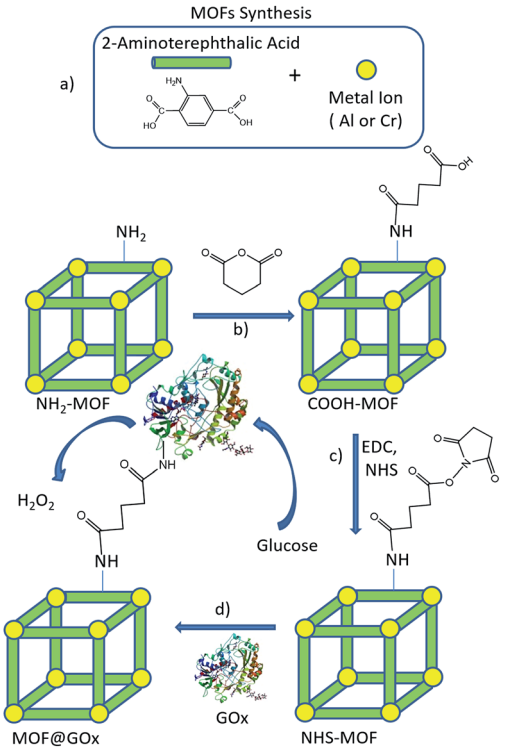

Fig. 1 Schematic synthesis showing the reaction steps for the preparation of GOx functionalized MOFs.

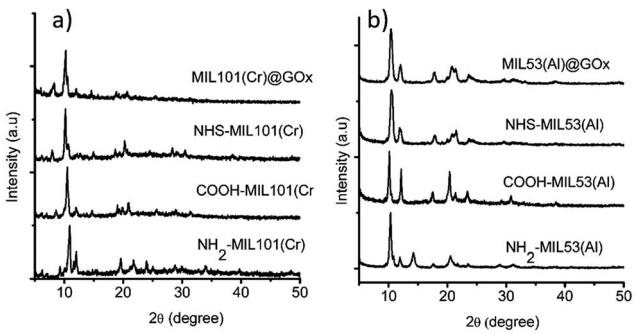

Fig. 2 XRD patterns of (a) MIL101(Cr) and (b) MIL53(Al) during the multi-step PSM reaction.

patterns of NH_2-MOFs are shown in Fig. 2, the crystal structures are comparable with those reported in literature.[19] The XRD patterns of the crystals during the several reaction PSM steps are also compared in Fig. 2 and show that the PSM occurs without degradation of the frameworks. The size of the nanocomposites was further confirmed by dynamic light scattering (DLS) (Fig. S3, ESI†), which is in good agreement with the results of the SEM images and indicates good dispersion of MOFs@GOx in phosphate buffer saline (PBS) (pH = 7.4).

Information on the PSM process was obtained through FT-IR measurements, Fig. 3 shows the comparison of FT-IR spectra before and after the enzyme attachment on the MOF in the range 3800–2500 cm^{-1}. The spectra of MIL53(Al)@GOx shows the appearance of intense bands due to the CH_2 symmetric and

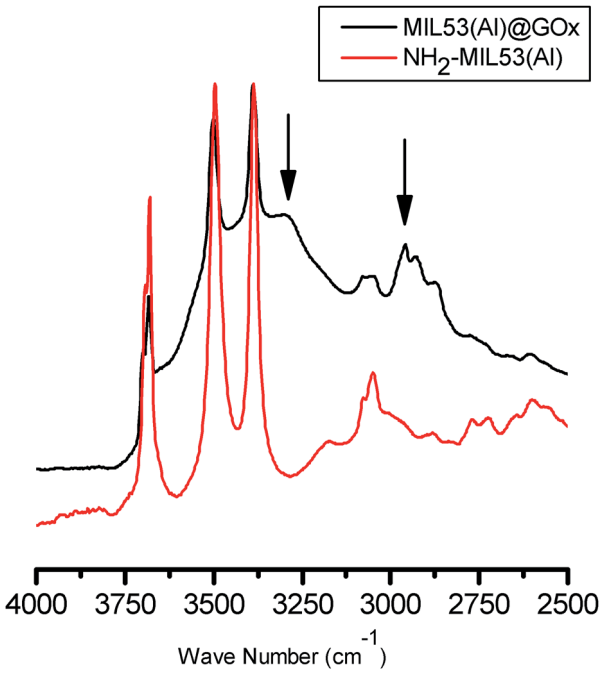

Fig. 3 FT-IR absorption spectra of NH_2-MIL53(Al) and MIL53(Al)@GOx.

antisymmetric stretches[20] at 2856 cm^{-1} and 2926 cm^{-1} and the presence of a new band at 3305 cm^{-1}, which can be assigned to amide stretch (GOx–CO–NH).[21] This evidence, combined with the absence of the same bands in the NH_2-MIL53(Al) spectra, confirms the grafting of the GOx on the surface.

The presence of different functional groups, such as $-NH_2$ and –COOH, and the successful attachment of the enzyme would result in the change of surface charges on MOFs after each reaction step. Therefore, zeta-potential (ζ) analysis was employed to further verify the covalent coupling of GOx on the surface.

Table 1 reports the ζ value after each reaction step measured in PBS (pH = 7.4). The NH_2-MOFs show positive ζ values due to both metal ion and the protonation of the amino groups. After the reaction with the glutar anhydride, the surface charge changed to negative due to the presence of the introduced carboxyl groups. After the reaction with GOx the ζ value further decreases because the GOx carries a net negative charge on its surface,[22] confirming the attachment of the enzyme on the MOFs surface.

Table 1 Zeta potential values of NH_2-MOFs, COOH–MOFs and MOFs@GOx

	ζ^a (mV)		
	NH_2	COOH	GOx
MIL53(Al)	14.2	−9.9	−21.9
MIL101(Cr)	32.5	−23.1	−41.0

[a] Measured in PBS (pH = 7.4).

Thermogravimetric analysis (TGA) of the samples (Fig. S4 and S5, ESI†) also confirms the presence of GOx on the surface. TGA exhibits similar pattern during the PSM steps and reveals no evident decrease of the thermal stability after PSM. As expected the total weight loss increases during the reaction steps. The first step up to roughly 200 °C for all considered materials is associated with loss of the guest molecules (water and organic solvents) from the cavities and unreacted reagents. The weight losses, starting at roughly 380 °C for all MOFs, mark the degradation of the framework. This degradation temperature in air compares well to the value reported in the literature.[19] The MOFs@GOx thermograms show a greater weight loss; a deeper drop in the range 300–400 °C compared to the parent material is attributed to the presence of GOx on the surface. The amount of GOx was estimated to be around 4.6 wt% for MIL53(Al)@GOx and 4.1 wt% for MIL101(Cr)@GOx.

The catalytic activity of MIL53(Al)@GOx and MIL101(Cr)@GOx was evaluated by spectrophotometric detection of the formed hydrogen peroxide during glucose oxidation using *o*-dianisidine as a chromogenic substrate. GOx converts glucose into gluconic acid and generates hydrogen peroxide (H_2O_2) that, in presence of horseradish peroxidase (HRP), oxidizes the *o*-dianisidine, which is detectable at 500 nm (see ESI†).[23] The activity was estimated by comparing the absorbance of free GOx and MOFs@GOx (Fig. S6, ESI†) as a function of time at a fixed wavelength (500 nm). The concentrations of the active GOx on the MOFs were estimated to be about 18 U mg^{-1} for MIL53(Al)@GOx, and 17 U mg^{-1} for MIL101(Cr)@GOx. The GOx attached on MOFs effectively preserves 46% for MIL53(Al)@GOx and 44% for MIL101(Cr)@GOx, of the total activity per unit mass compared to the free enzyme. This decrease may be attributed to a partial loss of enzyme structure during the functionalization steps. As a result, it can be stated that the GOx remains active on all MOFs, the activity is very similar which is in agreement with the TGA results, that shown similar weight losses for all MOFs@GOx. Using a similar method to calculate the catalytic activity, we obtained a calibration curve for the glucose detection. Fig. 4 shows the dependence of the absorbance at 450 nm on the concentration of glucose and there is a good linearity ($R^2 = 0.998$ and $R^2 = 0.991$ for MIL53(Al)@GOx and MIL101(Cr)@GOx, respectively) between the absorbance and the concentration of glucose in the range 0–1000 μM. The percentage relative standard deviation (% R.S.D.) for the low concentrations of glucose, was calculated to be 10.3% and 10.5% for MIL53(Al)@GOx and MIL101(Cr)@GOx, respectively. These results indicate a good reproducibility for glucose sensing applications. The limit of detection (LOD), calculated by the standard deviation of response and the slope,[24] is around 10 μM, comparable to other reported colorimetric glucose sensors.[25] Moreover, the detection limit of MOFs@GOx is well within the range of human blood sample, as the general blood glucose level in human body is 4–7 mM.[26] Furthermore, the cheap and versatile method to incorporate these multifunctional MOFs into miniaturized devices,[27] such as lab-on-a-chip or micro-fluidic devices, makes it potential candidates for commercial applications. To evaluate, the stability of MOFs@GOx, the colorimetric detection of glucose (1 mM) was monitored over a period of 3 weeks. As shown in Fig. S7,† the response of MOFs@GOx exhibited a decrease in the activity during the first week (~30%), and it stayed almost at the same level for the remaining two weeks. The MOFs@GOx retained about 60% of initial response after a period of 21 days.

The same test for the glucose oxidation was performed on non-enzyme loaded NH_2-MOFs and showed no activity towards glucose, as reported in Fig. S8, ESI.†

The selectivity of the GOx functionalized MOFs for glucose was evaluated by measuring the absorbance at 450 nm in the presence of various interfering compounds including fructose, mannose, galactose, saccharose. As shown in Fig. 5, even at 10-times higher concentrations compared to the concentration of glucose, the MOFs@GOx did not show any obvious activity towards these interfering compounds, demonstrating its high selectivity towards glucose.

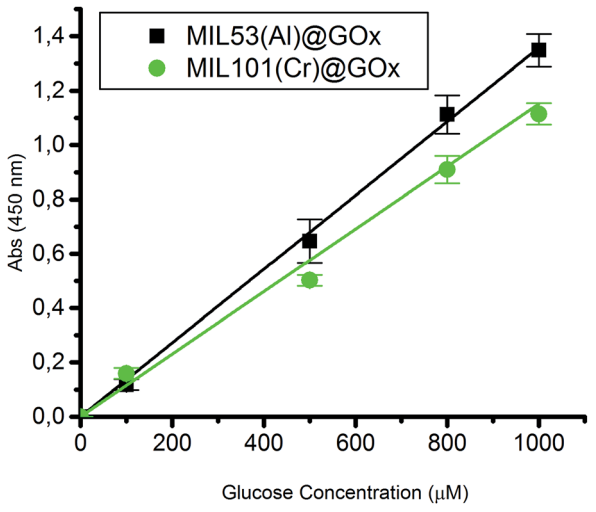

Fig. 4 Detection of glucose in concentration range of 0–1000 μM (absorbance at 450 nm was measured after incubation in the solution for 10 min at 37 °C of MIL-53(Al)@GOx (black line) and MIL-101(Cr)@GOx (green line)). The error bars indicated the standard deviation (S.D) of triplicate determinations for each concentration of glucose.

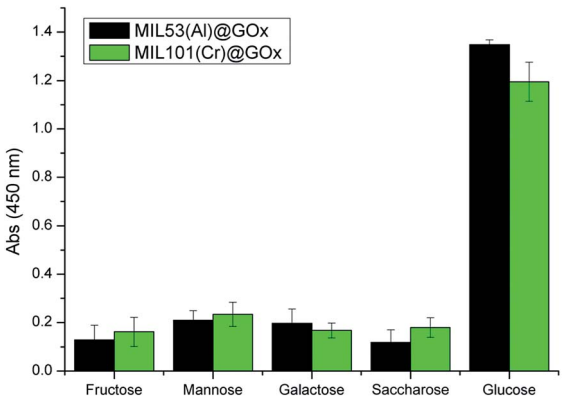

Fig. 5 Determination of the selectivity of MIL-53(Al)@GOx (black bar) and MIL-101(Cr)@GOx (green bar) (the solution contains 10 mM of D-fructose, 10 mM of D-mannose, 10 mM of D-galactose, 10 mM of D-saccharose and 1.0 mM of D-glucose, respectively).

In addition, to evaluate the role of unspecific interactions of the enzyme with the MOFs surface and to confirm the utility of PSM to attach the enzyme covalently to the surface, control samples were prepared by reacting directly the NH_2-MOFs with GOx without PSM. The colorimetric test for the glucose detection was carried out similarly to MOFs@GOx. In Fig. S9 (ESI†) we can observe that the absorbance measured for the blank samples (NH_2-MOFs@GOx) is much lower than MOFs@GOx, ruled out the possibility of physisorption on the surface and confirming the advantage to use the PSM to attach the GOx on NH_2-MOFs. The possibility to reuse the enzymes several times is another important aspect for immobilization of enzymes on solid supports. In the present case, a clear drop in activity is observed after the first catalytic use, followed by an almost constant activity over the following cycles. Although, the presented system shows less recyclability to other GOx/encapsulated MOFs,[17] it is possible reuse more times. We speculate that leaching of some weakly adsorbed enzyme occurs during the first catalytic use, leaving only the covalently bonded enzyme on the surface of the MOF, hence the initial decrease in activity (Fig. S10, ESI†).

In summary, we presented a PSM approach for carboxylate amino MOFs using GOx as a model enzyme. The prepared MOFs@GOx retained the enzymatic activity and showed selective properties for glucose. In spite of the less satisfactory recycling of the supported enzymes, we believe that our study offers a novel tool to immobilize biomolecules.

Acknowledgements

This work was supported by Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) through FIRB "RINAME Rete Integrata per la NAnoMEdicina" (RBAP114AMK).

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