Heterogenisation of Homogeneous Catalysts and Biocatalysts onto Carbon Materials

The development of efficient strategies for immobilising the enzyme L-asparaginase onto multi-walled carbon nanotubes and carbon xerogels by optimising several immobilisation conditions and tuning their surface chemistry is the focus of this topic.

Introduction

Immobilisation of L-asparaginase over carbon-based nanomaterials

L-asparaginase (ASNase, EC 3.5.1.1) is a biocatalyst widely used in industrial applications in a fast-growing global market. ASNase is mainly used for food, therapeutic, and biosensing applications. In the food industry, ASNase can reduce the production of acrylamide, a carcinogenic substance, without changing the flavour. However, the ideal ASNase to be used in the food industry must fulfill some requirements, such as improved stability, high substrate specificity, and high conversion rate. In the biopharmaceutical sector, ASNase is used in the treatment of acute lymphoblastic leukaemia (the most frequent type of leukaemia in children). Still, the bacterial origin of current ASNases commercially available may cause several adverse side effects. In addition, the purification processes required are costly, limiting their availability for industrial processes. ASNase immobilisation is a well-known technique that can help overcome these limitations, increasing enzyme stability and half-life without activity loss and allowing the reusability of the enzyme. Carbon-based nanomaterials have been successfully used for enzyme immobilisation due to their unique porous structure and size, the possibility of introducing numerous functional groups, high surface area, adsorption capacity, and biocompatibility, offering interesting properties for catalytic applications. Accordingly, multi-walled carbon nanotubes (MWCNTs) [1, 3, 4] and carbon xerogels (CXs) [2], with and without surface modifications, have been studied as supports for ASNase immobilisation, aiming to develop a reusable bioconjugate with enhanced stability and activity for future industrial applications.

Current Development

The presented works result from the collaboration with several institutions, namely CICECO – Aveiro Institute of Materials (University of Aveiro, Portugal), the School of Pharmaceutical Sciences of the University Estadual Paulista (Brazil), and LAQV-REQUIMTE, Department of Chemistry, Universidade NOVA de Lisboa (Portugal).

MWCNTs were investigated as a novel immobilisation platform for ASNase by physical adsorption [4]. This technique is a simple attachment method with low associated costs that allows easy enzyme immobilisation and reload along with facilitated support regeneration when compared to covalent binding methodologies. Different adsorption conditions were optimised (contact time, pH, and ASNase/MWCNTs ratio) to improve the enzyme loading and activity while characterising the ASNase-MWCNTs bioconjugate (Fig.1). In short, the immobilisation of commercial ASNase onto pristine MWCNTs was successfully achieved by adsorption, with an immobilisation yield (*IY*) and a recovery of free enzyme activity above 90% under the optimal conditions (45 min contact time, pH 8, and 1.5 mg mL^{-1} in 2 mg of MWCNTs). Under these conditions, a maximum enzyme loading capacity of 148 U g^{-1} was achieved.
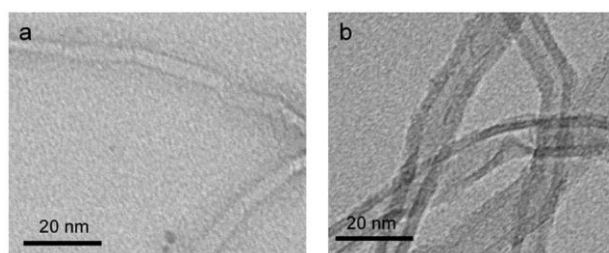

Fig. 1. TEM analysis of MWCNTs before (a) and after (b) ASNase immobilisation [3].

To fully explore the enzyme-MWCNT complex potential, the immobilisation of ASNase over modified MWCNTs [3] was investigated, along with the study of the optimal diameter of the MWCNTs [1]. Briefly, the MWCNTs functionalised with 0.3 M HNO$_3$ aqueous solution produced the highest *IY* and a relative recovered activity (*RRA*) of ASNase above 95% under similar conditions as the ones previously obtained for the pristine MWCNTs. In this case, the maximum predicted enzyme loading capacity of the MWCNTs was 158 U g^{-1} (higher than the obtained with the pristine MWCNTs). The hydrothermal treatment promoted a higher specific surface area of the material due to the creation of defect sites and holes on the sidewalls of the tubes, improving the support capacity to bind more enzyme particles and, consequently, achieve higher enzyme *RRA*. The immobilisation of ASNase onto pristine and functionalised MWCNTs (f-MWCNTs) with different size diameters (from <10 to 100 nm) also proved to affect the properties of the ASNase bioconjugate. The best *RRA* results of adsorbed ASNase were obtained with f-MWCNTs-10-20 and f-MWCNTs-20-40, showing the importance of the nanomaterial diameter size for the enzyme binding. MWCNTs with diameters smaller than 10 nm were shown to be too small for total enzyme binding, even presenting larger specific surface areas. MWCNTs with diameters larger than 40 nm revealed lower ASNase activity after immobilisation, probably due to the smaller surface areas and, consequently, fewer ASNase molecules adsorbed. Exceptional operational stability was achieved when immobilizing the ASNase onto f-MWCNTs-10-20, allowing its reuse for six reaction cycles with a retained activity of 74±2%, a value 1.3 times higher than when immobilized onto pristine MWCNTs (57±8%) (Fig. 2).
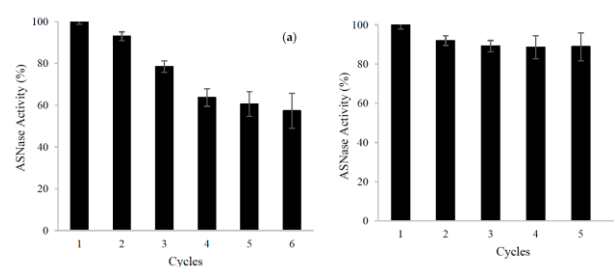
Fig. 2. Operational stability of immobilized ASNase onto (a) pristine and (b) f-MWCNTs-10-20. Error bars correspond to the standard deviation between replicates [1].

Other carbon-based nanomaterials were also explored as support for ASNase. CXs arise as a very interesting material for enzyme immobilisation of proteins due to the possibility of tailoring its mesoporosity and microporosity during the synthesis process by selecting suitable conditions. Therefore, CXs were also employed as a support for ASNase immobilisation through physical adsorption [2]. The affinity and interactions of a commercial ASNase from *Escherichia coli* with CXs with different pore sizes (4, 13, and 30 nm) were studied. An in-depth central composite experimental design and response surface methodology were used to optimize the immobilisation conditions (pH, contact time, and enzyme concentration) while minimising the number of necessary experiments. This allowed us to account for the effects of each studied factor on the response as well as the influence of their interactions. The parameter that revealed the most significant influence on the immobilisation efficiency was the ASNase concentration, as opposed to the contact time and the pH, which had no considerable impact. Fig. 3 shows that the activity of immobilized ASNase was sensitive even to small changes in the enzyme concentration, confirming this to be the most significant factor (with higher curvatures) for the ASNase immobilisation process over the three supports.

start performing applicability tests and scale up the immobilisation process.

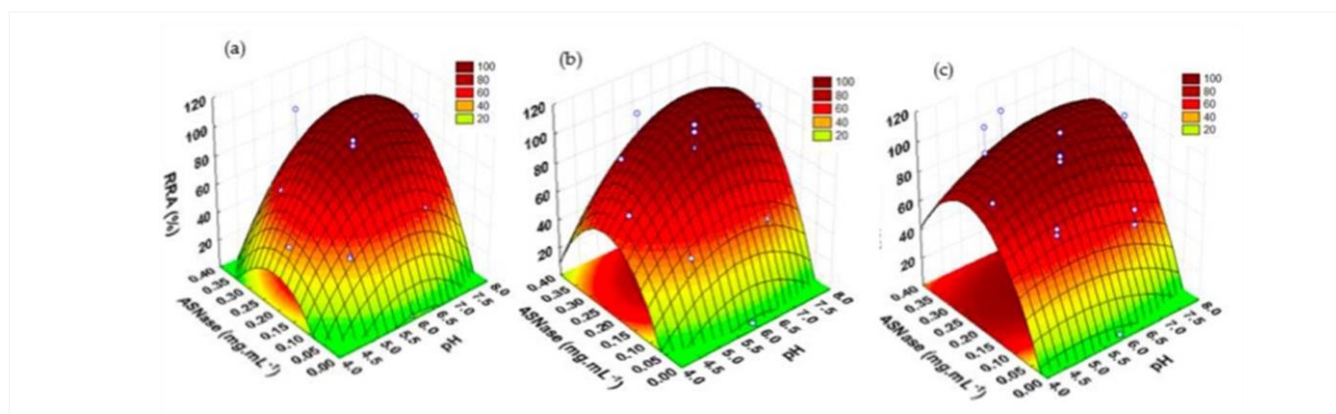**Related Sustainable Development Goals**

Fig. 3. Response surface plots for RRA of immobilized ASNase over CXs as a function of enzyme concentration and pH, for a contact time of 60 min. (a) CX-4; (b) CX-13; (c) CX-30 [2].

Moreover, CX-4 proved to be the most promising support for ASNase immobilisation, attaining exceptional *RRA* and *IY* values of 100% under the optimum conditions (contact time of 49 min, ASNase concentration of 0.26 mg mL^{-1}, pH of 6.73).
All these results show that MWCNTs and CXs are efficient supports for ASNase, opening new perspectives for the use of these bioconjugates in several application fields, e.g. as biosensors, in medicine, pharmaceutical and food industries.

Future Work

Research in enzyme immobilisation is still very much needed. Considering the promising results obtained with the surface modification performed in the MWCNTs, the most recent work includes the study of the change in surface chemistry of the CXs by increasing the number of oxygenated groups to enhance surface reactivity and hydrophilicity. Moreover, it is essential to