Nano-Bioproducts for Biomedical Applications

KEYWORDS: Anti-leukaemic biopharmaceutical, nanomaterials, purification, process integration

This project comprises the development of new strategies for L-asparaginase production and purification, for which precise control of the carbon-based nanomaterials' chemical surface will allow to immobilise and specifically purify the target enzyme from the complex matrix, with high purity and high yields.

Introduction

L-asparaginase (L-asparagine amidohydrolase EC 3.5.1.1 – ASNase) is an enzymatic biopharmaceutical used as a chemotherapeutic agent for the treatment of acute lymphoblastic leukaemia (ALL) (Fig. 1). Still, ASNase faces certain hindrances regarding its application. The major limitation when used as a pharmaceutical is the adverse reactions caused on patients, like fever, allergic reactions, skin rashes or even anaphylactic shocks. In addition, due to its non-human origin, it has a recognized rapid plasma clearance by native proteases. Apart from the adverse effects, the extremely high price of this drug is one of the major drawbacks for the ASNase widespread use in the treatment of ALL. Therefore, the demand for new biopharmaceuticals and new production/purification processes, within the downstream process, deserve special attention since these steps are the main limitations to launching the product into the market at a competitive cost.

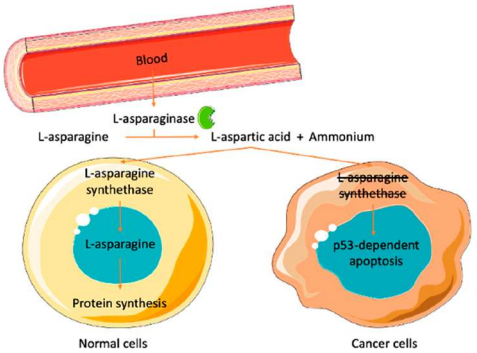

Fig 1. Schematic representation of the antitumoural outcome of L-asparaginase [1].

In this context, this work aims to develop novel sustainable technologies to produce and purify ASNase. Improvements in the upstream process will be attempted by using a novel recombinant ASNase to replace the usual bacteria that activate the immunological system, and thus with side effects. Since the ASNase purification step typically uses highly expensive and time-consuming methods, this project also comprises improvements in downstream processing, for which reusable pristine and functionalised carbon nanomaterials will be used. The presented outcomes result from the collaboration with several institutions, namely CICECO and LAQV-RWQUIMTE from Portugal, and UNESP from Brazil.

Current Development

ASNase immobilisation is a promising technique that can help overcome the problems associated with its use in the therapeutic sector. Increased stability without activity loss, reusability, and easier separation are the main advantages of enzyme immobilisation in this field. From the perspective of the development of a cost-effective and sustainable tool for biopharmaceutical application, enzyme immobilisation on solid supports can be a valuable mechanism for the isolation of these substances from a complex mixture of proteins. Therefore, the knowledge of the interactions between nanomaterials and the target biomolecule is essential, as it can reduce the number of purification steps and, hence, decrease operating costs.

Accordingly, multi-walled carbon nanotubes (MWCNTs) were explored as novel supports for ASNase immobilisation by a simple adsorption method [2]. The optimization process involved controlling the pH, contact time of immobilisation, and the concentration of ASNase to achieve maximum immobilisation yield (*IY*) and relative recovered activity (*RRA*). MWCNTs exhibited a notably high capacity for loading ASNase, with a maximum *IY* of 90%. The adsorbed ASNase maintained 90% of the initial enzyme activity under the optimized conditions (pH 8.0, 60 min, and 1.5×10^{-3} g mL^{-1} of ASNase).

In order to fully investigate the potential of the enzyme/MWCNT complex, the immobilisation of ASNase over functionalised MWCNTs was also studied [3], along with the influence of the diameter of the MWCNTs [4]. Envisioning the industrial application of ASNase/MWCNTs in the therapeutic sector, the reusability of the bioconjugate is mandatory. This issue was studied by analysing several cycles of L-asparagine hydrolysis using the most promising nanomaterial (MWCNTs functionalised with 0.3 M HNO_3) for the immobilisation of 1.5×10^{-3} g mL^{-1} of ASNase. The results provided in Fig. 2 show that, despite using a simple physical adsorption method (where relatively weak interactions are expected to be involved), the overall system reveals exceptional operational stability, without any loss of immobilised ASNase activity during 6 consecutive reaction cycles. These results also emphasize the improved ASNase bioconjugate stability acquired after attachment onto functionalised MWCNTs. This confirmed stability is a relevant acquired advantage, allowing the bioconjugate to be reused several times in batch processes, as well as the application in continuous processing systems [3]. The immobilisation of ASNase onto pristine and functionalised MWCNTs with different size diameters (from <10 to 100 nm) also demonstrated an impact on the characteristics of the bioconjugate. Maximum *RRA* values of adsorbed ASNase were specifically obtained for functionalised MWCNTs with diameters between 10–20 nm, and 20–40 nm, proving the importance of the nanomaterial diameter size for the enzyme binding [4].

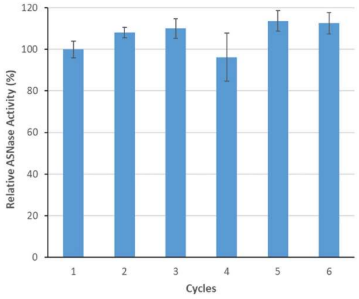

Fig 2. Operational stability of immobilised ASNase onto MWCNT-NC-0.3 during 60 min of contact time, pH 8.0, and with 1.5 × 10–3 g mL−1 of initial ASNase concentration [3].

Further, other carbon nanomaterials were also explored as supports for ASNase. Carbon xerogels (CXs) emerged as a

particularly interesting material for protein immobilisation due to their ability to tailor both mesoporosity and microporosity during synthesis by adjusting specific conditions. Hence, CXs were also employed as a support for ASNase immobilisation through physical adsorption [5]. CXs with different pore sizes (4, 13, and 30 nm) were studied were studied for the immobilisation of commercial ASNase from *Escherichia coli*. CX-4 showed the most promising results, achieving exceptional RRA and IY values of 100% under the optimized conditions (contact time of 49 min, ASNase concentration of 0.26 mg mL−1, and pH of 6.73). To confirm the immobilisation of the enzyme on the material, the CX-4 and the bioconjugate morphology were evaluated by SEM. After ASNase adsorption, the morphology of the CXs particles remains intact (Fig. 3c, d), very similar to their original structure (Fig. 3a, b), meaning that, possibly, the ASNase adsorbs mainly in the pore of the material. These results show that the entire enzyme immobilisation procedure did not damage the CXs structure, highlighting that the materials would be ready for further enzyme desorption and reuse. Nevertheless, through Fig. 3d, an additional organic layer between the spherical particles seems to be observed (red circle), attributed to adsorbed ASNase.

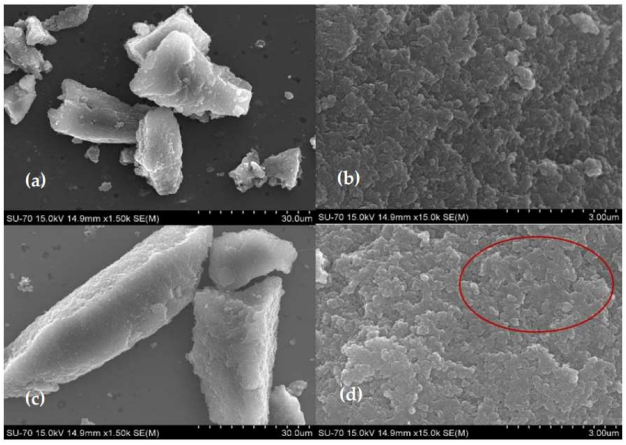

Fig 3. SEM images of CX-4 (a, b) and ASNase-CX-4 bioconjugate (c, d) [5].

All these results show that MWCNTs and CXs are efficient supports for ASNase, opening new perspectives for the use of these nanostructured materials for biomedical applications.

As previously mentioned, commercial ASNase (from *Escherichia coli* and *Erwinia chrysanthemi*) can cause toxicity, immuno-suppression, and the development of resistance by patients. Therefore, genomic innovation and the discovery of new microorganisms are of extreme importance for manufacturing ASNase with higher biological activity and fewer side effects. Studies performed by the Brazilian team involved in this project include the production of a fermentation broth containing ASNase expressed in *Bacillus subtilis*. To explore the use of CXs as a tool for the purification of ASNase, a series of batch tests were conducted, optimizing three key experimental parameters (fermentation extract concentration, CXs mass, and adsorption volume) to maximize the purification fold. A purification fold of 2.5 was reached during the batch adsorption of 3 mg mL^{-1} of fermentation extract onto 18 mg of CXs in 15 mL flasks. This purification value, corroborated by SDS-PAGE analysis, demonstrated CXs' ability to selectively isolate the target enzyme (ASNase) while adsorbing unwanted proteins from the solution. Despite testing various protein desorption methods (altering pH, adding NaCl, increasing temperature), these approaches were only able to achieve approximately 20% protein desorption, indicating incomplete regeneration of the CXs after use. However, continuous purification experiments using synthesized CXs in a column configuration resulted in an ASNase purification fold of 3.8 during a subsequent purification cycle. This value is comparable to, or even higher than, those achieved through conventional techniques. These outcomes prove the potential of this simple, rapid, and cost-effective single-step physical adsorption method using CXs to purify ASNase from fermentation extracts, representing a viable alternative to current chromatographic purification column practices in the pharmaceutical industry.

Future Perspectives

Research into enzyme immobilisation remains a critical area of study. Building upon the encouraging outcomes achieved through surface modification of MWCNTs, ongoing research now focuses on altering the surface chemistry of CXs to boost surface reactivity and hydrophilicity. The obtained results regarding the use of CXs for the purification of proteins are still limited to the laboratory scale. Therefore, to take this project to the next level it is necessary to evaluate the scalability of the method and to assess the economic feasibility of this process. The scale-up of the process will include both the production and the purification steps.

Moreover, the methodology developed for the enzymes can be also applied to other compounds, not only for pharmaceutical applications but also for diagnostics, analysis, etc. This project opened doors for the research of other carbon-based materials and enzymes with biomedical applications.

Related Sustainable Development Goals

Outputs

Master Dissertations

[1] Rita A. M. Barros, Carbon-based nanomaterials for the development of anti-leukemic drugs, MIB, FEUP, 2021
[2] Gonçalo Aguiar, Materiais de carbono para a purificação contínua de fármacos anti-leucémicos, MIEQ, FEUP, 2022

Selected Publications

[1] J. C. F. Nunes et al., Molecules 25, 5827 (2020)
[2] R. O. Cristóvão et al., RSC Adv. 10, 31205 (2020)
[3] M. R. Almeida et al., Scientific Reports 11, 21529 (2021)
[4] R. O. Cristóvão et al., Appl. Sci. 12, 8924 (2022)
[5] R. A. M. Barros et al., BioTech 11, 10 (2022)

Team

Joaquim L. Faria, Associate Professor; **Cláudia G. Silva**, Assistant Professor**; Raquel O. Cristóvão**, Assitant Researcher; **Rita A. M. Barros**, PhD student

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