Nanostructured Materials for Biomedical Applications

KEYWORDS: Protein Purification / Catalyst/ Photoluminescence/ Bioimaging

Carbon dots (CDs) with tunable fluorescence were developed and validated as potential fluorescent probes for bioimaging applications.

Carbon xerogels (CXs) were used as a cost-effective one-step process for the purification of L-asparaginase (ASNase) from a *Bacillus subtilis* fermentation extract.

Introduction

Bioimaging is a remarkable technique that allows real-time and non-invasive visualisation of biological events using probes and detectors. It plays a crucial role in advancing our understanding of biology, supporting medical diagnoses and treatments, and has been driving scientific discoveries in various disciplines. Carbon dots (CDs) exhibit strong fluorescence emission and excellent photostability, making them ideal for bioimaging applications. They can be used as fluorescent probes to mark and track specific cells, tissues, and biomolecules. The high brightness and low toxicity of CDs make them particularly useful in long-term imaging studies. Also, these fluorescent probes can be used to screen the crossing of cells and/or tissues. The main objective of this research topic is to develop CDs with good fluorescence properties for bioimaging and screen of the blood-brain barrier (BBB) permeability using brain-on-a-chip models.

L-asparaginase (ASNase, EC 3.5.1.1) is an enzyme that has been extensively used as a biotherapeutic agent for acute lymphoblastic leukaemia treatment. Still, current enzyme purification processes include a set of sequential techniques, that are highly costly (may account for up to 80% of its total cost) and time-consuming. Due to its simple, fast, and economical nature, enzyme immobilisation can be a cost-effective tool to isolate enzymes of interest from a complex biological media selectively. Therefore, considering their high surface area and customizable porosity, carbon xerogels (CXs) are highly attractive materials for protein immobilization and purification. The main goal of this research is to use CXs as an economically viable and simple platform for ASNase purification from a complex matrix.

Current Development

CDs have been studied as fluorescent probes for bioimaging under a collaboration with Dr Raquel O. Rodrigues (INL – International Iberian Nanotechnology Laboratory, Portugal, and Harvard Medical School – Brigham Women's Hospital, USA). Accordingly, CDs were synthesized through a microwave-assisted method employing citric acid, urea, and sodium fluoride as the precursors. The resulting samples were referred to as CD_X, in which *X* refers to the time of microwave irradiation (in min). The characteristics of the CDs were studied using UV-Vis spectrophotometry, Raman and Fourier transform infrared (FTIR) spectroscopy, and confocal microscopy. The CDs presented quantum yields up to 25.4%. Moreover, exposing the CDs to different wavelengths led to emissions at wavelengths corresponding to different colours (Fig. 1), which is a key feature in the development of CDs for bioimaging applications. CD_3 was then screened as a possible bioimaging tool to evaluate the permeability of a 3D BBB model constructed using a biomembrane seeded with human umbilical vein endothelial cells. The cell network in the hydrogel (BBB) is illustrated in Figure 2a. Next, a drop of a CD_3 suspension was added, and after 1 and 10 min, images Figure 2b and 2c were collected, respectively. Briefly, the results in Figure 2 suggest that CD_3 was absorbed by the hydrogel. For example, from Figure 2b to Figure 2c, it can be observed that the cells themselves become more fluorescent. Therefore, this work suggests that the synthesized CDs have great potential to be used as fluorescent probes for bioimaging applications. Nevertheless, more research is needed to validate our observations.

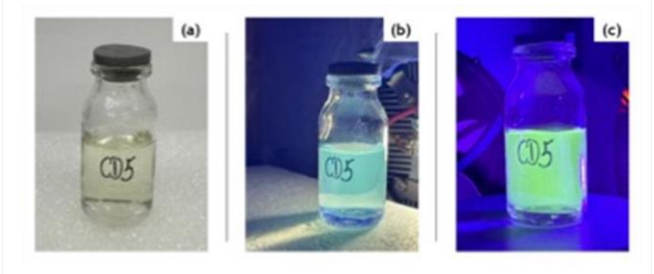

Fig. 1. Images of an aqueous suspension prepared with CD_5 when subjected to (a) natural light and (b) 365 nm and (c) 401 nm LED.

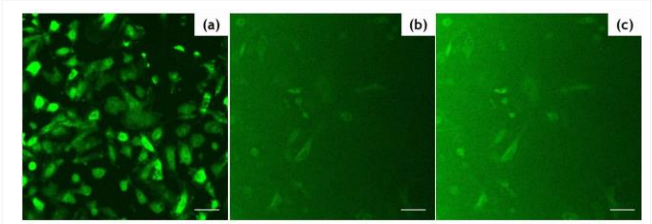

Fig. 2. Images obtained by confocal microscopy analyses, where (a) depicts HUVEC-GFP cells in biohydrogel after 7 days of culture, and (b) and (c) were obtained 1 and 10 min after the addition of CD_3 to the cells/biohydrogel, respectively. The laser with a wavelength of 488 nm was used for image collection, with a 10x objective. Images post-processed using Image J.

The study of CXs as a tool for the purification of ASNase has been developed in collaboration with CICECO – Aveiro Institute of Materials (University of Aveiro, Portugal), with LAQV-REQUIMTE (Universidade NOVA de Lisboa, Portugal), and the School of Pharmaceutical Sciences (UNESP – University Estadual Paulista, Brazil). CXs were obtained through a polycondensation reaction between resorcinol and formaldehyde. After CXs synthesis and characterization (pore radius = 6.3 nm; pore volume = 0.80 cm^3/g; BET surface area = 688 m^2/g; average particle size = 252 µm), batch tests were performed with the optimization of three experimental parameters (fermentation extract concentration, CXs amount, adsorption volume) to maximize the ASNase purification fold. An ASNase purification fold of 2.5 was reached during the batch adsorption of 3 mg/mL of fermentation extract onto 18 mg of CXs in 15 mL flasks. This purification value and the results of SDS-PAGE analysis showed that CXS could selectively isolate the target enzyme (ASNase) in solution while adsorbing the remaining uninteresting proteins (Fig. 3).

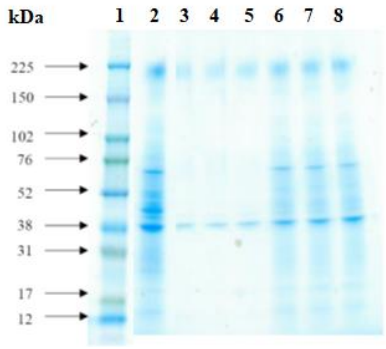

Fig. 3. SDS-PAGE analysis of several samples. Lane 1: Protein marker; Lane 2: ASNase fermentation extract 3 mg/mL; Lane 3-5: ASNase fermentation extract purified with 12 mg, 18 mg, and 24 mg of CXs on 15 mL; Lane 6-8: ASNase fermentation extract purified with 12 mg, 18 mg, and 24 mg of CXs, respectively, on 1.5 mL.

The protein desorption methods tested (pH change, NaCl addition, temperature increase) proved not to completely regenerate the CXs under use, reaching only around 20% of protein desorption. Nevertheless, the continuous purification experiments performed with the synthesized CXs packed in a column allowed us to obtain an ASNase purification fold of 3.8 in a second purification cycle, a value similar to or higher than those achieved with traditional techniques. All these results reveal that the ASNase purification from a fermentation extract by a simple, fast, and cost-effective one-step physical adsorption onto CXs has a high potential to be adopted by pharmaceutical, replacing the currently used chromatographic purification columns.

Therapeutic approaches based on nucleic acids to modulate cell activity have recently gained attention. These molecules arise from complex biotechnological processes, requiring effective manufacturing strategies, high purity, and precise quality control to be used as biopharmaceuticals. One of the most critical and time-consuming steps for nucleic acids-based biotherapeutics manufacturing is their purification, mainly due to the complexity of the extracts. We developed, in collaboration with the Health Sciences Research Centre of the University of Beira Interior (Portugal) and CICECO – Aveiro Institute of Materials (University of Aveiro, Portugal), a simple, efficient, and reliable method to isolate and clarify plasmid DNA (pDNA) from complex samples [1]. The method is based on the selective capture of RNA and other impurities using pristine carbon nanotubes (CNTs). Multi-walled CNTs (MWCNTs) with different diameters were studied to determine their adsorption capacity and to address their ability to interact and distinguish between nucleic acids. The results revealed that MWCNTs preferentially interact with RNA and that smaller MWCNTs present a higher adsorption capacity, as expected by the higher specific surface area. Overall, this study showed that MWCNTs significantly reduce the levels of impurities, namely RNA, gDNA, and proteins, by approximately 83.6 % compared to their initial level, enabling the recovery of clarified pDNA in solution while maintaining its stability throughout the recovery process. This method facilitates the pre-purification of pDNA for therapeutic applications (Fig. 4).

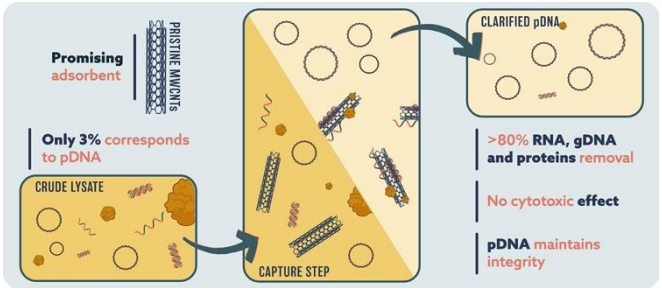

Fig. 4. Multi-walled carbon nanotubes for efficient plasmid DNA clarification.

Future Work

The ability of the CDs to cross the BBB is yet to be confirmed using fluorescence microscopy techniques and a brain-on-a-chip model currently being developed by our collaborator – Dr Rodrigues, at INL and Harvard Medical School. Moreover, the size and biocompatibility of the CDs must be thoroughly studied. Administering neuroprotective or therapeutic substances to particular regions of the brain poses a significant hurdle primarily because of the existence of the BBB (most drugs cannot cross this tight barrier). Therefore, successfully demonstrating the ability of our CDs to cross the BBB is expected to bring significant developments to neuroscience research.

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. Moreover, several microorganisms are also being studied for the recombinant production of ASNase with higher yields.

Related Sustainable Development Goals

Outputs

Master Dissertations

Catarina Araújo, *Development of carbon dots as fluorescence probes for bioimaging*, MIB, FEUP, 2023

Gonçalo Aguiar, *Materiais de carbono para a purificação contínua de fármacos anti-leucémicos*, MIEQ, FEUP, 2022

Selected Publications

[1] P. Ferreira et al., Separation and Purification Technology 320, 124224 (2023)

Team

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