Gelatin-graphene oxide nanocomposite hydrogels for the immobilization of *Kluyveromyces lactis*: novel high-performance encapsulates for probiotics and bioreactor packings

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Introduction

One of the most attractive routes for the small-scale bioproduction of metabolites enabled by microorganisms is to immobilize them in porous materials. This approach facilitates the exchange of nutrients and products while extending cell viability considerably. Some of the materials for immobilization include ceramic, metallic and polymeric matrices. In general, polymers offer the possibility for swellable 3D structures that can be tuned in terms of mechanical strength and stiffness. For biological applications, natural polymers stand out due to the possibility of facilitated degradation routes under physiological conditions and high biocompatibility. Examples of such polymers include agar, alginate, and gelatin. In the search for improving the mechanical properties and achieving extended life cycle without detrimentally impacting biocompatibility, these matrices have been modified with nanomaterials. This has been achieved by forming nanocomposites with an ample range of 1D and 2D nanomaterials including metal oxide nanoparticles, Graphene, layer double hydroxides, zeolites, carbon nanotubes and Graphene Oxide (GO).

The biotechnological production of bioproducts depends on the type of microorganism (bacteria or yeast), the immobilization and recirculation of the microorganism, the medium's pH, the temperature, the carbon source, the nitrogen source, the used fermentation mode, and the formation of by-products. In bioproduction, there are two ways to arrange the microorganisms inside the reactor. The first is with the free microorganism. A second alternative is to maintain the yeast or bacteria immobilized inside a matrix made of natural or synthetic materials. . Immobilization is advantageous because it helps overcoming inhibition due to the presence of substrate and metabolite and the decrease in microorganisms' activity as the bioproduction process progresses. As a result, the production yields are significantly reduced, limiting thereby the possibility of large-scale production .

One of the most challenging issues during functional food manufacturing is to ensure that the active components can maintain their structural stability during storage and consumption. This is mainly due to their pass through the gastrointestinal tract (GIT) where the pH of the environment continually changes, and enzyme activity may negatively impact these components. Different strategies have been developed to overcome this issue, including freeze and spray drying, emulsions, microencapsulation, nanoencapsulation, and encapsulation in polymeric matrices. Moreover, by controlling the encapsulation parameters, it is possible to maintain relatively high cell viability and stability at both the culture and storage stages. Despite some success cases over the past few years, material integrity issues as it passes through the GI tract are yet to be solved.

Graphene oxide (GO) has recently emerged as a new nanoscale carbon-based material that exhibits superior solubility in water and other solvents compared with graphene. This has enabled a number of applications and especially in the biomedical and biological fields where water is ubiquitous. Moreover, it offers high biocompatibility, antimicrobial properties and response to electrical fields in its reduced form. Finally, GO has been described to positively alter the mechanical properties after it is combined with several materials including polymers and ceramics.

The obtained nanocomposites exhibit tunable degradation rates, higher mechanical stability and integrity and relatively high biocompatibility. Here, we proposed to synthesize gelatin-GO nanocomposites for the encapsulation of the yeast *Kluyveromyces lactis* capable of tolerating continuous operation and changing environmental conditions. The synthesized hydrogels were characterized in morphological, physical-chemical, mechanical, thermal, and rheological properties. This comprehensive characterization allowed us to identify critical parameters to facilitate encapsulation, enhance cell survival, and study their application as probiotics. The gelatin-GO nanocomposite hydrogels showed that the compression values increased from 40 Pa to almost 187 Pa. Also, viability increased from 6 to 12% as well as in about 49% for the bulk porosity. Further rheological and thermal tests confirmed improvement in the physical integrity for the modified hydrogels.

Methods

For the manufacture of the hydrogels, type A gelatin was used, which was chemically crosslinked with different levels of glutaraldehyde (GTA) at 25% (v/v). For the reinforcement of the hydrogel, graphene oxide (GO) was used, which was obtained by following a modified version of the Turret's method.The microorganism selected for encapsulation was *Kluyveromyces lactis* GG799 wild type from *K. lactis* Protein Expression Kit (New England Biolabs, Ipswich, MA, USA). It was maintained in YPGlu plates [yeast extract 1.0% (w/v), peptone 2.0% (w/v), glucose 2.0% (w/v), agar 1.5% (w/v), ampicillin 100 ug/mL] and inoculated in YNB liquid medium [yeast nitrogen base (YNB) 0.68% (w/v), glucose 2.0% (w/v), lactose 2.0% (w/v), L-histidine 0.001% (w/v)]. For the yeasts' staining, propidium iodide was used, and they were washed with PBS (salt solution buffer). A single half-sphere hydrogel was placed in a 250 mL flask with 100 mL of different solutions simulating saliva, stomach, and small intestine conditions. The treatment began by exposing the hydrogel to the simulated saliva medium for 7 min, then to the simulated gastric fluid medium for two hours, and finally to the small intestine medium for two more hours. The whole process was performed with incubation at 37 °C and 150 RPM. The rheological analysis was performed in a frequency sweep in which we observed the storage (G') and loss (G") modulus. The procedure is carried out at 25°C, at an 1000 Pa stress, with a frequency range of 0.1-100 rad/s; a 2 cm diameter and 1 mm thick sample disk was prepared. Before and after bioreactor operation, the hydrogels were evaluated in a firmness test to determine changes in the mechanical response. This test measures force in compression at a 1.0 mm/s speed and 5.0 mm distance using the TA.HDplusC Texture Analyzer. The encapsulates with the yeast cells were packed in a milliliter scale (250 mL), external-loop airlift-bioreactor to test their performance. The system was designed and assembled in-house by 3D printing (Stratasys, USA) the base in polylactic acid (PLA) while manufacturing the body and lid from commercially available polypropylene. The external loop and connectors were cast in silicone rubber using 3D printed molds. The system was maintained at 30 C with aeration provided by an air pump (AC9904 RESUN, 8W) for 72 h.

Results and Discussion

Rheological testing allowed us to evaluate changes in the storage and loss moduli. As the contents of GO was increased in the hydrogels, the elastic modulus increased accordingly. Results show that there is a higher level of stiffness as the amount of GO increases. This may be due to the reinforcement made by the GO present in the hydrogel. In the firmness test, the gels

were compared at different concentrations of GO. Gels after72 hours of bioreactor operation showed a decrease in the compression force from 17 N to 12 N with respect to the material before operation. Firmness also increased with the GO contents going from 8 N for the pristine hydrogels to 17 N for the GO-modified ones at the highest concentration . Firmness was also reduced with the residence in the bioreactor. We can observe that at the beginning, the viability is similar between formulations concerning the target, with a difference of between 6% and 10%, respectively. After 72 hours of bioreactor residence, the cell viability declines with no statistical significance. The 3.0% or 5.0% (w/w) GTA hydrogels with encapsulated yeast cells were also exposed to simulated saliva, stomach, and small intestine media. Confocal imaging shows a progressive reduction in cell viability as encapsulates are exposed to the GIT simulated media. A quantitative analysis of the collected images demonstrated that the viable cells were reduced by about 20%, 35%, and 40% for simulated saliva, stomach, and small intestine media, respectively.