Formulation and Characterization of Gelatin-Based Hydrogels for the Encapsulation of *Kluyveromyces lactis* and Chitosan Nanoparticles: Biotech and Biomedical Applications †

Jorge Luis Patarroyo 1, Juan C. Cruz 2 and Luis H. Reyes 1,*

1 Department of Chemical and Food Engineering, Universidad de Los Andes, Bogotá 18a-12, Colombia; lh.reyes@uniandes.edu.co
2 Department of Biomedical Engineering, Universidad de Los Andes, Bogotá 18a-12, Colombia; jc.cruz@uniandes.edu.co
* Correspondence: lh.reyes@uniandes.edu.co
† Presented at the 2nd International Online-Conference on Nanomaterials, 15–30 November 2020; Available online: https://iocn2020.sciforum.net/.

Published: 15 November 2020

Abstract: A difficulty when orally administering microorganism-based probiotics is the significant loss of their bioactivity as they pass through the gastrointestinal (GI) tract. To overcome these issues, we propose to encapsulate the probiotic yeast *Kluyveromyces lactis* on chemically crosslinked gelatin hydrogels to protect the bioactive agents in different environments. Moreover, a challenge to obtain favorable results with therapeutic drugs and biomolecules is the inefficient cellular administration process. For this reason, we considered chitosan and gelatin nanoparticle loading systems to improve therapeutic efficacy. Also, we prepared hydrogels to encapsulate such nanoparticles by the chemical crosslinking of gelatin, an inexpensive and commercially available polymer. To explore changes in key physicochemical parameters and their impact on cell viability, we varied the concentration of the crosslinking agent (glutaraldehyde) and the gelatin. 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 and enhance system performance. Mainly due to pore size in the range of 5–10 µm, sufficient rigidity (breaking forces of about 1 N), low brittleness and structural stability under swelling and relatively high shear conditions, we selected hydrogels with a high concentration of gelatin (7.5% (w/v)) and concentrations of the crosslinking agent of 3.0% and 5.0% (w/w) for cell and nanoparticles encapsulation. Yeasts and nanoparticles were encapsulated and subsequently tested in bioreactor operation and GI tract simulated media, thereby leading to cell viability levels that approached 95% and 50%, respectively. After testing, the hydrogels’ firmness was only reduced to half of the initial value and maintained resistance to shear even under extreme pH conditions. These encouraging results indicate that the proposed encapsulates are suitable for overcoming most of the major issues of oral administration of drugs and probiotics and open the possibility to further explore additional biotech applications.

Keywords: Hydrogels; gelatin matrix; cross-linking; probiotics; nanoparticles; encapsulation

1. Introduction

Attention towards functional foods consumption in the general public has shifted because of the different long-term health benefits [1]. This is partly due to the incorporation of bioactive agents with proven activity towards mitigating impaired cellular functions. These bioactive agents have been
associated with different conditions, including cancer, diabetes, cardiovascular disease (CVD), hypertension, diarrhea, lactose intolerance, and some allergies [2,3].

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 [4]. This is mainly due to their pass through the gastrointestinal (GI) tract where the pH of the environment continually changes, and enzyme activity may negatively impact these components [5]. Different strategies have been developed to overcome this issue, including freeze and spray drying, emulsions, microencapsulation, nanoencapsulation, and encapsulation in polymeric matrices [2,6]. One aspect concerning hydrgels’ use is to tune their mechanical properties properly [7,8]. This has been achieved by implementing different strategies, including physical crosslinking, chemical modifications, and chemical crosslinking [7].

The protection provided by hydrogels to encapsulated microorganisms has been crucial for applications in bioremediation and metabolite production [9,10]. This has been the case due to their incorporation as packing materials into highly efficient bioreaction systems. With this approach, it has been possible to produce numerous commercial interest metabolites such as bioethanol, cellulose, biohydrogen, oxalic acid, gluconic acid, citric acid, malic acid, and lactic acid [11–15].

On another scale, nanoparticles (NPs) are being used to develop innovative biomedical technologies for therapeutics and drug-delivery systems. However, there are still many challenges to overcome due to human body characteristics, specifically the biological absorption barriers [16]. One of the major challenges of modern pharmacology is to ensure that the drugs can reach the tissue or organ target with relevant bioavailability [17].

This work is therefore dedicated to encapsulating the yeast strain K. lactis and gelatin/chitosan nanoparticles into gelatin type-A hydrogels to produce low-cost and highly stable probiotic vehicles. The capsules were made by covalent crosslinking of glutaraldehyde with the polymer. The thermal, rheological, and mechanical properties and the microscopic characteristics of the prepared matrices were evaluated before the encapsulating step. Upon encapsulation, successful proof-of-concept experiments were performed on a milliliter-scale bioreactor and a simulated gastrointestinal medium, as shown in Figure 1, where yields, cell viability, and biocompatibility were determined.

2. Materials and Methods

2.1. Microorganisms and Culture Media

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)] [18]. The inoculum was incubated in an orbital shaker at 30 °C and 200 RPM for 16 h.

2.2. Preparation of Gelatin Hydrogels and Probiotic Encapsulation

Sterile water was heated to 40 °C and mixed with gelatin Type A (food grade). The mixture was kept under constant stirring at 180 RPM for 30 min until a homogeneous mixture was achieved. Glutaraldehyde (GTA) solution 25% for synthesis (PanReac AppliChem, Spain) was added dropwise while stirring at 80 RPM (to ensure complete chemical crosslinking) in a water bath at 40 °C for 2 h. The yeast cells were resuspended in sterile water and carefully poured into the hydrogel solution to 5.0% (w/v). The process was conducted under low speed stirring for 30 min to avoid cell disruption. The mixture was cooled down to room temperature and poured into silicone molds. The hydrogels were stored at 4 °C for 24 h to complete the gelation process.
2.3. Performance of Encapsulates in a Milliliter Scale Bioreactor

The yeast cells encapsulates 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, Minnesota, USA) with a base in polylactic acid (PLA), while the body and lid were manufactured from commercially available polypropylene. The external loop and connectors were cast in silicone rubber using 3D printed molds. Aseptically, 15 half-sphere hydrogels were placed in the reactor, and the culture medium added to reach a 250 mL operation volume. The system was maintained at 30 °C with aeration provided by an air pump (AC9904 RESUN, 8W) for 72 h.

2.4. Performance of Encapsulates in the Simulated Gastrointestinal Tract Media

The simulated saliva medium was prepared according to the work of Li et al. [19], with slight modifications, and contained 1.4 mg/mL NaCl, 0.5 mg/mL KCl, 0.1 mg/mL CaCl₂, 0.15 mg/mL NaH₂PO₄, 0.025 mg/mL MgCl₂, 0.09 mg/mL CO(NH₂)₂, 0.2 mg/mL C₆H₁₂O₆, 2.5 units/mL α-amylase, 0.7 units/mL lysozyme, and pH adjusted to 7.0 a with solid NaOH. The stomach and intestine simulated media were based on the work described by Klein and collaborators [20] with slight modifications. The simulated stomach medium was prepared with 80 μM C₂₄H₄₀O₅Na₅, 0.16 mg/mL egg lecithin, 34.2 mM NaCl, and pH adjusted to 2.0 with a solution of HCl 37% (PanReac AppliChem, Spain). Finally, the small intestine was simulated with a medium containing 3 mM C₂₄H₄₀O₅Na₅, 1 mg/mL egg lecithin, 68.6 mM NaCl, 19.12 mM C₄H₄O₄, and pH adjusted to 7.0 a with solid NaOH. The treatment began by exposing the hydrogel to the simulated saliva medium for 7 min, then to the simulated gastric fluid medium for 2 h, and finally to the small intestine medium for two more hours. The whole process was performed with incubation at 37 °C and 150 RPM [4,21].

2.5. Survival Rate of Encapsulated Probiotics

The probiotic cells were stained with a fluorescent marker, observed under a confocal microscope, and counted to estimate the live/dead ratio. These analyses were carried out for the hydrogels after packed bioreactor operation and GI tract treatments. A propidium iodide solution was added to the gel surface for staining. The gel cross-sections were kept in darkness for 30 min to let the marker diffuse into the porous hydrogel. Propidium iodide stains red those cells that have compromised membranes (i.e., dead cells). Finally, image acquisition was performed in a Confocal Laser Scanning Microscope Olympus FV1000 (40X, 0.6 NA), and the live/dead ratio was calculated by processing images with the aid of Fiji-ImageJ software [22].

**Figure 1.** *K. lactis* encapsulating gelatin hydrogel for the airlift bioreactor application and the simulated human gastrointestinal tract.
2.6. Rheological Response

The rheological analyses were carried out in a Discovery Series Hybrid Rheometer-1 (TA Instruments, New Castle, DE, USA) by running a frequency scan between 0.1 and 100 Hz at a constant amplitude of 1.0% strain and 25 °C [23]. A parallel-plate (diameter 20 mm) geometry was used with a fixed gap distance (1.0 mm) between the plates [8]. This was followed for complete experimental design and the samples after the bioreactor GI tract treatments.

3. Results

3.1. Cell Viability Assay

Encapsulation of K. lactis proceeded with hydrogels containing 7.5% (w/v) gelatin made with either 3.0% or 5.0% (w/w) GTA. The confocal images in Figure 2 show a significantly lower number of dead cells in the packed hydrogels both before and after the milli-bioreactor operation for 72 h. A quantitative analysis of the images demonstrated that for 3.0% (w/w) GTA, the viable cells were reduced by about 2%, while for the 5.0% (w/w) GTA, the reduction approached 5%. 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. The confocal images in Figure 2 show a progressive reduction in cell viability as the 3.0% (w/w) GTA encapsulates are exposed to the simulated media. We found similar results for the 5.0% (w/w) GTA encapsulates. A quantitative analysis of the images demonstrated that for 3.0% (w/w) GTA, the viable cells were reduced by about 20%, 35%, and 40% for simulated saliva, stomach, and small intestine media. A similar analysis for the 5.0% (w/w) GTA showed a reduction of about 30%, 50% and 55%.

![Figure 2](image-url)

Figure 2. Confocal microscopy images. Dead cells are shown in blank color while live cells in green. Scale bar corresponds to 10 μm. (A): Live/dead K. lactis cells in the encapsulates made with 3.0% (w/v) GTA. (B): Live/dead K. lactis cells in the encapsulates made with 3.0% (w/w) GTA after 72 h of bioreactor operation. (C): Live/dead K. lactis cells in the encapsulates made with 3.0% (w/v) GTA after exposure to simulated saliva medium. (D): Live/dead K. lactis cells in the encapsulates made with 3.0% (w/w) GTA after exposure to simulated stomach medium. (E): Live/dead K. lactis cells in the encapsulates made with 3.0% (w/w) GTA after exposure to the simulated small intestine medium. (F): Live/dead K. lactis cells in the encapsulates made with 5.0% (w/v) GTA. (G): Live/dead K. lactis cells in the encapsulates made with 5.0% (w/w) GTA after 72 h of bioreactor operation. (H): Live/dead K. lactis cells in the encapsulates made with 5.0% (w/w) GTA after exposure to simulated saliva medium. (I): Live/dead K. lactis cells in the encapsulates made with 5.0% (w/w) GTA after exposure to simulated stomach medium. (J): Live/dead K. lactis cells in the encapsulates made with 5.0% (w/w) GTA after exposure to the simulated small intestine medium. (K): Yeast probiotic cell survival rate for encapsulates before and after bioreactor operation and after treatment with each of the gastrointestinal tract simulated media. Statistical significance calculated using analysis of variance (* p < 0.05).

3.2. Rheological Evaluation

The rheological response of the hydrogels after exposure to each simulated gastrointestinal tract media is shown in Figure 3. For the simulated saliva treatment, the 3.0% (w/w) GTA showed no
significant moduli changes concerning the control. In the case of the 5.0% (w/w) GTA, the variation is less subtle, and we identified a slight reduction in both moduli after treatment. We also evaluated the hydrogels’ rheological response after the operation in the milli-bioreactor for 72 h (Figure 3G–H). The storage module’s dominance over the loss module confirms that the elastic response is sufficient to maintain a solid-like structure. After the operation, the 3.0% (w/w) GTA showed no significant moduli changes concerning the control (Figure 3G). In the case of the 5.0% (w/w) GTA, there is a notorious effect on the rheological properties, as evidenced by a decrease of about five-fold for the storage module and up to two-fold for the loss module (Figure 3).

Figure 3. Storage and loss moduli for 3.0% w/w GTA (A) and 5.0% w/w GTA (B) hydrogels after exposure to gastrointestinal tract simulated media, and the comparison with a hydrogel in the absence of the treatment. Storage and loss moduli for 3.0% w/w GTA (C) and 5.0% w/w GTA (D) hydrogels after 72 h of milli-bioreactor operation, and the comparison with a hydrogel without this treatment. All the treatments have 7.5% w/v gelatin concentration.

4. Discussion

The results indicated that the encapsulating material is highly biocompatible for cell culture and demonstrated this strain’s high resilience during bioreactor operation. The difference in cell viability’s treatments is most likely due to the excess of unreacted GTA for the 5.0% (w/w) GTA hydrogels, which has been reported to be highly cytotoxic [24,25]. Additionally, due to the reduced pore size, in this case, mass transfer limitations and restricted space for proliferation are likely to play a significant role. As for the hydrogels after bioreactor operation, the simulated media’s differences might be related to mass transfer issues and restricted proliferation. These results are consistent with those obtained for the encapsulates of mouse embryonic fibroblast 3T3 cells in gelatin hydrogels [26] and fibroblasts in gelatin/chitosan hydrogels [27]. The fact that about 50-60% of the encapsulated cells remain active when reaching the intestine is encouraging to continue working on developing novel probiotic encapsulates from gelatin matrices.

The rheological results likely indicate a subtle reduction in the structural stability of the gel. Exposure to the simulated stomach medium led to a significant decrease in both moduli. These reductions reached about five-fold in the case of the 3.0% (w/w) GTA (Figure 3C) and of about ten-fold for the 5.0% (w/w) GTA (Figure 3D). Once again, this reflects marked altered structural stability and particularly the detrimental impact of low pH conditions. Finally, upon exposure to the small intestine medium, changes in the rheological behavior were insignificant for the 3.0% (w/w) GTA (Figure 3E), while a two-fold reduction was observed for the 5.0% (w/w) GTA (Figure 3F). Severe alterations of the structure were only observed at very high oscillation frequencies, which are not expected during the normal pass through the human gastrointestinal (GI) tract. Importantly, taken
together, these results indicate that during the pass through the GI tract, the material will continue to exhibit a solid-like rheological response, which is critical to assure that a large population of probiotics effectively reach the site of action. After the bioreactor operation, the loss modulus crossed the storage modulus at very high frequencies, indicating possible structural rearrangements during the 72 h of the bioreactor’s continuous operation. These results are comparable with those recently reported for a hydrogel-packed bioreactor [28,29].

Acknowledgments: The authors would like to thank the Departments of Chemical and Biomedical Engineering at Universidad de Los Andes for providing access to laboratory infrastructure. We also like to thank the microscopy core facility for access to the confocal microscope and technical assistance to collect the images.

Conflicts of Interest: The authors declare no conflict of interest.

References

**Publisher’s Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.