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Alginate assessment by NMR microscopy

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Alginate hydrogels have long been used to encapsulate cells for the purpose of cell transplantation. However, they also have been criticized because they fail to consistently maintain their integrity for extended periods of time. Two issues of critical importance that have yet to be thoroughly addressed concerning the long-term integrity of alginate/poly-L-lysine/alginate microcapsules are: (i) are there temporal changes in the alginate/poly-L-lysine interaction and (ii) are there temporal changes in the alginate gel structure. NMR microscopy is a non-invasive analytical technique that can address these issues. In this report, we present data to demonstrate the utility of ^{1}H NMR microscopy to (i) visualize the poly-L-lysine layer in an effort to address the first question, and (ii) to observe temporal changes in the alginate matrix that may represent changes in the gel structure.

1. Introduction

Alginate is a naturally occurring biopolymer that has been used extensively as a vehicle to encapsulate a variety of biological materials including enzymes and cells of both microbial and mammalian origin. In tissue engineering, and particularly in the development of a bioartificial pancreas, alginate has been used to encapsulate islets [1–4] and transformed β-cells [5–8] with considerable success. A layer of a polycation, such as poly-L-lysine (PLL), followed by an additional layer of alginate is commonly used to coat the central alginate matrix, providing mechanical stability to the matrix [9] and at least partial immunoprotection [10]. The ability to non-invasively monitor the integrity of the alginate/poly-L-lysine/alginate (APA) matrix as well as the viability and function of the encapsulated cells either *in vitro* or *in vivo* is critical to our understanding of how these tissue-engineered constructs function.

At present, our only means of assessing the efficacy of an implanted bioartificial pancreas is to measure blood glucose levels. It is obvious that recipient patients can be managed more effectively if one could predict implant failure while the recipient was still euglycemic. To achieve this, one needs to develop a non-invasive protocol to monitor the integrity of the implant and the viability/function of the implanted cells. NMR is an analytical technique [11, 12] that is uniquely suited to fulfill these requirements. It has the ability to simultaneously and non-invasively provide biochemical and structural information either under *in vivo* or *in vitro* conditions. In the field of tissue engineering, and particularly in the context of the bioartificial pancreas, the application of NMR has been limited. Over the past 10 years, our laboratory has applied NMR spectroscopic techniques to monitor *in vitro* [6, 7, 13–19] and *in vivo* [20, 21] the viability and metabolic activity of bioartificial pancreatic constructs.

Two issues of critical importance that have yet to be addressed about the long-term integrity of APA beads either *in vitro* or *in vivo* are: (i) are there temporal changes in the alginate/PLL interaction and (ii) are there temporal changes in the alginate gel structure. At present, it is not possible to non-invasively visualize the PLL layer or to monitor longitudinal changes in alginate structure. We hypothesize that ^{1}H NMR microscopy may be useful in developing a non-invasive protocol to evaluate the integrity of these constructs. This report highlights some of our latest research on the visualization of the PLL layer and temporal stability of the alginate matrix by using NMR microimaging techniques.

2. Methods

2.1. Alginate encapsulation

Various types of alginate were used in these studies, and they will be defined below in the description of the experiments. All alginate solutions were prepared

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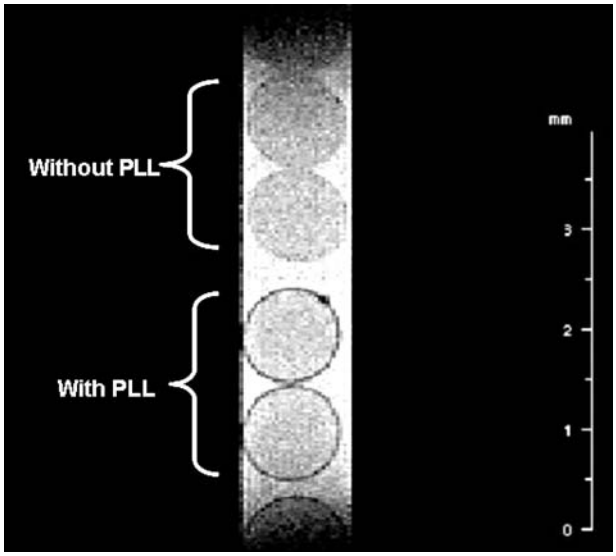

Figure 1 ^{1}H NMR gradient echo images of MVM alginate beads with and without PLL coating. Note the presence of the dark circle around the alginate beads that were coated with PLL. The pixel resolution of this image was 12.5 × 12.5 × 12.5 μm.

by dissolving alginate in physiological saline (0.85% NaCl) at a concentration of 2% (w/v). Alginate beads were generated with the aid of an electrostatic bead generator (Nisco, Basel, Switzerland). Completion of the APA beads was based on the initial protocol developed by Lim and Sun [3] and was used by our laboratory as previously described [6] for cell encapsulation. Microbeads were produced with and without a PLL coating to have a diameter of 800 μm at the time of the experiment. The PLL polymers used in these experiments had a molecular weight range of 15,000 and 30,000 (Sigma, St. Louis, MO) and was allowed to bond to the alginate beads for 6 min.

2.2. NMR microscopy

All MRI data were acquired using a vertical 17.6-T 89-mm bore cryopumped magnet equipped with a Bruker Avance console and Micro2.5 gradients (maximum strength of 1000 mT/m). Three to six beads immersed in DMEM were loaded into a capillary that was placed within a homebuilt solenoidal microcoil. Coupled with the high magnetic field, these small RF solenoids, which are susceptibility-matched to reduce field perturbations, greatly improve the sensitivity of the NMR experiment [22]. Given the size of the alginate beads and the length of the microcoil, several beads were analyzed simultaneously. Encapsulated cells were imaged in an unperfused state to avoid motion artifacts due to perfusion.

Images presented here were acquired using a gradient echo sequence (T_2* weighting) with a TE = 25 ms, TR = 2 s, matrix of 512 × 128 × 128 and field of view of 6.4 × 1.6 × 1.6 mm. Thus, the acquired spatial resolution of this image is 12.5 × 12.5 × 12.5 μm. In addition to high resolution images, a series of multiple slice spin echo images were acquired at different echo times for the purpose of measuring the T_2 value of the alginate as a function of time. These T_2-weighted images were acquired with a matrix of 512 × 256, field of view of 2.4 × 1.2 cm, slice thickness of 60 μm and TR of 2.5 s. The TE was varied from 15 to 120 ms to sample the T_2 value adequately.

3. Results and discussion

Fig. 1 illustrates a NMR microimage of four alginate beads (MVM alginate: 62% mannuronic acid content and 38% mannuronic acid content). The bottom two beads are coated with a PLL layer while the top two are not. A visual inspection of this image clearly shows a dark demarcation circle surrounding the alginate matrix. This circle is attributed to the presence of the PLL layer and is likely due to the magnetic susceptibility between the PLL layer and the alginate matrix. Using 3D volume reconstruction, we are able to segment the PLL layer and produce 3D images of the entire construct to assess its integrity, as seen is Fig. 2. We currently are in the process of characterizing this observation by varying the reaction time with the alginate matrix and the

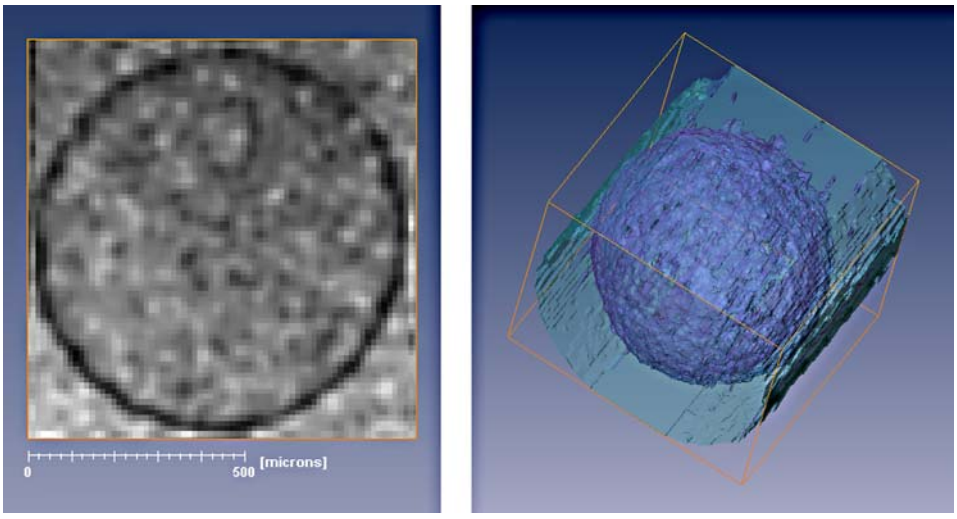

Figure 2 ^{1}H NMR gradient echo images of a single MVM alginate bead with a PLL coating applied during a 6-minute exposure. The dark circle around the alginate bead to the left (a single slice from a 3D dataset acquired with an echo time of 25 ms) has been segmented in the 3D rendering to the right. The darker blue of the 3D rendering represents the PLL shell while the aqua blue represents the surrounding media. Segmentation was performed based on pixel intensity values. Although a 3D Gaussian filter was applied to the image for the purposes of segmentation, the acquisition pixel resolution of the 3D dataset was 12.5 × 12.5 × 12.5 μm.

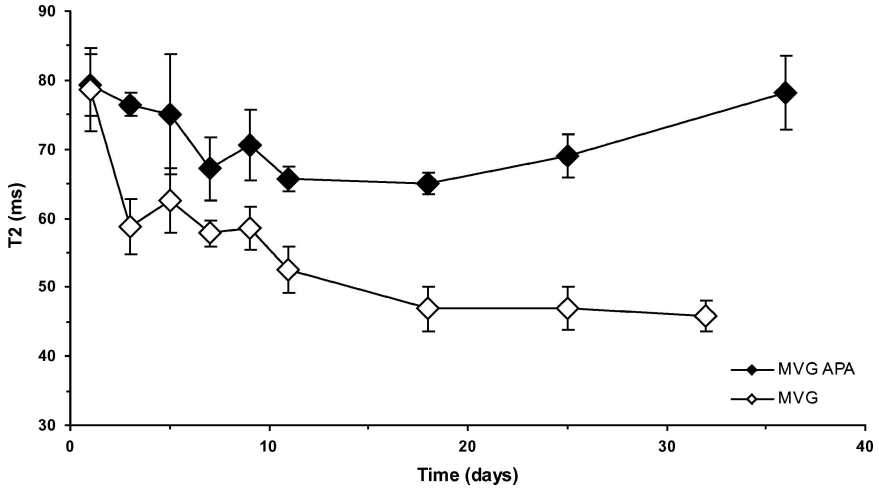

Figure 3 Temporal changes in the T_2 relaxation time of MVG alginate beads. Solid diamonds represent data acquired from MVG APA beads and open diamonds represent data acquired from MVG beads that were not coated with PLL. The error bars represent the standard deviation of the T_2 relaxation measurement.

molecular weight of the PLL polymer, which will alter the PLL thickness and the alginate/PLL interaction as has previously been reported [23, 24]. It is important to point out that the perturbations in the magnetic field that appear to be present with PLL should also be observable with other polycationic polymers that have been used to interact with alginate, such as poly-ornithin.

Another critical issue is the long-term stability of the alginate matrix during a prolonged culture. Given that alginate gel is formed by the ionic interaction between negatively charged alginate molecules and positively charged calcium (or other similarly charged cations), it is reasonable to hypothesize that during a prolonged culture the calcium ions may leak out of the alginate matrix. This process may cause the alginate/calcium bond to weaken, yielding a weaker gel that may dissolve or break. We recently have demonstrated that T_2 relaxation of alginate gels with the ^{1}H NMR imaging is a promising tool to non-invasively assess alginate gel microstructure [19]. We have continued these studies by monitoring changes in T_2 relaxation time longitudinally during a month long culture. Fig. 3 illustrates a temporal profile of T_2 measurements obtained from alginate beads made with an alginate rich in guluronic acid (MVG alginate: 73% guluronic acid content and 27% mannuronic acid content) with and without the PLL layer monitored over a month. The data show that: (i) the T_2 relaxation time of the alginate matrix decreases over time reaching a minimum within 10–14 days depending on preparation and (ii) MVG beads without PLL exhibited a bigger drop in T_2 than MVG based APA beads. Because a drop in T_2 correlates with a decrease in gel porosity, we can postulate that continuous culture of these beads (complete change of culture medium three times a week) has caused calcium ions to leak out of the bead, altering the alginate microstructure. Consequently, the pores of the MVG gel have decreased because the egg box configuration of the alginate [25] has collapsed with the decrease in available calcium. It is interesting to note that the minimum T_2 values measured during these experiments are similar to those reported previously for alginates rich in mannuronic acid [19]. Furthermore, metabolic studies with βTC3 cells encapsulated in MVG APA beads show that encapsulated cells begin to grow after only 14–21 days in culture, whereas βTC3 cells encapsulated in MVM grow continuously, unabated by the alginate matrix [26].

4. Conclusions

Using NMR microscopy and T_2 measurements, we have demonstrated that we can visualize the effects of the alginate/PLL interaction and monitor temporal changes in the structure of the hydrogel during a prolonged culture. These studies highlight the usefulness of ^{1}H NMR microscopy to provide valuable information about APA bead integrity. Future studies will investigate the use of MR microscopy to interrogate different alginates and culturing techniques to prevent the breakdown of the alginate matrix. Additionally, further work will be conducted on characterizing the PLL layer and its effect on the MR microimage. In summary, NMR microscopy has the potential to non-invasive assess biomaterials and tissue engineered constructs from both a cellular and structural perspective.

Acknowledgments

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