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Gelling kinetics and *in situ* mineralization of alginate hydrogels: A correlative spatiotemporal characterization toolbox

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ABSTRACT

Due to their large water content and structural similarities to the extracellular matrix, hydrogels are an attractive class of material in the tissue engineering field. Polymers capable of ionotropic gelation are of special interest due to their ability to form gels at mild conditions. In this study we have developed an experimental toolbox to measure the gelling kinetics of alginate upon crosslinking with calcium ions. A reaction–diffusion model for gelation has been used to describe the diffusion of calcium within the hydrogel and was shown to match experimental observations well. In particular, a single set of parameters was able to predict gelation kinetics over a wide range of gelling ion concentrations. The developed model was used to predict the gelling time for a number of geometries, including microspheres typically used for cell encapsulation. We also demonstrate that this toolbox can be used to spatiotemporally investigate the formation and evolution of mineral within the hydrogel network via correlative Raman microspectroscopy, confocal laser scanning microscopy and electron microscopy.

Statement of Significance

Hydrogels show great promise in cell-based tissue engineering, however new fabrication and modification methods are needed to realize the full potential of hydrogel based materials. The inclusion of an inorganic phase is one such approach and is known to affect both cell-material interactions and mechanical properties. This article describes the development of a correlative experimental approach where gel formation and mineralization has been investigated with spatial and temporal resolution by applying Raman microspectroscopy, optical and electron microscopy and a reaction–diffusion modeling scheme. Modeling allows us to predict gelling kinetics for other geometries and sizes than those investigated experimentally. Our experimental system enables non-destructive study of composite hydrogel systems relevant for, but not limited to, applications within bone tissue engineering.

1. Introduction

Alginates are a group of biopolymers widely used in biomedical applications and research, largely due to low immunogenicity, low toxicity and ease of forming stable hydrogels under cell compatible and near physiological conditions [1–8]. Alginate hydrogels with different physical forms, often made with encapsulated cells, have been fabricated, including micro- and macro-scale beads, alginate fibers, films, foams and 3D gels [9–15]. Recent research areas of particular interest and innovation include a wide variety of both hard and soft tissue engineering applications as well as cell encapsulation for the treatment of diseases such as diabetes [16,17]. Chemically modified alginates are also being investigated as extracellular matrix mimics [18,19]. To be able to design and fabricate new types of hydrogel based biomaterials, including hydrogel composites, a better understanding of the gel formation process and *in situ* modification strategies is needed [20,21]. When hydrogels are formed from polymers capable of ionotropic gelation, i.e. crosslinking by exogenous inorganic ions, the gel formation is a

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complex reaction–diffusion process, involving both transport of gelling and non-gelling ions, diffusion of the ungelled polymer and the formation of junction zones. For alginate, a polysaccharide consisting of mannuronic (M) acid and guluronic (G) acid residues, these junction zones consist of aligned polymers with repeating units of the G-monomer, termed the egg-box model [22,23]. In addition, it has been shown that MG-blocks also form stable junction zones [24]. A detailed understanding of these processes would not only allow for optimization of cell encapsulation, but also enable the formation of gels with complex geometries, such as fibers, films, microbeads and hollow spheres. In addition, if one is able to combine this knowledge with a detailed understanding of *in situ* modification methods, for example by incorporation of solid inorganic components, fibrous reinforcement or hydrogel-hydrogel composites, it would open new avenues for development of composite materials for application within tissue engineering and biomedical research and technology.

Modification of hydrogels with calcium phosphate (CaP) minerals to mimic the microenvironment of bone is one strategy to realize new classes of such composite materials [25–27]. We have focused on controlled mineralization of alginate hydrogels *in situ*, in a process which is compatible with cell encapsulation [28,29]. In such a system, CaP mineral is formed within the hydrogel network at the time of gelling. However the mineral phase undergoes several transformation and maturation steps, and both initial precipitation and subsequent storage will effect the properties of the final CaP phase. Processes such as crystal nucleation and growth within the gel network, solution mediated transformations and local changes in pH induced by gel formation and/or mineral precipitation are important [30–32]. Kinetics of alginate gelling have previously been investigated in non-mineralized systems and this knowledge has been applied to facilitate the formation of alginate structures with different geometries [33–37]. Mikkelsen and Elgsaeter developed a reaction–diffusion model which describes both gel formation kinetics, as well as the density distribution of alginate in calcium-alginate gels formed by the diffusion of gelling ions [36]. Thu et al. has estimated that the alginate gel front moves at a velocity of about 100 μm min^{-1} corresponding to 1.67 μm s^{-1} when 50 mM $CaCl_2$ solution was used [35]. However high spatial and temporal resolution experimental data is lacking, making progress in better understanding of the process difficult. High spatial and temporal resolution data on the gel front velocity combined with information about polymer concentration profiles, could be implemented in already existing numerical models, which in turn could be used to aid the rational design of hydrogel fabrication processes in other systems and geometries such as mineralized films, cell encapsulation matrices and microfluidic-based fabrication of alginate microbeads and fibers. Extensive knowledge of the gel system would allow for optimal process design. For example, one could maximize cell viability during cell encapsulation in microbeads, fibers or films, by finding the optimal ion concentrations and gelling times needed to make stable gels, at the same time minimizing cell exposure to potentially detrimental physicochemical conditions.

The situation is even more complex when a mineral phase, such as CaP, is precipitated within the hydrogel at the time of gelling. The process involves a complex interplay between many aspects, including fluxes of the reaction substrates and gelling ions, ion consumption due to gel formation, supersaturation controlled nucleation and growth of the mineral phase, spatial and temporal evolution of pH and transformation of the mineral phases [38]. A model experimental system in which these process could be studied *in situ*, in real time and with a range of physical methods would allow for a quantitative description of all these process. In the long run, this type of data could be used to construct complex models of the mineralization process, accounting for nucleation, growth and transformation rates of various CaP phases. This goal is however beyond the scope of this contribution, but it remains as a future challenge.

In this work we present an experimental framework for studying the spatially and temporally resolved gelling and *in situ* mineralization of alginate/calcium phosphate composites. To this experimental framework we apply a range of characterization techniques including dark field and confocal microscopy, scanning electron microscopy, and Raman spectroscopy to investigate the kinetics of gel formation and mineralization in real time. We then describe the development of methods to study the interplay between the hydrogel and mineral phases in hitherto undescribed detail.

2. Experimental

2.1. Alginate and gelling solution

All chemical reagents were purchased from Sigma–Aldrich, Norway unless otherwise stated. De-ionized water (DIW, 10–15 MΩcm) was used in all of the experiments. Sodium alginate (*Laminaria hyperborea*, Protanal LF 200S, $M_W = 2.74 \times 10^5$ g mol^{-1}, FMC BioPolymer AS, fraction of G-monomers: $F_G = 0.68$, $F_{GG} = 0.57$ and $F_{GM} = 0.11$) was used in the entire work, except for the alginate labeled with Fluoresceinamine (*Laminaria hyperborea*, Protanal SF60, $M_W = 2.2 \times 10^5$ g mol^{-1}, FMC BioPolymer AS, fraction of G-monomers: $F_G = 0.67$, $F_{GG} = 0.58$, $F_{GGG} = 0.52$) The fluorescently labeled alginate was made as described by Strand et al. [39].

Stock solutions of 1 M $CaCl_2$ and 1 M $CaCl_2$ and 0.9% (w/v) NaCl (VWR) were made in DIW. Buffering was done with 25 mM 3-(N-Morpholino) propanesulfonic acid (MOPS) at pH 6.5 and 7.4 for the gelling experiments and with 50 mM Tris(hydroxymethyl) aminomethane (TRIS) and 50 mM sodium acetate (NaAc) for mineralization experiments at pH 7 and 5 respectively. Gelling experiments refer to experiments where alginate solutions without a phosphate precursor have been used. Mineralization experiments refer to experiments where alginate solutions containing a phosphate precursor have been used. In all cases, gelling solution refers to a solution containing $CaCl_2$. The concentration of calcium and the pH in the gelling solution is specified where necessary.

For the gelling experiments, 2% (w/v) alginate solution was prepared by dissolving sodium alginate in DIW. For the mineralization experiments 1.8% alginate solutions containing 0.9% NaCl, with either 200 mM or 300 mM phosphate were prepared by dissolving sodium alginate and a mixture of $Na_2HPO_4 \cdot 7H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ (Thermo Fisher Scientific). The ratio of the phosphate precursors were chosen to give a final pH between 5 and 7. All sodium alginate solutions were stored at 4 °C between experiments.

2.2. Alginate flow cells

In order to conduct the experiments, a simple flow cell was constructed. The flow cell consisted of an alginate droplet sandwiched between two microscope slides separated by strips of 140 μm thick double-sided tape. A 1.5 μL droplet of alginate solution was placed onto the center of the slide and covered by either a second microscope slide or a cover slip as shown in Fig. 1a. Gentle pressure was applied in order to compress the droplet into a disc. 150 μL of gelling solution was then applied into the spacing between the slides to initiate the gelling.

2.3. Dark-field microscopy

The gelling reaction was studied in an Olympus IX-70 Inverted Microscope. The objective used was Olympus UPlanFl 4x/0.13NA in

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