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# Alginate Hydrogel Has a Negative Impact on in Vitro Collagen 1 Deposition by Fibroblasts

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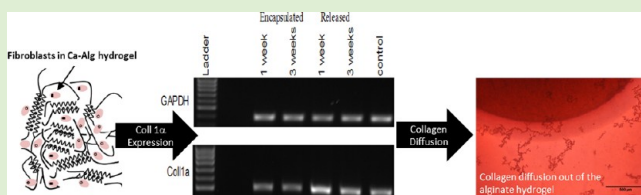
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**ABSTRACT:** Hydrogels have been widely investigated as 3D culture substrates because of their reported structural similarity to the extracellular matrix (ECM). Limited ECM deposition, however, occurs within these materials, so the resulting “tissues” bear little resemblance to those found in the body. Here matrix deposition by fibroblasts encapsulated within a calcium alginate (Ca-alg) hydrogel was investigated. Although the cells transcribed mRNA for coll I $\alpha$  over a period of 3 weeks, very little collagen protein deposition was observed within the gel by histology or immunohistochemistry (IHC). Although molecular diffusion demonstrated charge dependency, this did not prevent the flux of both positively and negative charged amino acids through the gel, suggesting that the absence of ECM could not be attributed to substrate limitation. The flux of protein, however, was charge-dependent as proteins with a net negative charge passed quickly through the Ca-alg into the medium. The minimal collagen deposition within the Ca-alg was attributed to a combination of rapid movement of negatively charged procollagen through the gel and steric hindrance of fibril formation.



## 1. INTRODUCTION

The formation of functional tissues from cells cultured with support materials in vitro is expected to help to meet the tissue regeneration needs of the aging population. Designing biomaterials that are suitable for both cell culture and tissue development is an important challenge in this area. Polymer-based hydrogels have been widely researched as 3D cell culture substrates due to perceived structural similarities with native extracellular matrix (ECM). Alginate hydrogels, in particular, have been extensively used for cell encapsulation and tissue engineering due to their mild gelation in the presence of calcium cations, low toxicity, and excellent mass transport properties. To date, problems that have been encountered when using alginate as a culture substrate have included: a lack of cell attachment, an unpredictable degradation rate, and difficulty in producing uniform structures. These problems have been addressed by covalently bonding RGD integrin recognition peptides to carboxylate groups of the alginate to promote cell attachment,<sup>1</sup> tailoring the degradation profile of alginate hydrogels by adjusting molecular weight and composition,<sup>2</sup> and using microfluidics<sup>3</sup> or soft lithography to produce gels of uniform size.<sup>4</sup> These modifications, however, can impact the microstructural architecture of the hydrogel, affecting important properties such as mechanical strength and molecular permeability. In particular, mass transport through cell culture substrates is a vital property for facilitating molecular diffusion of nutrients, waste molecules, and cell-signaling molecules. It is generally assumed that the high water content of hydrogels is advantageous for molecular diffusion.

Components of cell culture media, for instance, can diffuse into alginate hydrogels to an extent required to support the viability of encapsulated cells for several months. Indeed, it has been previously shown by the authors that 3T3 fibroblasts encapsulated in alginate hydrogel can remain viable for more than 150 days,<sup>5</sup> suggesting that flux of key molecules such as oxygen and glucose into alginate hydrogel is sufficient for cell survival. The rate and extent of diffusion of the components of cell culture media and molecules synthesized by the encapsulated cells important in tissue formation, however, is often neglected in the literature. Successful development of tissue requires the deposition of ECM as a structural support to replace the synthetic substrate as it degrades. In mammalian tissue, collagen synthesized by cells provides much of this support. For the synthesis of collagen to occur the cells require essential amino acids and other important facilitating molecules such as ascorbic acid, which, in vitro, are provided in the supplemented culture medium.

Type I collagen is synthesized as the soluble, negatively charged, precursor procollagen and consists of triple helices of collagen and carboxy terminal and amino terminal telopeptides. These telopeptides are enzymatically cleaved extracellularly from the procollagen by specific procollagen N-proteinase (PNP) and C-proteinases (PCPs), resulting in the formation of a tropocollagen. PCP, which is also known as bone

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morphogenetic protein 1 (BMP1),<sup>6</sup> catalyzes the rate-limiting step of collagen type I fibril formation; furthermore, the activity of BMP1 is specifically stimulated by the binding of procollagen C-proteinase enhancer 1 (PCPE1), a glycoprotein secreted by cells that produce fibrillar collagen. Once the telopeptides are removed, the resulting tropocollagen then interacts with neighboring tropocollagen molecules by an entropically driven process of intermolecular hydrophobic and electrostatic interactions to produce collagen fibrils with characteristic d banding periodicity. This process is known to be influenced by factors such as pH,<sup>7</sup> ionic strength,<sup>8</sup> temperature,<sup>9</sup> and the presence of sugars in the extracellular milieu.<sup>10</sup> It seems plausible to assume, therefore, that the presence of alginate, which is an anionic polysaccharide, may have an influence on collagen fibril formation. Moreover, the diffusivity of the precursor proteins through alginate may also have an impact. Indeed, recent work has shown irregular dense aggregates of smaller collagen fibrils formed in the presence of alginate.<sup>11</sup> Abnormal collagen type II assembly has also been reported in chick embryo chondrocytes when cultured in alginate beads.<sup>12</sup> In the present study, we have investigated the production of collagen type I in fibroblasts encapsulated in alginate. In addition, we have also examined the diffusivity through alginate of molecules of various sizes and charge in an attempt to understand further the formation of ECM or lack thereof in alginate cell culture substrates.

## 2. EXPERIMENTAL SECTION

**2.1. Materials.** Unless otherwise specified, all materials were obtained from Sigma–Aldrich (Poole, U.K.).

**2.2. Preparation of Alginate Hydrogels.** NIH 3T3 mouse fibroblasts were encapsulated in 2% w/v Ca-alg (MW 102 000–209 000, M/G ratio 1.56) beads by droplet exclusion and cultured in supplemented Dulbecco's modified Eagle's media, as previously described.<sup>35</sup> Acellular samples were prepared and cultured in the same manner, without the inclusion of cells.

**2.3. RT-PCR Analysis.** After 1 and 3 weeks of culture, Ca-alg beads containing 3T3 fibroblasts were homogenized, and mRNA was isolated from the suspension using an RNeasy Mini Kit (Qiagen, Crawley, U.K.). Reverse transcription of RNA to cDNA was performed using an Omniscript RT Kit (Qiagen). Polymerase chain reactions were performed using a red TAQ ready mix PCR mix, and resulting products were detected by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV illumination. GAPDH expression was used for normalization (Invitrogen, Paisley, U.K.), and the target gene analyzed was collagen  $I\alpha$ . The sequences of primers used for GAPDH were CCCATCACCATTCTCCAGGAGC (forward) and CCAGTGAGCTTCCCGTTCAGC (reverse) and AAAAGGGTTCATCGTGGCTTC (forward) and ACTCTGCGCTCTCCAGTCA (reverse) for Coll1 $\alpha$  (Invitrogen).

**2.4. Preparation of Samples for Histology and Immunohistochemistry.** After the desired culture time, acellular and NIH 3T3 cell-encapsulating Ca-alg beads were fixed in formal-saline, processed, and embedded in paraffin wax before sections of 5  $\mu$ m were cut and fixed to Superfrost plus slides (Laboratory Sales Limited, Rochdale, U.K.), as previously described.<sup>13</sup> A piece of adult rat skin was also embedded and sectioned according to this method for use as a positive control for HVG staining. Wax was removed from the slides by immersion in HistoClear (National Diagnostics, Hesse, U.K.) for 2  $\times$  20 min; then, the samples were rehydrated through graded alcohol prior to immunohistochemistry (IHC) or HVG staining.

**2.5. HVG Staining.** To detect the presence of collagen, we performed HVG staining of cell-encapsulating alginate hydrogel sections, as previously described.<sup>38</sup> Staining of acellular Ca-alg after 3 weeks of culture in supplemented culture media was done as a negative control and dermis of rat skin was done as a positive control.

HVG staining results in pink staining of collagen and blue/black staining of cell nuclei.

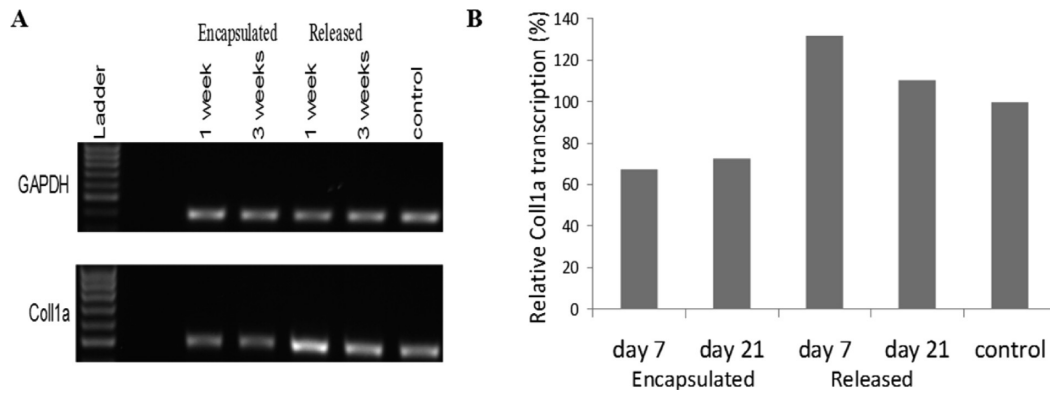
**2.6. IHC for Col-1: ICC and IHC.** IHC for Col-1 was performed on sections of cell encapsulating alginate hydrogels at 1 and 3 weeks. Cells were permeabilised by immersion in 0.5% Triton  $\times$  100 in Tris-buffered saline (TBS) for 10 min then washed 2  $\times$  5 min in TBS. Antigen retrieval was performed by immersion in 0.1% bovine trypsin in TBS at room temperature for 30 min. Slides were rinsed in tap water to stop digestion, then blocked for 30 min in 10% v/v fetal bovine serum (FBS)/TBS. Col-1 primary antibody (Abcam, Cambridge, U.K.) diluted in 2% v/v FBS/TBS was then applied and incubated overnight in a humidity chamber at 4  $^{\circ}$ C. The slides were then washed 3  $\times$  5 min in TBS before the red fluorescent Alexafluor secondary antibody (Invitrogen) diluted 1/800 in 2% FBS/was applied and incubated in a humidity chamber for 60 min at room temperature. Slides were washed 3  $\times$  5 min in TBS and mounted with Vectashield hard set mounting medium with DAPI (Vector Laboratories, Peterborough, U.K.). The mountant was allowed to dry overnight; then, samples were captured using a Axio Imager fluorescent microscope (Zeiss, Cambridge, U.K.) connected to a camera using Axiovision Rel 4.8 (Zeiss) software. Exposure times were kept constant between samples. All staining was performed in triplicate to ensure that presented images were representative. Negative controls were performed using sections with no primary antibody to see that the staining was specific. Primary antibodies used were anti-Collagen type I (Santa Cruz, Heidelberg, Germany) (1/200 dilution) and anti-vinculin (Sigma) (1/100 dilution). Green Alexafluor secondary antibodies (Invitrogen) were used at a dilution of 1/800. Rhodamine conjugated phalloidin (Invitrogen) was added with the secondary antibody for vinculin at a dilution of 1/50 to stain actin filaments red when visualized with a fluorescence microscope.

**2.7. Flux Experiments.** Alginate beads of  $4.00 \pm 0.50$  mm diameter were prepared by dropping a 5% w/v solution of Na-alginate into an excess of a 100 mM calcium chloride solution and left for 2 h at room temperature, then washed in deionized water three times and stored in an excess of 30 mM sodium bicarbonate solution. Flux of different molecules into Ca-alg beads was ascertained by placing 25 Ca-alg beads in 25 mL of stirred dye/protein solution. The change in the solutions' concentrations was assayed over time using the bicinchoninic acid assay for albumin or by spectrophotometer (Cecil, CE2040) for all other solutions. Flux was calculated by plotting a graph of concentration against time and then finding the gradient of the linear section. This was then divided by the total surface area of the beads and converted to give flux in units of mg/m<sup>2</sup>s.

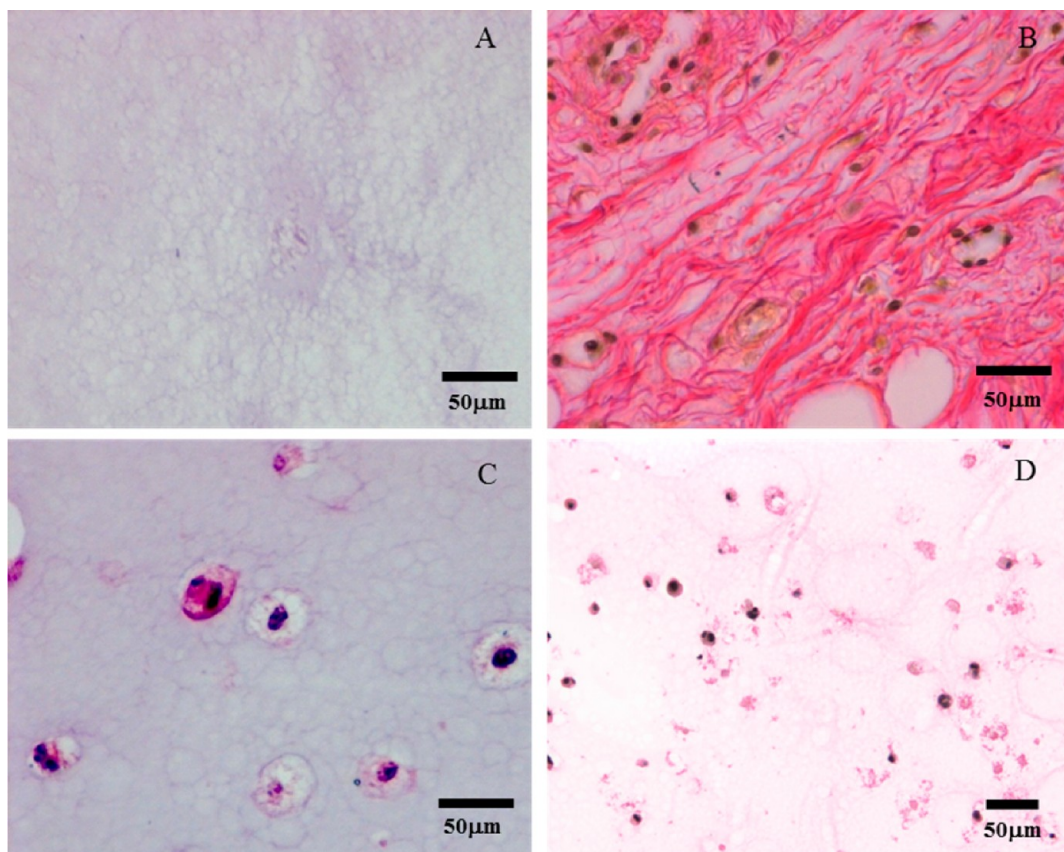
**2.8. Amino Acid Diffusion Experiments.** Alginate beads were prepared as described above; then, a single bead was stored for 2 h in a solution of either tryptophan or arginine prepared in HPLC-grade water (Millipore) at concentrations analogous to those in DMEM cell culture media. The beads were then removed from the amino acid solutions and washed three times in deionized water before they were placed in a 2% w/v solution of sodium citrate. Once the bead had dissolved, the sample was filtered using a centrifuge filter (4000 rpm for 5 min) with a 5000 Da molecular weight cut off.

**2.9. Amino Acid Detection Using HPLC.** Precolumn derivatization of filtered amino acid samples was performed by the addition of a 100  $\mu$ L sample to 400  $\mu$ L of the fluorescent marker *o*-phthalaldehyde (OPT), followed by thorough mixing using a vortex for 2 min. The sample was then injected onto a ODS C18 Ultrasphere Column 250 mm  $\times$  4.6 mm (Hichrom). Elution of the derivitized amino acids was monitored with a Shimadzu RF-535 fluorescence HPLC detector with an excitation wavelength of 340 nm and an emission wavelength of 455 nm. Two mobile phases were used for elution; Mobile phase A consisted of 0.05 M sodium phosphate buffer (pH 5.5), methanol, and THF (75:20:5 respectively). Mobile phase B consisted of 80% methanol and 20% of the 0.05 M NaH<sub>2</sub>PO<sub>4</sub> buffer. The pH of the phosphate buffer was adjusted to 5.5 using 1 N NaOH. The following gradient elution was used: 0–18 min, 40–50% B, 18–32 min 50–100% B.

**2.10. Ascorbic Acid Diffusion Experiments.** Ascorbic acid diffusion was assayed by submerging 10 alginate beads in 5 mL of



**Figure 1.** (A) RT-PCR analysis of GAPDH and Coll1a expression by 3T3 fibroblasts encapsulated in 2% w/v Ca-alginate hydrogel for 1 and 3 weeks. Expression is shown for encapsulated cells and those that are released and grown as monolayers for 48 h and control fibroblasts, grown as monolayers having never been encapsulated. (B) Relative Collagen 1 $\alpha$  transcription of these samples.



**Figure 2.** HVG staining of (A) acellular alginate gel, (B) rat dermis, and (C) encapsulated cells 1 week after encapsulation and (D) 3 weeks after encapsulation.

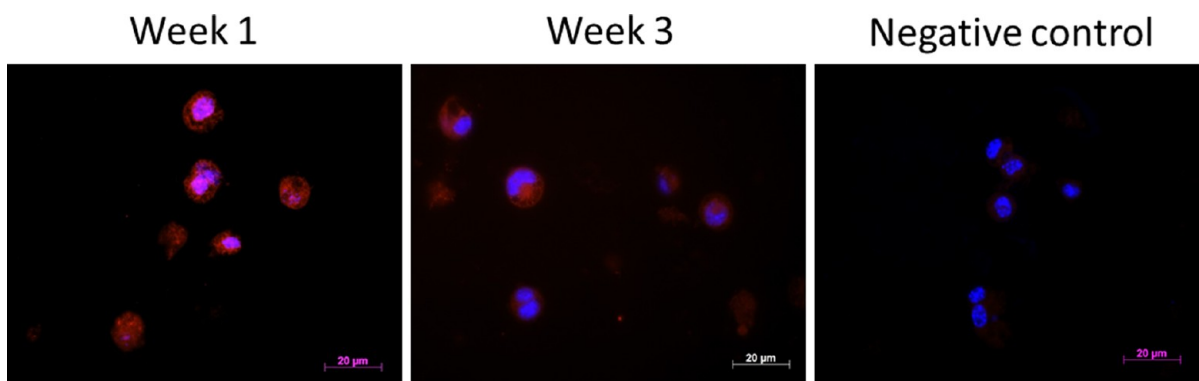
ascorbic acid solution 250 mM per time point. To recover the ascorbic acid imbibed by the alginate, we washed the beads three times with deionized water, then submerged them in 5 mL of 2% w/v sodium citrate. Once the beads were fully dissolved, 100  $\mu$ L was added to 900  $\mu$ L of DCPIP 1 mM; then, absorbance was measured at 470 nm.

**2.11. Sircol Collagen Assay.** Soluble rat tail collagen 2.1 mg/mL was adjusted to pH 4.5 using 1 M NaOH and then added to a solution of 5% w/v sodium alginate at a ratio of 10:1 alginate/collagen. The mixture was added dropwise into 100 mM CaCl<sub>2</sub>, and the resultant beads were allowed to cure for 2h. Twenty alginate beads were then added to 20 mL of unsupplemented DMEM, and samples of the media were taken at selected time points for up to 6 h. Each sample (100  $\mu$ L) was then analyzed for soluble collagen content using a Sircol collagen assay kit. In brief, 1 mL of Sircol dye reagent was added to each

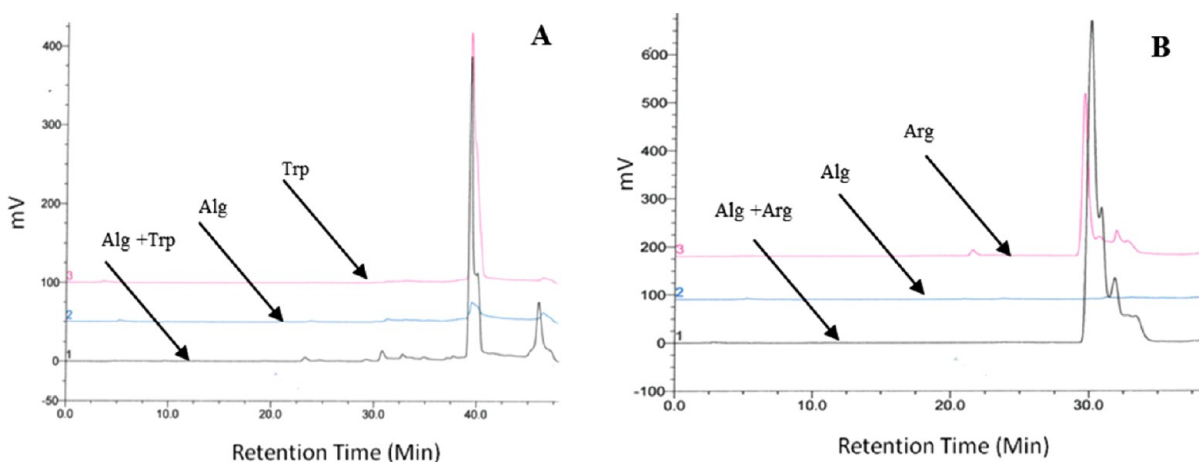
sample, and samples were shaken gently for 30 min. The samples were then centrifuged for 10 min at 12 000 rpm using a microcentrifuge. The supernatant was then drained and the pellet was washed with 750  $\mu$ L of acid-salt wash reagent and recentrifuged for 10 min at 12 000 rpm. The supernatant was removed and to the pellet 1 mL of alkali reagent was added and vortexed for 5 min before absorbance was measured at 540 nm.

### 3. RESULTS AND DISCUSSION

The transcription of collagen I $\alpha$  mRNA by 3T3 fibroblasts encapsulated in a 2% (w/v) Ca-alg hydrogel matrix for 3 weeks was analyzed using semi-quantitative reverse-transcription PCR (RT-PCR) and compared with that prior to encapsulation.



**Figure 3.** Immunohistochemistry for Collagen 1  $\alpha$  produced by encapsulated cells at 1 and 2 weeks and negative control.



**Figure 4.** Overlaid HPLC traces of amino acids alone (pink), alginate alone (blue), and alginate incubated in amino acid solution for 2 h (black) for (A) tryptophan and (B) arginine.

Analysis of the RT-PCR data revealed that collagen I $\alpha$  was transcribed by the encapsulated fibroblasts (Figure 1a) at a level that was only approximately 25–30% lower than before encapsulation (Figure 1b).

Because collagen I $\alpha$  transcripts were evident in the cultured cells, the hydrogel matrix was embedded and examined for matrix deposition after 1 and 3 weeks of culture using hematoxylin–Van Gieson (HVG) staining and using IHC with a fluorescent antibody for collagen I $\alpha$ . The HVG staining of sections is shown in Figure 2, and in the negative control there was very little background staining (Figure 2A), whereas the positive control (rat dermis; Figure 2B) consisted of matrix stained strongly pink and cell nuclei stained blue-black. After 1 (Figure 2C) and 3 weeks (Figure 2D) of encapsulation there was very little staining in the gel matrix, indicating minimal matrix deposition. After 3 weeks, there was a number of postmitotic nuclei present within the matrix, which may have indicated limited cell proliferation (Figure 2D), as has previously been reported.<sup>13</sup> The only matrix evident within the structure appeared to have been formed in the immediate vicinity of the cells (Figure 2C,D).

IHC did not detect collagen I $\alpha$  within the hydrogel matrix when cultured for up to 3 weeks and confirmed that the matrix immediately surrounding the cell was rich in collagen I $\alpha$  (Figure 3).

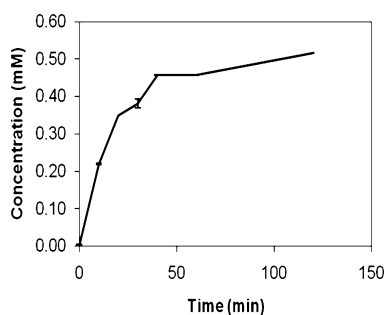
RT-PCR analysis seems to be the most popular method employed to demonstrate collagen synthesis by cells encapsulated in Ca-alg hydrogels and has been used to demonstrate

appreciable collagen expression by chondrocytes,<sup>14</sup> osteoblasts,<sup>15</sup> and bone marrow stromal cells<sup>16</sup> encapsulated in alginate hydrogels. Whereas analysis of actual collagen protein deposition has been assayed, rather than the transcript, it has, however, been reported that collagen expression by mammalian cells encapsulated in alginate hydrogels is limited. For example, Leddy et al. (2004) reported limited collagen protein expression by human adipose-derived stem cells when encapsulated in alginate hydrogels when compared with cells encapsulated in other hydrogels.<sup>17</sup> Alsberg et al. (2001) showed via histological staining that collagen production occurred when osteoblasts were encapsulated in alginate and cultured *in vivo* for bone tissue engineering, but collagen appeared to accumulate only in areas where the alginate had completely degraded and was therefore possibly only being produced by cells released from encapsulation.<sup>15</sup>

The transcription of collagen I $\alpha$  by the cell population but the absence of collagen protein within the gel matrix suggested that collagen was either not being translated or that fibril formation was not occurring within the gel matrix. It was initially hypothesized that the translation of collagen I $\alpha$  could have been hindered by a diffusional limitation imposed on the cells by the Ca-alg matrix. Although there was evidently sufficient flux of small molecules into the gel to maintain cell viability, perhaps flux of molecules required for collagen synthesis was insufficient, causing dysfunctional collagen synthesis. To assess the availability of amino acids essential for collagen production, we analyzed the passive loading into

alginate beads of the amino acids tryptophan (an essential hydrophobic amino acid) and arginine (a positively charged amino acid at physiological pH). Both tryptophan and arginine were detected using HPLC following derivatization of the amino acids with the fluorescent marker OPT. Diffusion of ascorbic acid was also measured by monitoring the reduction in absorbance of 2,6-dichlorophenolindophenol (DCPIP). Figure 4 highlights peaks for both tryptophan (Figure 4A) and arginine (Figure 4B) within the alginate gel following 2 h of exposure to each amino acid. This illustrated that both the hydrophobic tryptophan and positively charged arginine readily diffused into alginate.

Furthermore, negatively charged ascorbic acid was also shown to diffuse freely into alginate (Figure 5). This was most



**Figure 5.** Concentration of ascorbic acid imbibed by alginate beads as a function of time when exposed to 200 mM ascorbic acid solution. Vertical error bars represent the standard deviation of the reported mean values ( $n = 3$ ).

likely due to the relatively small molecular weights of the amino acids and ascorbic acid. Although the absence of collagen deposition within the gel could not be attributed to a limitation in the quantity of small molecules (MW <500) into the gel, it was possible that collagen deposition could have been prevented by limitations placed on the diffusion of larger molecules into the gel. Indeed, several authors have evaluated how molecule size can affect diffusion into Ca-alg gels, for which dextrans have been extensively employed because they are linear molecules with a low charge density at physiological pH. These studies indicated that the size limitation for flux in Ca-alg was ~2000 kDa.<sup>18–20</sup>

It has also been demonstrated that other molecules required for cell viability, such as glucose, effectively enter alginate hydrogels,<sup>21,22</sup> which is not surprising because glucose is a small molecule of no overall charge at physiological pH. The flux of cationic antibiotics through anionic alginate has also been studied due to its relevance to the treatment of lung infections in cystic fibrosis patients. Flux through the alginate and was seen to be restricted by means of the polyanionic alginate ionically trapping the cationic antibiotics.<sup>23,24</sup> Stewart et al. (1993) performed flux studies at a pH value of 6.2 because they were interested in the use of alginate hydrogel to encapsulate dairy microbes. They showed that alginate had noncovalent interactions with charged proteins, including isoelectric interactions. It was hypothesized that electrostatic repulsion of negatively charged molecules should be expected due to the negative charge of alginate but that local positive and negative charges may also alter the flux of molecules into alginate hydrogels.<sup>19</sup> To determine the influence of size and charge on flux into Ca-alg at physiological pH, we measured the diffusion of albumin, rose bengal, and trypan blue (which all carry a net

negative charge) at pH 7.4. None of these three molecules diffused into the Ca-alg matrix (Table 1). In comparison,

**Table 1. Charge and Molecular Weight and Mean Flux Rates of Methylene Blue, Hemoglobin, Rose Bengal, Trypan Blue, and Albumin into 5% w/v Ca-alg Beads at pH 7.4 ( $n = 3$ )<sup>a</sup>**

	methylene blue	hemoglobin	rose bengal	trypan blue	albumin
charge	–	0	–	–	–
MW	347.91	68000	1017.65	960.8	66430
flux (mg/m <sup>2</sup> ·s)	0.14	0.07	0	0	0

<sup>a</sup>Methylene blue and hemoglobin diffuse into alginate, whereas rose bengal, albumin, and trypan blue fail to diffuse in.

methylene blue and hemoglobin, which carry a positive and neutral charge, respectively, pass through the hydrogel matrix at neutral pH (Table 1). These results confirmed the findings of previous studies that methylene blue<sup>25</sup> and hemoglobin<sup>26</sup> enter Ca-alg under physiological conditions and that albumin does not.<sup>22</sup> The lack of albumin flux was, however, previously attributed to the size of the albumin molecule rather than the negative charge.

These results prompted further investigation into the role charge plays in diffusion into alginate. To achieve this, we measured the flux of hemoglobin (which has a molecular weight of ~68 000 Da, comparable to that of albumin) at pH 6, 7.4, and 9, where the net charge of the protein changes from positive to neutral to negative, respectively. This was compared with the flux of methylene blue across the same pH range, for which the overall charge remains positive under all three conditions. The results illustrated that hemoglobin entered the alginate when the overall charge was either positive or neutral but not when it was negative (Table 2). To be certain that the

**Table 2. Mean Flux Rate of Hemoglobin and Methylene Blue for Different pH Values ( $n = 3$ )<sup>a</sup>**

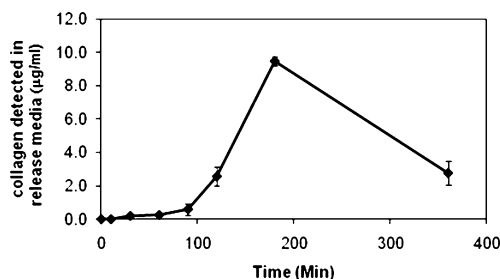
		pH 6	pH 7.4	pH 9
hemoglobin	charge	+	0	–
	flux rate (mg/m <sup>2</sup> ·s)	0.19	0.09	0
methylene blue	flux rate (mg/m <sup>2</sup> ·s)	0.13	0.13	0.11
	charge	+	+	+

<sup>a</sup>pH makes little difference to the flux of methylene blue, which remains positively charged at all three pHs, suggesting that the structure of the gelled alginate is not influenced by the small pH changes. Flux of hemoglobin does occur at pH 6 and 7.4 when the net charge of the molecule is positive and neutral, respectively. At pH 9, when the overall charge of the molecule was negative, there was no flux of hemoglobin.

change in flux was not a result of a change in size due to aggregation of the hemoglobin at the different pHs tested, the samples were sized by nanoparticle tracking analysis using a Nanosight LM10 apparatus. The particle size distributions obtained for the hemoglobin samples at pH 6, 7.4, and 9 were in a similar range with no significant evidence of aggregation ( $D_{50}$  at pH 6 173 nm, pH 7.4 194 nm, and pH 9 177 nm). It is known that alginate is susceptible to both acid and alkaline degradation, which could have an impact on the gel structure and subsequent diffusivity. Degradation, however, is at its minimum between pH 5 and 10 and increases substantially below pH 5 and above pH 10 due to acid hydrolysis and  $\beta$ -

elimination, respectively.<sup>27,28</sup> Because the flux of the methylene blue remained largely unchanged by pH, it can be concluded that there was no significant change in the structure of the Ca-alg and that the limited flux of hemoglobin at pH 9 was due to the negative charge of the protein at this pH value. The higher flux of hemoglobin into Ca-alg at pH 6, when the charge of the protein was positive, could be attributed to the electrostatic attraction between negatively charged alginate and positively charged hemoglobin. This potential electrostatic attraction between positively charged peptides/proteins and alginate has been previously described.<sup>29–31</sup>

These results could provide the explanation for the lack of matrix found in our alginate constructs. Because procollagen molecules have a net negative charge, procollagen may have been weakly retained by the gel and so dispersed into the culture medium before fibril formation could occur. Furthermore, BMP-1 and PCPE-1 (BMP1 (Pi 6.48) and PCPE-1 (Pi 7.41) Source www.phosphosite.org), which have net negative and neutral charges, respectively, at physiological pH, also have the potential of diffusing out of the alginate gel. Consequently, the diffusion of soluble collagen out of alginate hydrogel was investigated. The results revealed that small amounts of soluble collagen diffused from the alginate gel into the surrounding media. This was shown to increase with time before decreasing (Figure 6), indicating that the collagen is released by the

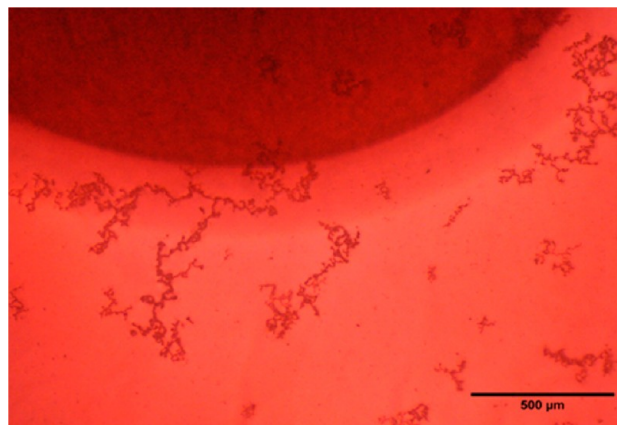


**Figure 6.** Soluble collagen detected in DMEM following release from alginate beads. Vertical error bars represent the standard deviation of the reported mean values ( $n = 3$ ).

alginate gel in the soluble form before converting to the insoluble form. To determine if this was the case, we treated a sample of the 6 h media with acetic acid for 2 h to resolubilize the collagen, and the sample was remeasured for soluble collagen and there was a five-fold increase to levels obtained at 90 min following incubation.

It should be noted, however, that although there was collagen released into the media, there was no strong evidence of ordered fibril formation (Figure 7). Rather, a network of material was formed that radiated from the periphery of the bead and was evident in the media around the beads. It was also noted that a raft of material eventually formed at the air–liquid interface.

We believe that this diffusion of collagen also occurs from cell synthesized collagen when cultured in alginate. When secreted by the cells, procollagen molecules consist of a triple helix (composed of three  $\alpha$ -chains) of  $\sim 300$  nm in length and 1.5 nm in diameter,<sup>32</sup> and at either end there is the N-terminal and C-terminal telopeptides that give the molecule a net negative charge at physiological pH. Alginate hydrogels are known to have a wide pore size distribution from 5 to 150 nm<sup>33</sup> with the larger pore sizes found in alginates containing a high proportion of guluronate residues (like those used in this



**Figure 7.** Micrograph showing collagen released from an alginate bead into cell culture media (DMEM) following 6 h of incubation at 37 °C.

study). In addition, cavities, fractures, and shafts can exist within alginate gel beads, which allow for an easier diffusion of substrates and products throughout the gel matrix.<sup>25</sup> Furthermore, Mu et al. (2007) have previously reported that the hydrodynamic radius of solubilized collagen is in the region of 125 nm.<sup>34</sup> This combination favors, therefore, some collagen diffusion from the alginate in the soluble form prior to fibril formation. Furthermore, favorable diffusion conditions are also apparent for the negatively charged BMP-1 ( $\sim 100$  kDa) and PCPE-1 ( $\sim 50$  kDa). The rate of permeation through the gel is, however, likely to be different for each of the proteins.

This simplistic idea that procollagen and the proteins involved in the cleavage of the procollagen telopeptides diffuse out of alginate hydrogels, preventing collagen fibril formation, does not explain the pericellular collagen deposition shown in Figure 2C,2D. We postulate that the pericellular space between the cells and the alginate increased over the first 7 days of culture as a result of the cellular requirement for calcium, which caused localized degradation of the alginate. Indeed, we have previously shown that degradation of alginate occurs rapidly in fibroblast-loaded alginate hydrogels within these first 7 days.<sup>35</sup> Within this space it is likely that a small amount of collagen begins to assemble as the procollagen and BMP-1 are secreted by the cells. However, lateral fibril growth is prevented from proceeding by steric inhibition when approaching the alginate gel network. Furthermore the increased entropy of the system would favor lateral association of the collagen producing irregular and dense aggregates of smaller fibrils, which supports previous reports of abnormal collagen formation in the presence of alginate.<sup>11,12,36</sup> It is known that the presence of glycosaminoglycan polyanions can have a striking effect on the precipitation of collagen, and it has been postulated that the presence of such polyanions helps determine the size and rate of fibril formation *in vivo*.<sup>37</sup> It is therefore likely that alginate (a polyanion) may have a similar effect retarding fibril formation *in situ*. It is our opinion that the porous, negatively charged, hydrated alginate gel network and the BMP-1/PCPE-1-dependent negatively charged procollagen with its intrinsic environmentally sensitive, self-assembly mechanism generates a situation that is favorable for procollagen, BMP-1, and PCPE-1 to diffuse from 3D alginate cell culture substrates into the culture medium, preventing *in situ* fibril formation. Routine media change would not allow sufficient time for the concentration of collagen in the media (which is present in excess volume) to reach the critical concentration required for

nucleation and growth of the fibrils. This provides an explanation why no collagen fibrils are found in the media when alginate is used as a cell culture substrate.

#### 4. CONCLUSIONS

Here it has been shown that although collagen type 1 $\alpha$  is transcribed by encapsulated fibroblast cells, collagen protein is not deposited within the Ca-alg matrix, with only small quantities being deposited immediately around the entrapped cells. The diffusion of collagen precursor molecules out of the alginate gel before fibrillization occurs is thought to cause this observed lack of matrix formation. This finding questions the wisdom of using RT-PCR to evaluate the deposition of ECM by encapsulated cells. It also suggests that a paradigm shift is needed in the way tissue culture scaffolds are designed. Although biopolymeric hydrogel matrices do resemble the ECM from a morphological point of view, they are likely to influence significantly matrix deposition and could result in the formation of tissue unlike that found in the body. Indeed, Ca-alg is likely to be better employed as a cell delivery vehicle, capable of disintegration and cell release to a desired site in vivo. Furthermore, this work highlights the need for a thorough understanding of molecular diffusion properties in hydrogel tissue culture substrates and its potential effects on tissue development, and the realization of this is extremely important.

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##### Notes

The authors declare no competing financial interest.

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