Hindawi Publishing Corporation
Research Letters in Nanotechnology
Volume 2009, Article ID 614301, 4 pages
doi:10.1155/2009/614301

Research Letter

Protein Fibrillar Hydrogels for Three-Dimensional Tissue Engineering

Hui Yan,[1] Antti Nykanen,[2] Janne Ruokolainen,[1] David Farrar,[3] and Aline F. Miller[1]

[1] *Manchester Interdisciplinary Biocentre & School of Chemical Engineering and Analytical Science, University of Manchester, 131 Princess Street, Manchester M60 1QD, UK*
[2] *Department of Engineering Physics and Mathematics and Centre for New Materials, Helsinki University of Technology, P. O. Box 2200, TKK 02015, Finland*
[3] *Smith and Nephew Research Centre, York Science Park, Heslington, York YO10 5DF, UK*

Correspondence should be addressed to Aline F. Miller, aline.miller@manchester.ac.uk

Received 8 April 2009; Accepted 17 June 2009

Recommended by Maryam Tabrizian

Protein self-assembly into highly ordered fibrillar aggregates has attracted increasing attention over recent years, due primarily to its association with disease states such as Alzheimer's. More recently, however, research has focused on understanding the generic behavior of protein self-assembly where fibrillation is typically induced under harsh conditions of low pH and/or high temperature. Moreover the inherent properties of these fibrils, including their nanoscale dimension, environmental responsiveness, and biological compatibility, are attracting substantial interest for exploiting these fibrils for the creation of new materials. Here we will show how protein fibrils can be formed under physiological conditions and their subsequent gelation driven using the ionic strength of cell culture media while simultaneously incorporating cells homogeneously throughout the gel network. The fibrillar and elastic nature of the gel have been confirmed using cryo-transmission electron microscopy and oscillatory rheology, respectively; while cell culture work shows that our hydrogels promote cell spreading, attachment, and proliferation in three dimensions.

Copyright © 2009 Hui Yan et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Hydrogels have recently attracted increasing attention as tissue engineering scaffolds for repairing and regenerating tissues [1–4]. There is a wide variety of natural and synthetic materials currently being employed to create such materials but recent research effort has focused on using natural materials as they are cheap, abundant, and require limited functionalization [5–7]. With this in mind we have previously demonstrated that hen egg white lysozyme (HEWL) protein can form hydrogels at physiological pH simply by adding a small quantity of the reductant dithiothreitol (DTT) which encourages the protein to gel under mild conditions [8, 9]. HEWL was selected as it is a small globular protein and contains both α-helix and β-sheet in its secondary structure and has high solubility in water. We went on to show that the mesh size of the hydrogel and its mechanical properties could be controlled by varying concentration and our two-dimensional cell culture work demonstrated that these hydrogels are cytocompatable [9, 10]. However, before these materials can find any tissue engineering application, cells need to be incorporated throughout the hydrogel and their viability explored.

In this letter we will outline a novel method for the incorporation of cells in 3-dimensions directly in a HEWL hydrogel by using the ionic strength of cell culture media to drive gel formation while simultaneously incorporating cells homogeneously within the hydrogel. A schematic for this process is given in Figure 1. The resulting gel morphology and mechanical behavior have been explored using cryo-transmission electron microscopy and oscillatory rheology respectively, and their potential for use as viable supports for three-dimensional cell growth has been explored by analyzing cell distribution and viability within the hydrogel scaffold.

The influence of ionic strength on HEWL gelation behavior was investigated with 20 mM DTT over a range of protein concentrations (0–3 mM) in the presence of NaCl (0–100 mM). NaCl was selected as the salt due to its physiological presence in the body and in cell culture media. These

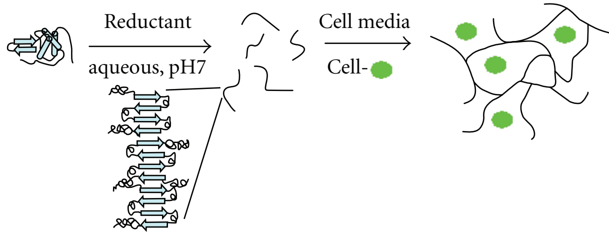


Figure 1: A schematic of the protein unfolding, fibrillation, and gelation induced by ionic strength in the presence of cells.

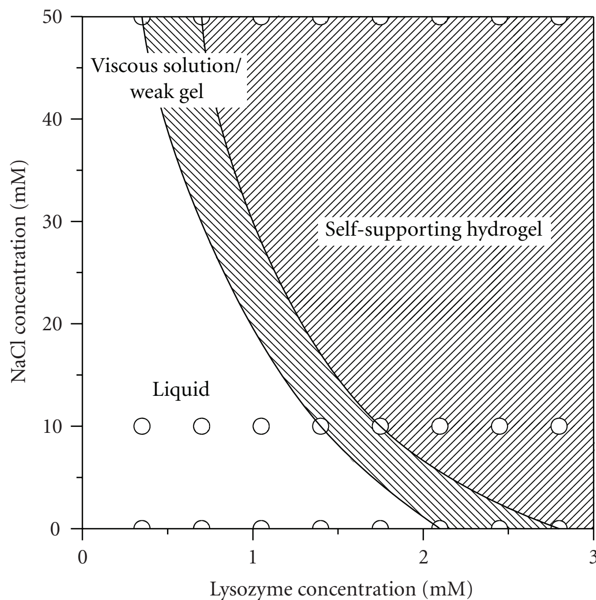


Figure 2: Phase diagram of HEWL gelation as a function of protein concentration and NaCl concentration (empty circles represent sample points).

samples were subjected to one heating/cooling cycle and the changes of their macroscopic appearance were recorded and used to map out a detailed phase diagram (Figure 2). Transparent hydrogels are obtained at 1.5 mM HEWL with 10 mM NaCl. This is in contrast to the freely flowing viscous liquid obtained with no NaCl [10]. When the NaCl concentration is increased to 50 mM, turbid gels form, and when the NaCl concentration is increased further to 100 mM, turbid solutions form where the protein precipitates. Similar gelation behavior was observed for HEWL at 2.0 and 3.0 mM. It is clear that the presence of salt encourages gel formation as the critical gelation concentration decreases to 1.0 mM at 20 mM NaCl in comparison to 3.0 mM with no NaCl. It is believed that the salt screens the charges carried by lysozyme therefore making them more attractive to each other [11, 12]. This enhanced attraction will consequently encourage their unfolding and subsequent incorporation into fibrils, which in turn will lead to an increase in fibril number, and possibly length, thus facilitating entanglement to form a macroscopic, self-supporting hydrogel at lower protein concentrations.

Cells are cultured in vitro in the presence of media containing various organic salts, therefore we aim to capitilize on the ionic strength of cell media to trigger the gelation of lysozyme and at the same time incorporate cells homogeneously within the hydrogel. Figure 2 shows that a final salt concentration between 10 and 50 mM is optimum as higher salt concentration causes the protein to precipitate and no gel is formed below 10 mM of salt. The ionic strength, I_c, of a typical human dermal fibroblast (hDF) basal media (Dulbecco's Modified Eagles Medium (DMEM) containing 10% foetal bovine serum (FBS) and 1% penicillin/ streptomycin) was calculated to be 189 mM using (1) and the formulation obtained from Invitrogen's product data sheet,

$$I_C = \frac{1}{2}\sum_{B=1}^{n} c_B z_B^2, \tag{1}$$

where c_B is the molar concentration of the ion and z_B is the charge that ion carries. It is evident therefore that a 5-fold dilution of cell media will give a hydrogel with a final ionic strength of ~37.8 mM, which is within the preferred range of 10–50 mM. Solutions of 1 mM lysozyme were subsequently prepared in the presence of 20 mM DTT. The samples were heated to 85 °C, incubated for 10 minutes and cooled gradually to 37 °C where a viscous solution was obtained. A volume of 200 μL of this solution was transferred gently into a millicell insert and 50 μL of the cell culture media was then added quickly and mixed gently using the micropipette. The millicell inserts were then placed in a 24 wellplate and incubated at 4 °C. The samples were checked by inverting the millicell every 15 minutes. Transparent, self-supporting gels formed with the addition of cell culture media after ~1 hour.

To confirm the fibrillar nature of our hydrogels we used Cryo-transmission electron microscopy (Cryo-TEM) to compare the morphology of a 1 mM lysozyme/DTT solution in pure water and a 1 mM lysozyme/DTT gel in cell culture media (Figure 3). Fibril structures with a diameter of ~6–8 nm and ~200–300 nm in length are present in the sample in pure water. Such short fibrils exist as isolated entities, hence the macroscopic sample remains a liquid and flows when inverted. In contrast, the morphology of the gel formed in cell media is characterized by a densely entangled homogeneous fibrillar network structure. The fibrils have a similar radius of ~6–8 nm but the number and length of the fibrils have increased significantly. Such observation confirms previous speculation that more protein molecules have been incorporated into fibrils in the presence of salt.

Figure 4 compares the mechanical spectra performed at room temperature for the viscous solution prepared in the presence of DTT in pure water and the gel prepared in DTT and culture media. The samples were all prepared in situ to preclude any artifacts that may be introduced when samples are transferred to the rheometer due to the extra shear applied.

In each case, the storage modulus, G', is found to be approximately an order of magnitude larger than the loss modulus, G'', indicative of an elastic rather than viscous

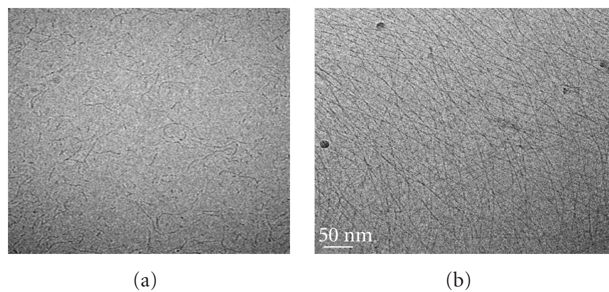


Figure 3: Cryo TEM images of 1.0 mM lysozyme/DTT mixture in pure water (a) and in hDF cell culture media (b). The scale bar in each case represents 50 nm.

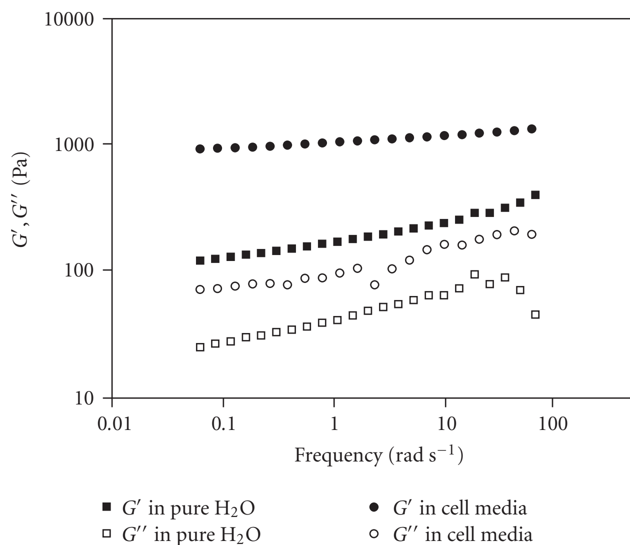


Figure 4: Elastic, G' and viscous, G'' moduli of 1 mM cured lysozyme solutions/hydrogels in pure water and in hDF culture media as a function of shear frequency obtained at 25 °C.

material. Also both G' and G'' are essentially independent of frequency over the range 10^{-2}–10^{2} rad s^{-1}, which indicates the dominant viscoelastic relaxations of the network are at lower frequencies, that is, the relaxation time, τ, of the network is long. Such rheological behavior matches the characteristic signature of a solid-like gel. It is clear that G' increases when cell culture media is present; G' is [$\sim$100] Pa for the lysozyme/DTT mixture and [$\sim$1000] Pa for the lysozyme/DTT/hDF mixture. These results confirm that the addition of salt can be used to enhance the mechanical strength of the sample.

However hDFs were used to test the potential of our method and material for use as a three-dimensional tissue engineering scaffold. In particular, we monitored how well our method led to the incorporation of cells homogeneously throughout the fibrillar network and also the biocompatibility of the hydrogel. hDFs were chosen for two reasons: firstly they provide information on hydrogel interaction with human cells and secondly fibroblasts have a relatively fast growth rate in comparison to other cells, for example,

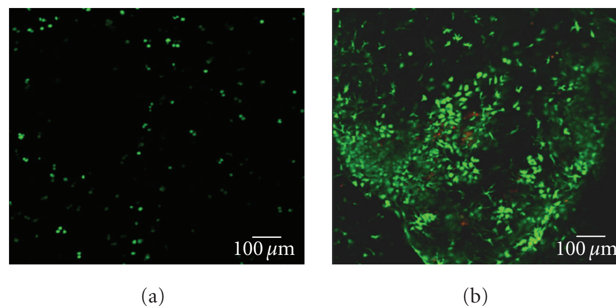


Figure 5: Confocal micrographs of live/dead staining of 1 mM lysozyme hydrogels in hDF culture media 24 hours (a) and 48 hours (b) after seeding. The scale bar represents 100 μm.

mature chondrocytes. Cells were suspended in hDF media and lysozyme hydrogels prepared as described earlier using a 4 : 1 ratio of lysozyme solution to cell media. The final density of cells in the hydrogel was 5×10^{6} cells mL^{-1}. It should be noted that due to the requirement of cell growth, 1 mL of loaded hydrogel was added into each well. Replicates of 3 gels were evaluated during 3 sets of experiments. Typical fluorescent micrographs of live/dead staining 24 and 48 hours after seeding are shown in Figure 5. These images were both taken of the same region at a depth of $\sim$50 μm.

These show that the cells are rounded and display bright green fluorescence, indicating the cells are alive and that the HEWL hydrogel provides a viable support for hDF cells. Furthermore cells with variable degrees of brightness are observed due to their presence in different focal-planes, which indicates the cells are distributed throughout the hydrogel. This is confirmed by imaging through different optical sections and our results show that cells are distributed homogeneously throughout the scaffold. It is also clear that the number of cells increases over time as there are a greater number of cells present after 48 hours of culture. This was semiquantified using Proplus software by counting the number of red (dead) and green (live) spots and taking the average from five images of different parts of the hydrogel. Each image was circa $1 \times 1\,\mu$m and the scaffold as a whole was circa 1 cm in diameter. The number of dead cells present was of a very low number and did not increase significantly over time: 3 ± 1 after 2 hours, 8 ± 3 after 24 hours and 19 ± 4 after 48 hours. Interestingly slightly more cell death was observed in the middle of the scaffold (35 ± 8 after 48 hours) in comparison to the surface, presumably due to the nutrients not being able to migrate through the scaffold in a time-efficient manner. In contrast the live cell number increased from 180 ± 50 after 2 hours to 605 ± 140 after 24 hours and to 1510 ± 260 after 48 hours. This number and trend did not change significantly throughout the scaffold confirming our hydrogels also support cell proliferation in 3-dimensions. Further cell culture work with matrices with a larger mesh size is now underway which will also include immnohistochemical staining of Collagen type II.

In summary, this research letter sets out a novel method for the preparation of self-supporting, fibrillar hydrogels from lysozyme with hDF cells distributed throughout the

hydrogel. This was achieved simply by using a reductant to induce protein self-assembly and the ionic strength inherent in cell culture media to trigger slow gelation after cells have been incorporated in the gel matrix. Moreover the hydrogels prepared have been shown to be cytocompatible, promoting cell attachment and spreading without the need for the additional incorporation of cell-adhesive peptide sequences. Such results indicate an alternative strategy of using proteins as building blocks to construct novel materials, thus making such protein hydrogels promising candidates as three-dimensional tissue engineering scaffolds.

Acknowledgments

The authors gratefully acknowledge financial support from the EU 6th Framework Marie Curie Early Stage Training Programme (ExPERT - 305730).

References

[1] M. R. Hynd, J. N. Turner, and W. Shain, "Applications of hydrogels for neural cell engineering," *Journal of Biomaterials Science, Polymer Edition*, vol. 18, no. 10, pp. 1223–1244, 2007.

[2] U. Hersel, C. Dahmen, and H. Kessler, "RGD modified polymers: biomaterials for stimulated cell adhesion and beyond," *Biomaterials*, vol. 24, no. 24, pp. 4385–4415, 2003.

[3] N. A. Peppas and R. Langer, "Origins and development of biomedical engineering within chemical engineering," in *AICHE Journal*, vol. 50, no. 3, pp. 536–546, 2004.

[4] B. Baroli, "Hydrogels for tissue engineering and delivery of tissue-inducing substances," *Journal of Pharmaceutical Sciences*, vol. 96, no. 9, pp. 2197–2223, 2007.

[5] J. F. Mano, G. A. Silva, H. S. Azevedo, et al., "Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends," *Journal of the Royal Society Interface*, vol. 4, no. 17, pp. 999–1030, 2007.

[6] P. B. Malafaya, G. A. Silva, and R. L. Reis, "Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications," *Advanced Drug Delivery Reviews*, vol. 59, no. 4-5, pp. 207–233, 2007.

[7] A. Saiani, A. Mohammed, H. Frielinghaus, et al., "Self-assembly and gelation properties of δ-helix versus β-sheet forming peptides," *Soft Matter*, vol. 5, no. 1, pp. 193–202, 2009.

[8] H. Yan, A. Nykanen, J. Ruokolainen, et al., "Thermo-reversible protein fibrillar hydrogels as cell scaffolds," *Faraday Discussions*, vol. 139, pp. 71–84, 2008.

[9] H. Yan, A. Saiani, J. E. Gough, and A. F. Miller, "Thermoreversible protein hydrogel as cell scaffold," *Biomacromolecules*, vol. 7, no. 10, pp. 2776–2782, 2006.

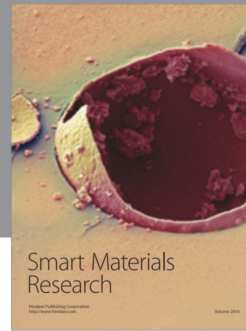
[10] H. Yan, H. Frielinghaus, A. Nykanen, J. Ruokolainen, A. Saiani, and A. F. Miller, "Thermoreversible lysozyme hydrogels: properties and an insight into the gelation pathway," *Soft Matter*, vol. 4, no. 6, pp. 1313–1325, 2008.

[11] B. Ozbas, J. Kretsinger, K. Rajagopal, J. P. Schneider, and D. J. Pochan, "Salt-triggered peptide folding and consequent self-assembly into hydrogels with tunable modulus," *Macromolecules*, vol. 37, no. 19, pp. 7331–7337, 2004.

[12] K. L. Niece, C. Czeisler, V. Sahni, et al., "Modification of gelation kinetics in bioactive peptide amphiphiles," *Biomaterials*, vol. 29, no. 34, pp. 4501–4509, 2008.

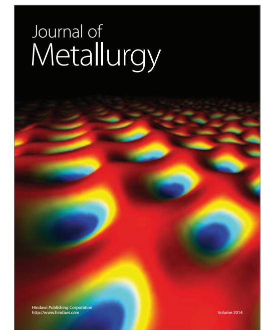
Journal of
Nanotechnology

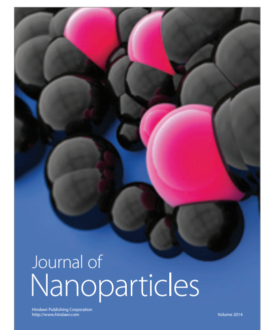
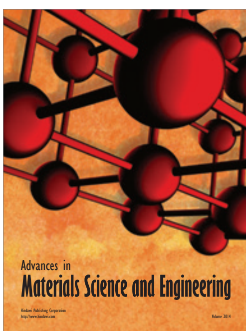
International Journal of
Corrosion

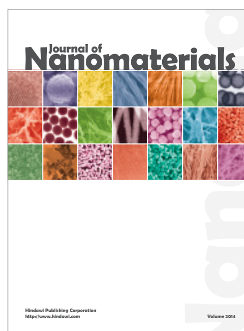
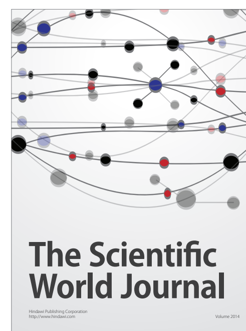
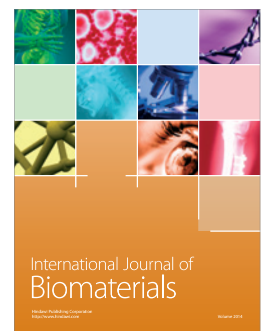
International Journal of
Polymer Science

Smart Materials
Research

Journal of
Composites

BioMed
Research International

Hindawi
Submit your manuscripts at
http://www.hindawi.com

Journal of
Metallurgy

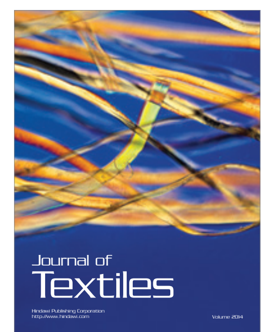
Journal of
Materials

Journal of
Nanoparticles

Advances in
Materials Science and Engineering

Scientifica

Journal of
Nanomaterials

The Scientific
World Journal

International Journal of
Biomaterials

Journal of
Nanoscience

Journal of
Coatings

Journal of
Crystallography

Journal of
Ceramics

Journal of
Textiles