Controlling stiffness in nanostructured hydrogels produced by enzymatic dephosphorylation

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Abstract

In the present paper, we report on enzyme-initiated self-assembly of Fmoc (fluoren-9-ylmethoxycarbonyl)-tyrosine hydrogels by enzymatic dephosphorylation under physiological conditions and provide evidence for the ability to control the modulus. Upon enzyme action, a self-assembling network of interconnecting fibres is formed, observed by cryo-SEM (scanning electron microscopy) and TEM (transmission electron microscopy). The concentration of alkaline phosphatase added to the Fmoc-tyrosine phosphate ester precursor solution had a direct effect on the gelation time, mechanical properties and molecular arrangements as determined through oscillatory rheology, fluorescence spectroscopy and CD spectroscopy. This highly tuneable cost-effective gel system may have applications in three-dimensional cell culture.

Introduction

Self-assembled hydrogels from small molecular building blocks provide highly tuneable materials for both biological and non-biological applications [1–5]. Biological applications of these systems include biosensing, controlled drug release, three-dimensional cell culture and tissue engineering [6,7]. In recent years, self-assembling peptide hydrogels have received significant attention as they are believed to have potential as next-generation biomaterials [8]. For these materials to be successful in tissue engineering and three-dimensional cell culture, self-assembly ideally occurs under physiological conditions (37°C, pH 7–7.5 and an ionic strength of 0.15 M) [6,9].

A number of approaches to formation of self-assembled hydrogels have been described, depending on the trigger used to initiate self-assembly. These can be broadly divided into two systems, the first type are those where the self-assembly is initiated by an overall change in environmental conditions, such as pH [9,9a] or ionic strength [10]. The second type are those that start off as non-assembling precursors that form self-assembling building blocks in response to a locally applied stimulus, such as light [11] or the catalytic action of enzymes [12]. The latter type operate under otherwise constant conditions and may provide better control over the self-assembly process and its kinetics. Enzyme-triggered self-assembly is especially attractive as it adds biological selectivity to the self-assembly trigger [12]. A range of enzymes have been utilized to initiate self-assembly, including transglutaminase [13], thermolysin [6,14,15], subtilisin [6], penicillin G amidase [16] and a combination of kinase and phosphatase [17,18]. Recent studies have demonstrated that the concentration of enzyme could also determine the mechanical properties of the hydrogels with high enzyme concentrations producing gels more rapidly, resulting in stiffer gels [19]. The ability to predetermine, and vary, the mechanical properties of the hydrogels is of particular interest to cell culture, particularly for stem cells. The elastic moduli of materials has been shown to have a direct effect on the differentiation pathways chosen by mesenchymal stem cells [20,21].

The majority of the enzyme-initiated systems utilize relatively non-specific enzymes, such as proteases, which may interfere with or degrade cell-surface proteins. Therefore, for cell culture applications, we favour specific non-destructive enzymes, such as phosphatases. Phosphatases have been used to initiate self-assembly in a number of studies [1,2,18,19,22–24]. They are a family of enzymes that dephosphorylate hydroxylated amino acid residues and, in conjunction with kinases, regulate protein function in vivo [25,26]. Two types of phosphatase have been utilized in the context of self-assembly: alkaline [1,2,24] and acidic [2,19,23]. Alkaline phosphatase is of special interest as it is one of the cell-surface markers for pluripotent stem cells [27]. It is also found throughout the body predominantly within the liver and bones and is also expressed in the intestine, placenta and kidneys [28,29].

The non-self-assembling precursors reported to date have varied significantly in size and molecular structure. The simplest precursor was demonstrated by Yang et al. [1,2]. The precursor Fmoc (fluoren-9-ylmethoxycarbonyl) linked to a single amino acid, a phosphorylated tyrosine residue, does not self-assemble due to electrostatic repulsion by the charged phosphate groups. On addition of a phosphatase, self-assembly could occur as the precursor was dephosphorylated, forming Fmoc-Y-OH (Fmoc-tyrosine-OH). In the present paper, we demonstrate further development of this system which combines three features: (i) self-assembly within

Key words: cell culture, hydrogel, nanostructure, phosphatase, stiffness.

Abbreviations used: Fmoc, fluoren-9-ylmethoxycarbonyl; Fmoc-Y-OH, Fmoc-tyrosine-OH; Fmoc-tyrosine (phosphate)-OH; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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physiological conditions; (ii) control of the mechanical properties; and (iii) control of the molecular order.

Studies
First, we investigated whether phosphatase-triggered self-assembly of Fmoc-tyrosine could occur under physiological conditions. To this end, 40 mM Fmoc-Y(p)-OH [Fmoc-tyrosine (phosphate)-OH] was dissolved in an alkaline phosphate buffer and the pH was adjusted to neutral. Self-assembly occurred under physiological conditions (pH 7.4, 0.15 M ionic strength and 37 °C) on addition of alkaline phosphatase, as illustrated in Figure 1. A second hydrogel system, Fmoc-Y-OH (Fmoc-tyrosine-OH), where self-assembly is initiated through gradual lowering of the pH from 9.8 to 7.4 was also investigated. These two systems allow for a comparison between chemically identical hydrogels prepared via different routes within physiological conditions.

Secondly, we investigated the effect of enzyme concentration on the hydrogels’ mechanical properties. The effect of enzyme concentration on the gelation time and fibre size was also studied, as these are known to have a direct effect on the mechanical properties.

As expected, the concentration of enzyme added to the Fmoc-Y(p)-OH solution controlled the rate of hydrogelation [19] (Figure 2A). One enzyme unit equates to 1 unit of alkaline phosphatase hydrolysing 1 μmol of 4-nitrophenyl phosphate per min at pH 9.8 and 37 °C. At the two highest concentrations, 10 and 3 units · μl⁻¹, gelation occurred within 60–120 min, whereas, at the lowest enzyme concentration, 0.1 unit · μl⁻¹, gelation took over 24 h. The hydrogel formed through lowering the pH has a significantly higher rate of gelation with a stable self-supporting hydrogel forming within 5 min. The ability to predetermine the gelation time through varying the enzyme concentration is of particular interest to a number of applications in three-dimensional cell culture and tissue engineering [19].

The conversion into Fmoc-Y-OH by alkaline phosphatase was followed by reverse-phase HPLC. For all enzyme concentrations, with the exception of 0.1 unit · μl⁻¹, the molecules were fully dephosphorylated by 24 h. On addition of alkaline phosphatase at 0.1 unit · μl⁻¹, only 35.5 ± 1.8% Fmoc-Y(p)-OH had been converted by 24 h (Figure 2A). The low conversion rate observed on addition of 0.1 unit · μl⁻¹ explains the significantly longer gelation time discussed above.

The fibre size and network were studied by TEM (transmission electron microscopy) and cryo-SEM (scanning electron microscopy) where they were observed to form an interconnecting network (Figures 2B and 2D). The fibre size was found to be independent of alkaline phosphatase concentration, indicating that there was a favoured structural organization of the fibrils (Figure 2A). The fibres within the hydrogel formed through lowering the pH were larger than those within the enzymatic hydrogels. The differences within fibre size are thought to be due to the different structure.
Electron microscopy, rheology and the effect of enzyme concentration on the hydrogels’ properties

Figure 2 | Effects of alkaline phosphatase concentration on the gelation time, conversion into Fmoc-Y-OH (after 24 h), fibre size and mechanical properties. One unit corresponds to the amount of enzyme that hydrolyses 1 μmol of 4-nitrophenyl phosphate per min at pH 9.8 at 37°C. (B) Cryo-SEM image of a hydrogel formed from Fmoc-Y(p)-OH on addition of alkaline phosphatase (10 units · μl⁻¹). Scale bar, 1 μm. (C) Oscillatory rheology of hydrogels formed from Fmoc-Y(p)-OH on addition of alkaline phosphatase (10 units · μl⁻¹) (n = 3). (D) TEM of hydrogels formed from Fmoc-Y(p)-OH on addition of alkaline phosphatase (10 units · μl⁻¹). Scale bar, 500 nm.

<table>
<thead>
<tr>
<th>Enzyme concentration (μl⁻¹)</th>
<th>Gelation Time (mins)</th>
<th>Conversion (%)</th>
<th>Fibre Size (nm)</th>
<th>G’ (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>60 - 120</td>
<td>100</td>
<td>31.0 ± 13.3</td>
<td>34 ± 13</td>
</tr>
<tr>
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<td>60 - 120</td>
<td>100</td>
<td>24.9 ± 8.2</td>
<td>16 ± 7</td>
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<tr>
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<td>120 - 240</td>
<td>100</td>
<td>33.0 ± 11.0</td>
<td>9 ± 8</td>
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<tr>
<td>0.1</td>
<td>&gt; 1440</td>
<td>35.5 ± 1.8</td>
<td>34.3 ± 10.3</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>pH</td>
<td>5</td>
<td>-</td>
<td>58.9 ± 22.7</td>
<td>0.6 ± 0.1</td>
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nucleation and growth pathways during self-assembly. In the hydrogels formed by enzymatic initiation, the molecules are dephosphorylated in a spatiotemporally confined manner (i.e. at the enzyme’s active site [15]) which may lead to more uniform production of the fibrils, whereas, in pH-triggered systems, nucleation and structure growth is less controlled. This disordered nucleation and gelation process in comparison with the enzyme system may lead to the observed variation in fibre size.

The viscoelastic properties of the hydrogels were assessed by oscillatory rheology 72 h after enzyme addition. All of the hydrogels were solid elastic materials, demonstrated by the storage modulus (G’) being an order of magnitude larger than the loss modulus (G’’) (Figure 2C). The samples exhibited a weak dependence on frequency; for example on addition of alkaline phosphatase at 10 units · μl⁻¹ G’∝(frequency)⁰.¹¹². This had been observed in previous studies of Fmoc-Y(p)-OH [1,2]. The concentration of enzyme was found to have a direct effect on the mechanical properties of the hydrogels (Figure 2A). The hydrogel formed on addition of the highest enzyme concentration had the largest elastic modulus (34 ± 9 kPa). Lowering the concentration of alkaline phosphatase added to Fmoc-Y(p)-OH lowered the mechanical properties of the hydrogels. Although the elastic modulus for 0.1 unit · μl⁻¹ is greater than that for 1 unit · μl⁻¹, there is no significant difference between the two (P = 0.607 for a two-tailed Student’s t test). The lowest G’ was recorded for the hydrogel formed through lowering the pH, therefore the rapid disordered gelation that occurs within these samples and larger fibre size produces hydrogels with lower mechanical properties [19]. Therefore we have shown it is possible for the enzyme concentration to control the mechanical properties of this hydrogel system. In future, it may be possible to predetermine the mechanical properties of the hydrogel through careful selection of the enzyme concentration, which would be beneficial in cell culture for matching mechanical properties to cell type.

Finally, we investigated whether the supramolecular arrangement within the fibres was affected by the enzyme concentration (Figure 3). The molecular environment of the fluorenlyl group was studied by fluorescence spectroscopy 24 h after enzyme addition. The solution of the non-self-assembling monomer, Fmoc-Y(p)-OH, exhibited a peak centred at 320 nm attributed to the Fmoc group, and a shoulder between 350 and 370 nm which will be discussed further. On addition of the alkaline phosphatase at
The molecular environments of the fluorenyl group and tyrosine residue have been determined previously using CD spectroscopy [15,33,36]. The hydrogel systems were assessed using CD 24 h after enzyme addition. The fluorenyl groups are believed to be in a chiral orientation on the basis of the peaks in the region 300–320 nm [15,33]. The peak minimum around 316 nm is thought to be an indication of the structural order of the fluorenyl groups with increasing order related to the increase in enzyme concentration. The hydrogel formed through lowering the pH had a comparable order of the fluorenyl group with the enzyme-initiated samples between 1 and 3 units · μl⁻¹.

It is known that the tyrosine side chain can be observed within the near-UV region (250–300 nm) [36], additionally it is a region that also has contributions from the Fmoc group [37]. For hydrogels formed on addition of alkaline phosphatase at concentrations greater than 1 unit · μl⁻¹, there are two peaks present, with their centres at 285 and 290 nm. For the hydrogel formed through lowering the pH, only one peak is present, with a centre at 290 nm. These peaks could be due to the tyrosine residues or the Fmoc group, and may indicate that they are within multiple environments or configurations within the hydrogels; however, it is not possible to accurately determine the cause or the environments. Overall, through the use of fluorescence and CD spectroscopy, we have shown that the molecular order is controlled through enzyme concentration, with higher apparent order observed for higher enzyme concentrations.

**Conclusions**

In summary, we have demonstrated that Fmoc-Y-OH can self-assemble within physiological conditions on enzyme initiation. We have also shown that the concentration of enzyme can determine the mechanical properties of the hydrogel and give control over the molecular order. Owing to its simplicity and low cost, we believe that this self-assembling system has potential applications within three-dimensional cell culture and controlled drug release. Work is currently underway to fully characterize these gels and to assess their suitability as a scaffold for the culture of embryonic stem cells.

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**References**


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