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Cations influence the cross-linking of hydrogels made of recombinant, polyanionic spider silk proteins

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ABSTRACT

Hydrogels made of polyanionic recombinant spider silk proteins (spidroins) were prepared either in the presence or the absence of Dulbecco's Modified Eagle Medium (DMEM). Mono- and divalent cations present in DMEM severely affected the self-assembly process of the spidroins. Although the addition of DMEM had no apparent effect on secondary structure formation, there was a significant effect on the kinetics as well as on the hydrogel network; in the presence of DMEM, gelation occurred more rapidly. Additionally, the hydrogels were stiffer; however, the hydrogels were still shear-thinning. In summary, it can be concluded that there is a significant impact of ionic cross-linking on recombinant spidroin-based hydrogels.

1. Introduction

Traditional tissue engineering techniques have a significant disadvantage: the placement of different components which are used to prepare tissue-like constructs (cells, biomaterials and biochemical factors) is imprecise [1,2]. To overcome this disadvantage, researchers have developed several techniques which allow for co-processing of cells and biomaterials into specific structures; this sub-type of tissue engineering is referred to as biofabrication [3,4]. Of these techniques, one of the most promising is 3D bioprinting; layer-by-layer manufacturing of cell-encapsulating biomaterials into 3D scaffolds [5]. Natural biomaterials which have been used in 3D bioprinting are collagen, gelatin and alginate; however, all of these have some sort of disadvantage i.e. poor mechanical properties [1–3,6–14]. As an alternative to these common bioinks, the recombinant spider silk protein (spidroin) eADF4(C16) and its modified variant eADF4(C16)-RGD have been recently introduced [15].

The polyanionic spidroin eADF4(C16) consists of 16 repeats of module C (sequence: GSSAAAAAAAASGPGGYG PENQGPSGPG-GYGPGGP), which mimics the consensus sequence of the repetitive core of the European garden spider *Araneus diadematus* dragline silk fibroin 4 (ADF4) [16,17]. The RGD variant thereof contains an RGD integrin-binding motif introduced by genetic engineering at the C-terminus, which was previously shown to enhance mammalian cell attachment on spider silk films [18]. These proteins can self-assemble from a disordered structure in solution into β-sheet rich fibrils [19]. Self-assembly is triggered by temperature, kosmotropic phosphate ions and increased protein concentration [20,21].

Bioinks naturally require the use of cell culture media in the fabrication process. As various ions severely affect the self-assembly process [22], the aim of this research was to characterize the material properties of the hydrogel prepared in the presence of cell culture media. Through various assays, stages of the network formation were observed: basic protein structure (FTIR), fibril morphology and association (TEM), hydrogel network formation kinetics (turbidimetry). Additionally, an effect of the formed network on a critical bulk property (i.e. mechanics) of the hydrogel was also observed (rheology).

2. Experimental

2.1. Transmission electron microscopy (TEM)

For TEM analysis, 3% w/v eADF4(C16) and 3% w/v eADF4(C16)-RGD hydrogels, in the presence or absence of 15% v/v DMEM, were diluted to 1 mg/mL. 5 μL of the diluted hydrogel was scattered on 100-mesh Formvar-coated copper TEM grids (Plano GmbH, Germany), incubated for 10 min, washed two times using 5 μL of double distilled water (ddH_2O), and fibrils were negatively stained

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using 5 μL 2% uranyl acetate solution. Samples were allowed to dry for at least 24 h at ambient temperature before imaging. TEM imaging of dry samples was performed with a JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV and equipped with a 4000 × 4000 charge-coupled device camera (UltraScan 4000; Gatan, Pleasanton, CA).

2.2. Analysis of gelation kinetics

For gelation analysis, 100 μL of concentrated eADF4(C16) and eADF4(C16)-RGD solutions in the presence or absence of 15% v/v Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Life Technologies, USA) were added to 96-well plates (Nunc, Germany). Phenol red-free DMEM was used to prevent false measurement or background noise that might be introduced by this pH indicator. The hydrogels were incubated at 37 °C and analyzed at various time points for changes in turbidity. Turbidity changes upon gelation were monitored at 570 nm using a Microplate Reader (Mithras LB 940, Berthold Technologies, Germany) in absorbance mode. A sample number of 4 (n=4) was used for each experimental group.

2.3. Fourier-Transform Infrared (FTIR) spectroscopy

Secondary structure content of the eADF4(C16) and eADF4(C16)-RGD hydrogels in the presence or absence of 15% v/v DMEM (Biochrom, Berlin, Germany) was evaluated after freeze-drying hydrogel samples with a Bruker Tensor 27 spectrometer (Bruker, Germany). Spectra were detected by attenuated total reflection (ATR) with a resolution of 4 cm^{-1}, and 120 scans were averaged. Analysis of the amide I band (1595–1705 cm^{-1}) was performed by Fourier self-deconvolution (FSD) to determine individual secondary structure elements as described previously [23–26]. A sample number of 3 (n=3) was used for each experimental group.

2.4. Rheology

Stress-strain curves of eADF4(C16) and eADF4(C16)-RGD in the presence of different ions were measured using a flow measurement mode at the Rheometer AR-G2 (TA Instruments, New Castle, DE, USA) with a 25 mm plate-plate geometry and a 0.5 mm gap and a sample volume of 600 μL at room temperature. The shear rate was kept constant at 3.0^{-3} 1/s. To analyze the influence of 15% v/v DMEM on the viscosity behavior of these hydrogels, the hydrogels were measured using additionally the steady state flow measurement mode. Here, the shear rate was increased from 0.1 to 100 s^{-1}. For all measurements a solvent trap with a wet sponge was used to minimize evaporation. All rheological measurements were performed with pre-formed hydrogels. The highly concentrated spider silk solutions were gelled for 24 h at 37 °C before rheological measurements. A sample number of 2–3 (n=2–3) was used for each experimental group and one representative curve shown per group.

3. Results and discussions

3.1. Concentration of relevant ions in hydrogel formulations

Dulbecco's Modified Eagle Medium (DMEM) contains numerous salts, sugars and proteins, which could influence the charge-charge interactions between the proteins. In this context, salts like $CaCl_2$, NaCl and KCl have already been identified as important in the formation of spider silk threads in nature, and are therefore of particular interest for the hydrogel formation as well [27]. These ions are classified as either kosmotropic or chaotropic [28]: Ions such as Ca^{2+}, are highly chaotropic, Cl^- is neither kosmotropic of chaotropic, and K^+ and Na^+ are kosmotropic, with K+ being slightly more kosmotropic.

In the final formulation of the eADF4(C16) and eADF4(C16)-RGD hydrogels prepared with DMEM, the molarity is 16.43 mM for NaCl, 0.80 mM for KCl, and 0.27 mM for $CaCl_2$. An example calculation for NaCl is shown below in Eq. (1). The entire value is multiplied by 0.15 to account for the fact that the final concentration of DMEM is 15% v/v.

$$6.4g/L/58.44g/mol*1000\ mM/1\ M*0.15 = 16.43\ mM\ \ NaCl \quad (1)$$

3.2. Structural characterization of eADF4(C16) and eADF4(C16)-RGD hydrogels

The consensus motif (C-module) comprises 35 amino acids with one $(Ala)_8$ stretch able to form β-sheets as well as glycine/proline rich GPGXY repeats remaining disordered or helical in solution [26,29,30]. When the protein converts from the soluble to the insoluble state, there is an increase in the amount of β-sheet rich structures [21,26]. Therefore, the gelation process of eADF4(C16) and eADF4(C16)-RGD can be characterized by the formation of nanofibrils accompanied by this change in secondary structure. Here, it was investigated if the presence of 15% v/v cell culture media influences the secondary structure of eADF4(C16) and eADF4(C16)-RGD hydrogels using FTIR spectroscopy. Fourier self-deconvolution (FSD) of the amide I band allowed assignment of individual secondary structure elements (Table 1) [23–26].

The hydrogels fabricated in the absence or presence of DMEM were indistinguishable concerning their secondary structure composition; all hydrogels showed an overall β-sheet content between 45% and 47%.

3.3. Morphological analysis of the fibrillary network

The morphology of the fibrils and fibrillary network of the 3% w/v eADF4(C16) and eADF4(C16)-RGD hydrogels in presence of cell culture media were evaluated using transmission electron microscopy (TEM) (Fig. 1A and B).

3% w/v eADF4(C16) hydrogels were organized by nanofibrils with a diameter of around 10 nm as shown previously, while 3% w/v eADF4(C16)-RGD hydrogels showed slightly thinner nanofibrils with a diameter of around 7 nm [32]. In addition, the fibrillary network of eADF4(C16)-RGD hydrogels was more densely packed in comparison to that of eADF4(C16) hydrogels. However, the presence of DMEM had no apparent influence on the gross morphology of the hydrogels; although there appeared to be a change in opacity, as confirmed by turbidity measurements, likely originating from the slightly denser packing.

Table 1
Secondary structure elements of 3% eADF4(C16) and 3% eADF4(C16)-RGD made in the absence or the presence of DMEM (15% v/v). Structural contents were calculated using Fourier self-deconvolution (FSD) of the amide I bands.

		Secondary structure content/%			
Secondary structure[a]	*Wavenumber range/* cm^{-1}	3% C16	3% C16, 15% DMEM	3% C16-RGD	3% C16-RGD, 15% DMEM
α-helices	1656–1662	8.9 ± 0.3	8.5 ± 0.1	8.7 ± 0.6	7.5 ± 1.1
β-sheets	1616–1637, 1697–1703	44.7 ± 1.3	47.1 ± 1.8	45.1 ± 0.8	46.1 ± 2.5
Random coils	1638–1655	22.5 ± 0.9	21.7 ± 0.3	23.0 ± 0.2	21.9 ± 0.2
Turns	1663–1696	21.3 ± 0.4	21.4 ± 0.5	20.7 ± 0.6	21.9 ± 0.7
Side chains	1595–1615	2.6 ± 1.1	1.3 ± 2.2	2.5 ± 0.4	2.6 ± 1.0

[a] Peak assignment taken from literature [23,31].

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