Rational design and application of responsive α-helical peptide hydrogels

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Abstract

Biocompatible hydrogels have a wide variety of potential applications in biotechnology and medicine, such as the controlled delivery and release of cells, cosmetics and drugs; and as supports for cell growth and tissue engineering. Rational peptide design and engineering are emerging as promising new routes to such functional biomaterials. Here we present the first examples of rationally designed and fully characterized self-assembling hydrogels based on standard linear peptides with purely α-helical structures, which we call hydrogelating self-assembling fibres (hSAFs). These form spanning networks of α-helical fibrils that interact to give self-supporting hydrogels of >99% water content. The peptide sequences can be engineered to alter the underlying mechanism of gelation and, consequently, the hydrogel properties. Interestingly, for example, those with hydrogen-bonded networks melt upon heating, whereas those formed via hydrophobic interactions strengthen when warmed. The hSAFs are dual-peptide systems that only gel on mixing, which gives tight control over assembly. These properties raise possibilities for using the hSAFs as substrates in cell culture. We have tested this in comparison with the widely used Matrigel substrate, and demonstrate that, like Matrigel, hSAFs support both growth and differentiation of rat adrenal pheochromocytoma cells for sustained periods in culture.

As our understanding of sequence-to-structure relationships in proteins improves, so does our ability to rationally design new proteins and protein-based materials. Unlike discrete peptide and protein objects, the design of biomaterials requires additional rules for self-assembly to allow the nano-to-micron scale regimes to be bridged. In these respects, synthetically accessible peptides—which can be programmed to fold into prescribed structures, and to self-assemble into larger architectures—offer routes to rationally designed peptide and protein-based biomaterials. Indeed, a variety of peptide-based self-assembling fibres, tapes and hydrogels have been produced. Much of this effort has been directed to the assembly of β-structured systems, though α-helix-based fibrous and α-helix-containing gelling materials have been explored to some extent.
Previously, we have described a number of fibrous biomaterials based on the α-helical coiled coil. These so-called SAFs (self-assembling fibers) comprise two 28-residue peptides designed to co-assemble, resulting in an offset α-helical dimer with complementary sticky ends. The ends promote longitudinal assembly into α-helical coiled-coil fibrils, which bundle to form matured fibers. Elsewhere, we have demonstrated that specific interactions—fostered by features on the surface of the coiled coil—lead to crystallization of the peptides within the fibers, Figure 1a. Consequently, fibers are highly ordered, thickened, and settle out of solution. For the work presented here, we reasoned that replacing these few specific interactions with many more-general interactions would lead to networks of non-covalently cross-linked fibrils and, hence, physical hydrogels.

The design rules for the hydrogelating SAF (hSAF) peptides initially followed those for the SAFs. That is, they were two-component systems based on the coiled-coil heptad sequence repeat, abcdefg. As the a, d, e and g positions are responsible for directing the dimer interface, Figure 1, these were maintained from the original SAF design. Where the hSAFs differ from the original designs is at the b, c and f positions, which are exposed on the surfaces of the coiled-coil assemblies. For the hSAFs these positions were made combinations of alanine and glutamine residues: alanine was chosen to promote weak hydrophobic interactions between fibrils; and glutamine for its propensity to hydrogen bond. Initially, three hSAF designs were investigated: variants hSAF$_{AAA}$, hSAF$_{QQQ}$ and hSAF$_{AAQ}$. Table 1, where subscripts denote amino acids at b, c and f, respectively. In hSAF$_{AAQ}$, which serve as a control, the pattern of alanine and glutamine residues was the same as for canonical positions in previous SAF designs.

Complementary hSAF peptides were mixed on ice, and either allowed to assemble at this temperature for 30 minutes, or removed after 5 minutes and incubated for 25 minutes at 20 °C. After these times, to test for gel formation, sample vials were inverted and incubated for a further 30 minutes without changing the temperature (Figure S1, Supporting Information). Through this simple test, hSAF$_{AAA}$ and hSAF$_{QQQ}$ both formed self-supporting gels. hSAF$_{QQQ}$ formed a gel at low temperature, which melted on warming; whereas, the hSAF$_{AAA}$ appeared to form a weak gel at low temperature that strengthened on warming. Moreover, this gel did not melt on heating up to 95 °C. The control, hSAF$_{AAQ}$ did not form gels.

To confirm and quantify the gel strengths, the storage (G’) and loss (G”) moduli were recorded as a function of temperature using both microrheology (Figure S2, Supporting Information) and bulk oscillatory rheology, Figure 2a. For both hSAF$_{AAA}$ and hSAF$_{QQQ}$, G’ was greater than G” at low temperatures, confirming gel formation. However, whereas hSAF$_{AAA}$ showed a slight increase in gel strength with temperature, hSAF$_{QQQ}$ showed a transition to a liquid state between 16 and 19 °C, followed by a switch back to a gel state at higher temperatures.

The formation of fibrils within the hydrogels was confirmed by low-temperature field emission scanning electron microscopy, Figure 2b-e. For both hSAF$_{AAA}$ and hSAF$_{QQQ}$, the samples prepared on ice showed interconnected fibers with polydisperse widths, but without uninterrupted networks, Figures 2b&c. Interestingly, on warming to room temperature, hSAF$_{AAA}$ samples showed a homogeneous uninterrupted network of thinner fibers, Figure 2e. The images for the control peptide, hSAF$_{AAQ}$, revealed no fibrous structures or networks, Figure 2d, though unconnected fibers were visible by standard, negative-stain transmission electron microscopy of samples prepared at 20 °C (Figure S3, Supporting Information).
The peptide secondary structure and its packing in the fibril assemblies was probed by circular dichroism (CD) spectroscopy and x-ray fiber diffraction (XRD), respectively. CD spectra recorded at 4 °C and 20 °C for both hSAF_{AAA} and hSAF_{QQQ} were characteristic of α-helical structure, Figures 3a&c. That for the hSAF_{AAA} was the more intense and did not change upon heating to 20 °C, while the spectrum for hSAF_{QQQ} lost intensity upon heating and demonstrated distortion due to light scattering20. These data are consistent with the gelation experiments described above.

XRD was performed on hSAF_{AAA} at 20 °C and on hSAF_{QQQ} at 4 °C, Figures 3b&d. In both cases, the diffraction patterns were similar to those presented for the other SAF systems, although the unaligned fibers within the hSAF gels resulted in more-diffuse patterns with strong circular rings from water. hSAF_{AAA} and hSAF_{QQQ} gels both gave diffraction patterns with meridional reflections at 5.15 Å (M), corresponding to the 5.4 Å helical repeat of an α-helix supercoiled within a coiled coil.

The sharper meridional arc (M2) suggested some cross-β structure in the hSAF_{QQQ} sample. We posit that this is likely due to the high glutamine content of this sequence, which favors amyloid-like assemblies in other systems.22, 23 However, cross-β structure normally gives a stronger signal in XRD; thus, the comparatively weak reflection in Figure 3d, together with the predominantly α-helical CD spectrum, Figure 3c, indicate only very small levels of β-structure in the hSAF_{QQQ} hydrogel.

Regarding the structural organization within the α-helical fibrils of the hSAF_{AAA} and low-temperature hSAF_{QQQ} gels, we have reported previously that for the standard, non-gelling SAFs the equatorial reflections in the XRD, Figures 3b&d, relate to the packing of the coiled coils on a hexagonal lattice. Due to overlap of some the reflections in the XRD data for the hSAF gels, however, it was not possible to index these arcs completely. Nonetheless, by comparison with our foregoing studies, it was possible to assess the packing distances between coiled coils in the gels. In the standard SAFs, coiled coils are 18.2 Å apart. From the new data for hSAF_{AAA} and hSAF_{QQQ} the corresponding separations were 17.3 Å and 21.5 Å, respectively. These spacings correlate with the changes to the sequences: for hSAF_{AAA} closer packing is expected because of the shorter alanine side chains; whereas, in hSAF_{QQQ} an increase might be expected because of (1) the replacement of predominantly alanine residues at b and c with the larger glutamine, and (2) the likely additional solvation of these hydrophilic residues. CD spectra and XRD patterns, consistent with these assertions were obtained for the hSAF_{AAQ} control fibres, (Figure S4, Supporting Information).

To probe the utility of the hSAF_{AAA} gels as a substrate for cell growth, we tested for peptide cytotoxicity and cell differentiation using rat adrenal pheochromocytoma (PC12) cells. First, however, we had to further stabilize the fibril-fibril interactions and the resulting gels. This was because, though hSAF_{AAA} gels could be washed and soaked in both phosphate-buffered saline (PBS) and standard cell-culture media, they did not persist for sufficient time to allow sustained cell-culture experiments. To stabilize the gels, in each of the hSAF_{AAA} peptides we replaced one of the surface-exposed alanine residues at an f position with the more-hydrophobic tryptophan, Table 1. This also allowed easy quantification of peptide concentration. In all respects—spectroscopic, microscopic and gel formation—the hSAF_{AAA-W} combination behaved similarly to the parent peptides (Figure S5 Supporting Information). Moreover the new peptides gelled at room temperature and the gels were stable in PBS and cell-culture media at 37 °C for more than two weeks, which permitted cell-biology studies as follows.

In Alamar Blue cell-proliferation assays24, PC12 cells seeded on collagen and then treated with increasing concentrations (0.5 - 2.5 mM, equivalent to 1.5 - 7.5 mg/ml, total pepide) of...
hSAF\textsubscript{AAA-W} peptides and gels proliferated, and were statistically no different to controls without peptide. This was in contrast to similarly prepared cells treated with staurosporine, a known inducer of apoptosis, which died (Figure S6, Supporting Information). Moreover, PC12 cells seeded on hSAF\textsubscript{AAA-W} gels (without collagen) could be induced to differentiate into neural cells using nerve growth factor at 100 ng/ml medium\textsuperscript{25}, as judged by the presence of neurite projections from the cell bodies, Figure 4a. As shown by phase-contrast microscopy, Figures 4a&b, the appearance of cells seeded on the hSAF\textsubscript{AAA-W} gels was similar to those seeded on the widely used, but more-complex and \textit{ex vivo} Matrigel\textsuperscript{26} substrate. Despite also using NS-1 cells, which are believed not to form aggregates, many of the induced cells ingressed the gels clustered in three dimensions and both with hSAFs and Matrigel; \textit{i.e.}, achieving 3D cell cultures. \textit{N.b.} multiple images from the first 10 days of these comparative cell-culture experiments are given in Figure S7 of the Supporting Information.

To compare cell differentiation within the hSAF and Matrigel substrates semi-quantitatively, we followed neurite extension with time, Figure 4c, and gauged overall differentiation in each culture, Figure 4d. A cell was defined to have differentiated if it had axodendritic processes longer than 2-cell body diameters in length, \textit{i.e.} processes longer than 20 μm\textsuperscript{27}. Though there was a lag in process growth and, consequently, cell differentiation in hSAF gels compared with Matrigel, on both counts the hSAF substrate performed at ~75% of Matrigel by 10 days. In making this comparison, it is important to bear in mind that hSAF is a well-defined de novo substrate without any of natural structural proteins and associated cell-recognition motifs, or growth factors inherently present in Matrigel. Therefore, the performance of cells on hSAFs is particularly encouraging. In principle however, defined functionalities and additional factors could be engineered or added in known and controlled ways in future.

The hSAF peptides presented here gel at a peptide concentration of 1 mM (\textasciitilde 3 mg/ml) in each peptide; that is, they have >99% water content. Moreover, as we have shown, changing the nature of the outer surfaces of the coiled coils—and, therefore, the inter-fibril interactions—allows temperature-responsive hydrogel properties to be engineered. This interesting and potentially useful behavior warrants further comment. The hSAF\textsubscript{QQQ} peptides, which have surface polar residues—\textit{i.e.}, glutamine residues at the \textit{f} positions of the coiled-coil repeat that have amide side chains and hydrogen-bonding potential—assemble to weak gels at low temperature and melt on warming. This is consistent with the breaking of weak hydrogen-bonded cross-links between fibrils in a wet peptide gel. Whereas, hSAF\textsubscript{AAA} peptides—which present only methyl side chains on their outer surfaces—form gels that become stronger on warming and are stable up to at least 95 °C. This is consistent with hydrophobic cross-links between peptide fibrils. In contrast, the control peptides, hSAF\textsubscript{AAQ}, in which the chemical symmetry of the outer surfaces is broken, do form fibers, but these do not form uninterrupted inter-fibril interactions and do not gel. Similar thermally responsive behavior has been reported for an entirely different single-peptide hydrogelating system; namely, MAX3 and variants from the Schneider and Pochan team\textsuperscript{28}. In this case, the peptide is designed to form an antiparallel β-hairpin, which folds unimolecularly upon heating and then assembles into amyloid-like fibrils that gel; the process reverses upon cooling. Our observations of gelation by the hSAF system are fully consistent with our initial design principles for modifying the fibril surfaces, and further demonstrate that the rational design of increasingly complex biomaterial systems is possible through different routes.

Finally, our demonstration that hSAFs support cell growth and differentiation is encouraging for the application of these gels as straightforward, chemically defined and engineerable scaffolds for cell culture and tissue engineering. The hSAF systems also carry the distinct
advantage that they have two peptide components, and, therefore, gel only upon mixing. Thus, these new designs encompass unprecedented control, and represent an exciting addition to the available arsenal of biomaterials and gels.

METHODS

Peptide synthesis

Peptides were synthesized on a CEM “Liberty” peptide synthesizers using standard solid-phase Fmoc chemistry. Amino acids were purchased from Novabiochem and other reagents from Rathburn Chemicals, unless otherwise stated. Peptides were cleaved using 95 % TFA (Sigma), 2.5 % TIS (Sigma) and 2.5 % 18.2 MQ ultra-pure water, purified by reverse-phase HPLC using acetonitrile (Fisher)-water gradients with 0.1% TFA, and confirmed by MALDI-TOF mass spectrometry. Pure peptides were freeze dried from acetic acid, weighed and dissolved in ultra-pure water to give 3 mM (~ 9 mg/ml) stocks.

CD spectroscopy

hSAF gels were prepared in 10 mM MOPS (Sigma) pH 7.0, at 1 mM (~ 3 mg/ml) of each peptide and incubated as described. All CD experiments were carried out in 0.01 cm quartz cells (Starna) using a Jasco J-810 circular dichroism spectrometer fitted with a Peltier temperature controller. Spectra were recorded between 190 and 260 nm with a 1 nm data pitch and bandwidth, 4 s response time, 50 nm.min\(^{-1}\) scanning speed and averaged over 2 accumulations. Baselines recorded using the same buffer, cell and parameters were subtracted from the data.

Electron microscopy

Low Temperature Field Emission Scanning Electron Microscopy was carried out using a JEOL 6301F microscope and Gatan Alto 2500 low temperature equipment. Samples were mounted on brass rivets of 1 mm internal diameter. At set times, samples were frozen in nitrogen slush, and stored at -80 °C. These were mounted into a cooled holder (-196 °C), plunged into liquid nitrogen, and transferred to the preparation chamber. These were then fractured using a cold scalpel tip and warmed to -90 °C for 30 seconds to remove a layer of ice. After re-cooling to -110 °C, samples were coated with 2 nm Pt/Pd and transferred to the microscope stage. For TEM experiments, 6 μl samples were placed on carbon-coated 400-mesh copper grids (Agar) on filter paper and dried; stained with 6 μl of filtered 1 % uranyl acetate solution; and examined in a Philips CM 100 microscope at 80 kV. Images were recorded using a Kodak Megaplus Camera 1.4i digital camera.

Rheology

Rheological measurements used an Anton Paar Physica RC 301 with 8 mm parallel plate geometry, Peltier plate, and environmental hood, with a 200 mm gap setting. G’ and G” were recorded using non-destructive oscillatory measurements at a strain of 0.5%, and over 3 - 45 °C. The measuring plate was surrounded by water to prevent drying of the sample. Samples were mixed \textit{in situ} on the lower plate at 3 °C to total volumes of 300 μl, the geometry was lowered into position and samples incubated for 30 minutes.

XRD

Samples for X-ray fibre diffraction were prepared as previously described\textsuperscript{19}. 10 μl of the hydrogels were suspended between two wax-filled capillaries, spaced ~1 mm apart and allowed to dry in air. The capillaries were gently separated and the fibre samples placed on a goniometer head. Diffraction data were collected using an R-Axis IV++ detector and Rigaku
rotating anode with CuKα radiation. The specimen-detector distance was 160 mm, and exposure time 15 mins. X-ray patterns were examined using Mosflm29 and CLEARER30.

Cell biology

Rat adrenal pheochromocytoma (PC12) cells and the subclone Neuroscreen-1 (NS-1) (ThermoFisher Scientific Cellomics) were used. Cells were maintained at 37 °C, 5% CO2 in cell-culture medium comprising: Dulbecco’s Modified Eagle Media (DMEM, Invitrogen) supplemented with 10% (v/v) horse serum (Sigma), 5% (v/v), fetal bovine serum (Sigma), 1% (v/v) penicillin/streptomycin solution (Sigma), and 2mM L-glutamine (Invitrogen). For the proliferation assays, cells were seeded at 1 × 10^4 cells cm^-2 in 96-well plates pre-coated with collagen solution (4 mg rat-tail type VII collagen (Sigma) dissolved in filter-sterilized solution of 0.8 ml glacial acetic acid, 100 mls ultra-pure H2O; and activated before coating with 40 μl sterile 3.7% (w/v) NaCl per ml of collagen solution). After incubation for 24 hours, the medium was replaced, and peptide (0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM total) added. After 72 hours, Alamar Blue dye (Serotec) was added (10% (v/v)) and incubated for 8 hrs. The fluorescence (excitation, 560 nm; emission, 590 nm) of 100 μl samples was measured. The ratio of the fluorescence intensities to that for a no-peptide control wells was taken as a measure of cell proliferation. Another control used staurosporine (Sigma) to establish cell death. For the differentiation studies, ~1 cm^2 × 2 mm hydrogel samples 1 mM in each peptide were prepared in phosphate-buffered saline (10 mM phosphate, 137 mM NaCl) and DMEM in 48-well dishes and left overnight. The gels were washed (x6) with cell-culture medium before seeding at 2 × 10^4 cells/well and leaving for 24 hours. The medium was replaced and nerve growth factor (Sigma) added at 100 ng/ml in DMEM. Matrigel (BD Biosciences) was prepared following manufacturer’s instructions, before washing and seeding similarly. Cells were followed for 2 weeks, replacing with fresh medium and NGF every 3 - 4 days.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. hSAF design principles
(a) In previous SAF designs, specific charged interactions between certain b and c positions lead to peptide alignment and fiber thickening. (b) For the hSAFs, we replaced these specific interactions with weaker, more-general interactions at all b, c and f sites, to result in smaller, more flexible, bundles of thinner fibres.
Figure 2. Gel strength and network formation by the hSAFs

(a) Gel strength gauged by nondestructive oscillatory rheology. Key: $G'$ (solid symbols) and $G''$ (open); hSAF\textsubscript{AAA} (discs) and hSAF\textsubscript{QQQ} (squares). Measurements were made at a strain of 0.5 \%. (b - e) Network formation as observed by cryoSEM for: hSAF\textsubscript{QQQ} (a), hSAF\textsubscript{AAA} (b), and hSAF\textsubscript{AAQ} (c), all assembled on ice for 15 minutes; and (d) for hSAF\textsubscript{AAA} assembled on ice for 3 minutes and then at room temperature for 12 minutes. All images are at the same magnification.
Figure 3. α-helical secondary structure and packing within the fibrils and gels
CD spectra (a and c) and x-ray fiber diffraction patterns (b and d) for hSAF_{AAA} (a and b)
and hSAF_{QQQ} (b and d).
Figure 4. Cell growth and differentiation on hSAF hydrogels
Phase-contrast microscopy of differentiating rat adrenal pheochromocytoma (PC12) cells in hSAF_{AAA-W} hydrogel (a), and matrigel (b). These images were taken 10 days after adding nerve growth factor. Images from the full 14-day time course are given in the Supplementary information. Cell differentiation as observed by neurite outgrowth was semi-quantified over time by: (c) the lengths of the processes; and (d) the percentage of cells showing processes. In both plots, error bars show the standard error of the mean for measurements from ≥ 100 cells/cell clusters across 10 different fields of view in three different triplicate wells.
Table 1

hSAF amino-acid sequences

| Peptid name | Sequence | Heptad repeat | g | abcdefg | abcdefg | abcdefg |
|-------------|----------|---------------|---|---------|---------|---------|
| hSAF<sub>AAA</sub> p1 | K IAALKAK IAALKAE IAALEAE NAALEA |
| hSAF<sub>AAA</sub> p2 | K IAALKAK NAALKAE IAALEAE IAALEA |
| hSAF<sub>QQQ</sub> p1 | K IQQLKQK IQQLKQE IQQLEQE NQQLEQ |
| hSAF<sub>QQQ</sub> p2 | K IQQLKQK NQQLKQE IQQLEQE IQQLEQ |
| hSAF<sub>AAQ</sub> p1 | K IAALKQK IAALEQE IAALEQE NAALEQ |
| hSAF<sub>AAQ</sub> p2 | K IAALKQK NAALKQE IAALEQE IAALEQ |
| hSAF<sub>AAA-W</sub> p1 | K IAALKAK IAALKAE IAALEWE NAALEA |
| hSAF<sub>AAA-W</sub> p2 | K IAALKAK NAALKAE IAALEWE IAALEA |

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