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Assessing Cellular Response to Functionalized α-Helical Peptide Hydrogels

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For applications in 2D and 3D cell cultures and tissue engineering, there is a need to develop biocompatible scaffolds that support cell and tissue growth therefore mimicking the biochemical and morphological properties of the natural extracellular matrix (ECM). In order to support cellular growth, the scaffold must provide mechanical stability, promote cellular attachment, proliferation and differentiation, permit diffusion of gases, nutrients and waste and allow control of the degradation rate of the temporary support while minimizing cytotoxic side effects in vivo.[1]

Hydrogels have been extensively investigated and used clinically for cell support in vitro and in vivo in regenerative medicine; their underlying structure mimics the interconnected fibrous network of the ECM,[2] the hydrated and porous nature of the gels allows diffusion of nutrients into the scaffold and waste to diffuse out,[3,4] and bioactive molecules can be incorporated into the fabric of the gels via passive uptake, direct incorporation during material synthesis, or conjugation after synthesis and/or assembly.[5–8]

While natural and ex vivo materials such as agarose,[9] alginate,[10] carrageenan,[11] gelatin,[12] collagen,[13] and Matrigel[14] are common current choices for such scaffolds due to their availability and established cellular responses, there is often a lack of control over their formation, degradation, mechanical properties, and chemical modification. Furthermore, ex vivo scaffolds, such as collagens and Matrigel,[15] show batch-to-batch variation and can potentially introduce disease. Synthetic scaffolds, such as poly(2-hydroxyethylmethacrylate) [16] poly(vinyl alcohol),[17] and polypeptide-based protein anchors[18] address some of these issues, and provide partially favorable environments for 2D and 3D cell cultures. However, their reduced complexity often fails to mirror native tissue and some degradation by-products can cause unwanted cellular responses.[19] The advantages and limitations of various tissue engineering scaffolds are reviewed by Chan and Leong.[20]

In principle, bottom-up scaffolds generated and engineered via biomolecular design allow the desired traits from the natural and synthetic scaffolds to be combined into one construct, and so create platforms for guiding cell growth and inducing specific biological responses. With this in mind, a number of peptide-based systems have been reported that utilize amyloid-like assemblies,[21–23] helical assemblies,[24–26] and peptide amphiphiles[27–30] as building blocks. A challenge in this area is to build complexity and control into these systems, ideally in a modular or pick-and-mix way; some of the systems reported to date lend themselves better to this ambition than others.[31]

Using a bottom-up design approach, we have reported a two-component peptide system for making hydrogels, termed hSAFs (hydrogelating self-assembling fibers).[32] The peptides (hSAF-p1 and hSAF-p2) are designed de novo using principles for peptide self-assembly. When mixed the two peptides form coiled-coil α-helical fibrous structures, which subsequently interact to form percolated gels. These gels support 2D cell culture. Here, we show that the original two-peptide hSAF system can be supplemented with other components to bring cell-binding functions to the system, hence building up complexity and functionality.

To achieve this functionalization, we developed a variant of hSAF-p1 harboring an azide moiety (Figure 1A,B, Figure 1, Supporting Information). This peptide, hSAF-p1(N3), was mixed with hSAF-p2 and after overnight gelation an alkyne-bearing peptide containing the cell adhesion motif Arg-Gly-Asp-Ser (alk-RGDS) was added and appended to the hydrogel via copper-catalyzed azide-alkyne cycloaddition (CuAAC; hereafter referred to as the “click reaction”) by overnight reaction in the presence of Cu(I) (Figure 1B).[33] Alk-RGDS was used in this study as it promotes cellular attachment via integrin binding.[14] The use of RGD to promote cellular adhesion in other peptide-based fibrous and hydrogel systems has been reported.[25,35,36] We argue here that we gain added utility and control over assembly and functionalization using a modular, dual-peptide system, that is, the α-helical de novo-designed hSAFs. The RGDS-decorated hSAF assemblies were α-helical to an extent comparable to the parent system (see Figure 2, Supporting Information); and electron microscopy (EM) showed that the decoration and subsequent washing procedure did not perturb the gel structure (Figure 2A–D). For this work, we incorporated azidonorleucine at the N-terminus of hSAF-p1, although successful decoration was
also achieved by substitution at the C-terminus (Figure 3, Supporting Information). Gel formation with predecorated p1(N3) and p2 was not successful (Figure 4, Supporting Information). Analysis of the decorated gels by high-performance liquid chromatography (HPLC) showed that the RGDS functionality extended entirely through 2 mm thick...
gels (Figure 5, Supporting Information); and an absorbance-
based copper assay showed that the copper used to drive the 
reaction was successfully removed by subsequent washing 
(Figure 6, Supporting Information).

To assess and compare cellular responses of undecorated and 
RGDS-decorated hSAFs, we constructed a “half-moon model” 
(Figure 1C), in which the two gels were prepared side-by-side 
in the same tissue-culture well, a similar model to that recently 
presented by Chan et al.\textsuperscript{[37]}

As a model for neuronal differentiation, we seeded PC12 
cells\textsuperscript{[38]} on both sides of the half-moon hSAF gels. The experiments 
were followed by light and fluorescence microscopy 
(Figure 3A–F), and after 14 days cell morphology indicated that 
cells had attached to both the undecorated hSAF- and RGDS-
decorated hSAF sides. However, the number of cells attached to 
the latter appeared considerably greater (Figure 3A,D).

The proliferative activity of the cells growing on the RGDS-
decorated side of the gels was \( \approx50\% \) greater than that of cells 
on the undecorated side. The higher rates of metabolic activity 
and proliferation\textsuperscript{[39]} on the decorated hSAF side were similar to 
those observed for PC12 cells seeded on commercially available 
Matrigel (Figure 3G,H; Figures 7 and 8, Supporting Information).

The above-mentioned experiments were conducted without 
novel growth factor (NGF), which terminates mitosis 
and induces primary neural outgrowth in PC12 cells.\textsuperscript{[40]} In parallel 
experiments, the introduction of NGF promoted cell differen-
tiation, as defined by the presence of neurite-like extensions, 
by day 3 on both sides of the gel (Figure 3I). Again the degree 
of differentiation was higher on the hSAF-decorated side: 
\( (11 \pm 4.6)\% \) cells showed processes on this side, compared with 
\( (6 \pm 2.6)\% \) on the undecorated side; and the mean number of 
near processes extending from the cell body was more than 
twice as high on the decorated versus undecorated hSAF gel 
by day 14 (Figure 3J). However, the projection length did not 
only significantly between the decorated hSAF and undecorated 
hSAF halves of the gel (Figure 3K). An assessment of PC12 
cells on gels with alk-RGDS attached via the \( C \)-terminus of 
hSAF-p1 showed that the effects were similar to those observed 
with the ligand attached via the \( N \)-terminus (Figure 9, Sup-
porting Information).

To test the specificity of the peptide–cell interactions, we 
compared hSAF gels decorated with alk-RGDS and alk-RGES. 
The latter reduces the efficacy of cell attachment considerably 
compared with alk-RGDS-based sequences.\textsuperscript{[41]} We found this to
be the case in the hSAF system: changing the aspartic acid (D)
to a glutamic acid (E) reduced cellular attachment by approxi-
mately 50% (Figure 10, Supporting Information).

The above studies used hSAF gels with every hSAF-p1(N$_i$)
decorated. It would be advantageous to reduce this per-
centage to reduce reagent costs and to allow combinations of functionality to be added via addition of cocktails of modi-
fiers. To begin testing this, we prepared gels with 1%, 10%,
and 100% hSAF-p1(N$_i$) in hSAF-p1 and performed the click reaction with alk-RGDS. The cellular responses to undecor-
rated hSAF and 1% incorporation were similar. However, the
behavior of the 10% and 100% decoration was also similar,
showing that considerably less reagent can be used (Figure
11, Supporting Information).

Finally, a separate assessment of 3T3 fibroblast cells with
RGDS-functionalized hSAFs showed that although the attach-
ment of the cells appeared greater on RGDS-decorated gels than on the undecorated gels, the proliferative activity of the
cells on RGDS-decorated hSAF gels was comparable to that on tissue-culture-treated poly(styrene) (TCP; Figures 12–14,
Supporting Information). Thus, not all cells respond signifi-
cantly to our hSAF gel system.

In summary, we have conjugated a cell-adhesion motif to a
rationally designed self-assembling peptide hydrogel system,
resulting in stable functional scaffolds suitable for cell culture.
Utilization of a “half-moon” protocol allows functionalized and
non-functionalized gels to be compared directly in the same
tissue-culture well. The morphology, viability, and prolifera-
tive activity of PC12 cells seeded on the scaffold surface were
demonstrated over 14 days, showing enhanced cellular growth
and differentiation on RGDS-modified hSAF gels, highlighting
the potential for adding cell-specific motifs to more closely
mimic ECM biochemistry. This novel functionalized system
offers complex functional scaffolds with tight control over mor-
phology and biochemistry, and with the potential to engineer
cell cultures, cell therapy delivery systems, and tissue matrices
that closely reflect the in vivo environment and thereby enhance

**Experimental Section**

**Scaffold Formation**: Peptides were synthesized using standard solid-
phase peptide synthesis protocols on a CEM “Liberty” microwave-
assisted peptide synthesizer. Peptides were purified by reversed-phase HPLC and their masses confirmed by MALDI-TOF mass spectrometry. Typically, hSAF gels were prepared by mixing separate 1 × 10$^{-3}$ M stock solutions for each parent peptide (hSAF-p1 and hSAF-p2), which were made up in 20 × 10$^{-3}$ M MOPS (3-(N-morpholino)
propanesulfonic acid) buffer at pH 7.4. This gave final solutions of 0.5 × 10$^{-3}$ M in each peptide. These were left on ice for 5 min followed by 30 min incubation at 20 °C, resulting in gels, which we refer to as 0.5 × 10$^{-3}$ M gels. (n.b., For the C-terminally modified peptide, the stock solutions were prepared at 2 × 10$^{-3}$ M, giving “1 × 10$^{-3}$ M gels”.) For decoration experiments, hSAF-p1 was substituted for hSAF-p1(N$_i$).

After, gel formation was performed by addition of 2 × 10$^{-3}$ M alk-RGDS and CuSO$_4$ and ascorbic acid each at 4 × 10$^{-3}$ M final concentration at 20 °C overnight. The gel was then washed with 10 × 10$^{-3}$ M ethylenediaminetetraacetic acid (EDTA) buffer, phosphate buffered saline (PBS), and supplemented-Dulbecco’s Modified Eagle Medium (S-DMEM). The presence of remaining copper after decoration was assessed by bichromonic acid assay (see Figure 6, Supporting Information). The extent of clicked alk-RGDS was analyzed by analytical HPLC followed, with peak identity confirmed by mass spectrometry. Half-moon gels were formed in 24-well cell-culture plates using sterile glass coverslips as temporary separators for the undecorated hSAF- and RGDS-decorated hSAF gels.

**Biophysical Measurements**: Peptide secondary structure was
determined via circular dichroism spectroscopy using a jasco J-810 CD spectrometer. Fiber morphology was visualized using a JEM 1200 EX MIK transmission electron microscope with a MegaViewII digital camera. Gel scaffold morphology was determined by fixing the sample with glutaraldehyde, removing the moisture via a critical point drying method and imaging using a Jeol JSM-6330F field-emission scanning electron microscope.

**Cell Studies**: PC12 cells, kindly gifted by Prof. Jeremy Henley at the University of Bristol, were seeded onto gels. Cellular morphology was assessed using a light microscope. For live cell imaging, the cells were stained with calcine-AM, their nuclei highlighted with DAPI (4′,6-diamidino-2-phenylindole) and imaged using a Leica DM IRBE inverted epifluorescence microscope. The metabolic activity, and therefore the proliferation rate, of the cells was evaluated by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) absorbance assay (see §1.14, Supporting Information). These data were supported by a DNA quantification assay (see §1.15, Supporting Information). Differentiated PC12 cells were imaged using light microscopy, and Image$^*$ was used to count the number of differentiated cells (where differentiation is defined as one or more

**Author Contributions**

E.A., M.A.B., N.M., and D.N.W. conceived the project. All authors
designed the various experiments. E.A., K.L.H., N.M., A.R.T., and A.W.
made the peptides and performed the biophysical work. N.M. and E.A.
conducted the cell-culture experiments. M.A.B. and D.N.W. supervised
the work. N.M., A.W., and D.N.W. wrote the paper.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or
from the author.

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