We report the surface nucleated growth of self-assembled dipeptide films. The seeding-layer was a thin dipeptide film with a globular structure. Placing the seeding-layer in contact with dipeptide led to growth of fibres overnight. Active enzymes were incorporated into the gel by adding them to the growth solution.

Low molecular weight hydrogelators (LMWGs) are small molecules that can self-assemble into complex hierarchical structures, trapping water inside the matrix to give hydrogels\textsuperscript{1-4}. LMW hydrogels are currently being investigated as energy transfer materials\textsuperscript{5, 6} and as 3-D scaffolds for cell growth and wound healing\textsuperscript{7-9}. One subset of the LMWG is the dipeptide gelator. A range of aromatic functionalised dipeptides have been shown to self-assemble into β-sheet like structures that further coordinate into fibres. Fibril formation can be induced in dipeptide-based LMWG systems enzymatically,\textsuperscript{10-12} thermally,\textsuperscript{13} by changing solvent composition,\textsuperscript{14} or via pH drop.\textsuperscript{15-21} LMWG gelators that assemble at surfaces due to electrostatic interactions or ligand-receptor interactions have also been reported.\textsuperscript{22, 23} In 2010, we showed that ultra-thin gel films and membranes could be grown by inducing a localised pH drop at the surface of an electrode.\textsuperscript{24} The pH was decreased by the release of protons that accompanied the electrochemical oxidation of 1,4-hydroquinone to 1,4-benzoquinone.

Fmoc-LG (Figure 1) is a LMWG that forms gels when the pH drops below the pK\textsubscript{a} of the terminal carboxylic acid (pK\textsubscript{a} = 5.8 \textsuperscript{16}). In this communication, we report the nucleated growth of a fibrous N-(9-Fluorenylmethoxycarbonyl)-L-leucine-glycine (Fmoc-LG) gels on top of an 80-100 nm thick electrochemically grown seeding layers. The thicker gel layer grew over 48 hours if the seeding layer was placed in contact with a solution of Fmoc-LG at pH 7 (Fig 1S in the ESI shows photos of a seeding layer and the thicker layer formed after 48 hours). The initial gel layer (‘seeding layer’, thickness 80-100 nm; Figure 2S) was grown on the surface of a gold electrode by electrochemically generating a surface localised pH drop following protocols described previously (see ESI for full experimental detail). The nanometre thick layer was invisible to the naked eye, but its presence was confirmed by surface plasmon resonance spectroscopy (SPR, Figure 1S) and transmission electron microscopy (TEM, Figure 2). TEM showed a continuous film that contained numerous spherical aggregates which were only a few nanometres in diameter (Figure 1(a)). The seeding layer was gently rinsed with NaCl (0.098 mol dm\textsuperscript{-3}, at pH 7) and then left in contact with ∼ 0.5 cm\textsuperscript{-3} of Fmoc-LG (2.4 mmol dm\textsuperscript{-3}, either in NaCl or phosphate buffered saline both at pH7) for 48 hours without the application of any further current.

![Figure 1](image1.png)

Fig 1. (a) Schematic showing the surface initiated growth of an Fmoc-LG film, (b) the structure of Fmoc-LG, (c) a photo of a surface initiated gel layer after 48 hours in solution (The gel was formed within an area delineated by the circular o-ring of an electrochemical SPR cell).

![Figure 2](image2.png)

Fig 2 (a) shows a TEM image of the seeding layer grown electrochemically onto the TEM grid (the grid was used as the electrode); (b) shows the development of fibrous structures after the seeding layer has been left in contact with Fmoc-LG-OH in solution for 48 hours. The scale bar on the left hand image is 20 nm and the scale bar on the right hand image is 200 nm.

The seeding layer was not removed by very gentle rinsing, a fact that was checked by both SPR and TEM. After 48 hours a thick gel layer formed on top of the seeding layer, TEM (Figure 2(b)) showed that fibres were now present in the gel. It is important to note that the scale bar in Figure 2(a) is 20 nm and in Figure 2(b)
is 200nm. The thicker film (Fig. 2(b)) still contained some spherical aggregates, although the diameters were much larger (tens of nanometres). The film also contained fibres with a range of diameters, which were up to several microns in length.

A nucleation and growth mechanism for Fmoc-LG gels has previously been reported in mixed DMSO/water systems. Using a pH trigger, fibre growth was imaged, although the nucleation step was below the temporal and spatial resolution of confocal microscopy. Spheres were initially formed and then replaced by fibres that appeared to grow from a small number of nucleation sites. In amyloid plaque formation, a nucleation and growth type mechanism is frequently suggested. The kinetics of amyloid formation show a time lag while nuclei are created, then a growth phase in which the nuclei are extended into fibrillar structures. The nuclei can be small oligomers, spherical aggregates or micellar species. If pre-formed nuclei are added to a protein solution, there is no time-lag and fibre formation rapidly follows. Fibre formation by the miss-folding of silk-like proteins has also been induced by the presence of a mica surface.

In the experiments described above it is likely that two mechanisms are contributing to the nucleated growth of the thicker film. Firstly there may be enough protons remaining inside the seeding layer to create a local pH close to the pK_a of the Fmoc-LG. As mentioned above the final pH of the gel was 6.5, even though it had been sitting in a solution at pH 7 for 48 hours. This suggests that some protons remained trapped within the film. Secondly there is evidence in the literature that the pK_a of dipeptides and other acids may be strongly modified by their environment. Dipeptides with environment specific or ‘apparent’ pK_a considerably higher than the pK_a of the free dipeptide have been reported. Surface induced pK_a shifts have also been reported for a range of self-assembled monolayers (SAMs) and surface bound molecules. Impedance spectroscopy has been used to measure surface pK_a values for a range of functionalised SAMs; it was found that the pK_a of both acid and amine functionalised monolayers shifted about 2 pH units more alkaline when compared to the pK_a in solution. Burris et al. showed that there was a relationship between surface roughness and apparent pK_a for 3-mercaptopropionic acid monolayers on gold electrodes with shifts of up to 4 pH units relative to the solution pK_a possible. Abiman et al. investigated the reasons behind pK_a shifts of up to 2 pH units for benzoic acid covalently bound to different carbon surfaces. They concluded that the pK_a shift was entropically controlled due to changes in the solvent ordering at the surface upon ionisation. The hydrophobicity/hydrophilicity of the surface also played a role. The pK_a of Fmoc-LG in 0.1 molar NaCl was measured to be 6.1 by a pH titration (Fig 3S), already slightly above the pK_a in pure water (5.8). It would only take a small increase in the pK_a of the molecules at the surface for nucleated growth to occur.

In a final experiment, surface-templated gel formation was used to incorporate horse radish peroxidase (HRP) inside the gel films, by simply adding it to the second growth solution (See ESI for experimental details). HRP has previously been incorporated into diphenylalanine nanotubes and the authors found that encapsulation enhanced the stability of the enzyme. Enzymes and bacteria have also been incorporated in Fmoc-F/gelatin films. After 48 hours, a 1-2 mm thick gel layer formed, the layer was removed from the growth solution and rinsed. The incorporation of HRP in the gel was proved by carrying out a simple colorimetric assay, namely the conversion of o-
phenylenediamine (OPD) to 2,3-diaminophenazine (DAP) in the presence of HRP and H₂O₂ (ESI). DAP has a strong orange colour and its appearance is easily monitored by UV-Vis. The gel was placed in a solution containing OPD and H₂O₂ and the colour change in the gel was monitored by UV-Vis in reflectance mode.

The solution containing the OPD and H₂O₂ was not observed to change colour, but the gel turned a uniform orange, suggesting that active HRP was evenly distributed. Interestingly the conversion of OPD to DAP was ~10 times slower inside the gel than in solution (Fig 4S).

In conclusion, the nucleated growth of a hydrogel layer on top of a nanometre thick seeding layer has been shown. It is likely that the nucleated growth occurs due to some protons trapped within the seeding layer lowering the local pH and a modification of the apparent pKₐ of the Fmoc-LG due to the surface environment. The growth of gel layers in contact with a pH 7 solution has been used to allow the enzyme HRP to be incorporated into the gel.

Notes and references

PJC acknowledges the EPSRC for funding (EP/H026304/1). DJA and LC acknowledge the EPSRC for funding (EP/G012741/1).

Department of Chemistry, University of Bath, BA2 7AY. E-mail: p.j.cameron@bath.ac.uk

Department of Chemistry, University of Liverpool, L69 7ZD, U.K.

† Electronic Supplementary Information (ESI) available: Experimental detail, SPR curves for growth of hydrogel layers, Fresnel fitting of layers, calibration calculations for enzyme turnover. See DOI: 10.1039/b000000x