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Communication

Surface nucleated growth of dipeptide fibres

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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[1-4]. LMW hydrogels are currently being investigated as energy transfer materials[5, 6] and as 3-D scaffolds for cell growth and wound healing[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 co-ordinate into fibres. Fibril formation can be induced in dipeptide-based LMWG systems enzymatically[10-12], thermally,[13] by changing solvent composition[14], or via pH drop. [15-21] LMW gelators that assemble at surfaces due to electrostatic interactions or ligand-receptor interactions have also been reported.[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.[17] 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_a of the terminal carboxylic acid (pK_a = 5.8 [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^{-3}, at pH 7) and then left in contact with ~ 0.5 cm^{-3} of Fmoc-LG (2.4 mmol dm^{-3}, either in NaCl or phosphate buffered saline both at pH7) for 48 hours without the application of any further current.

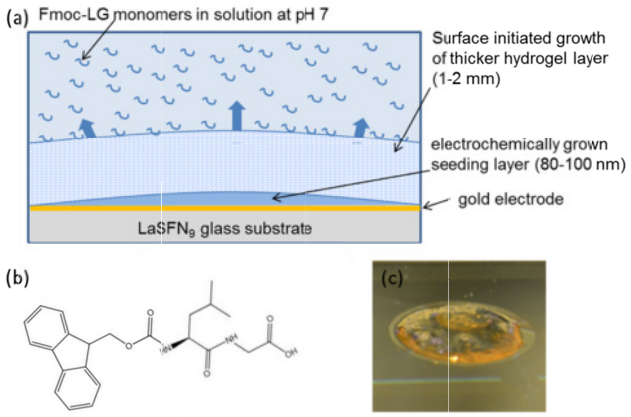

Fig 1. (a) Schematic showing the surface initiated growth of an Fmoc-LG film, (b) the structure of Fmoc-LG, (c) a photo of a surface initiated gel layer after 48 hours in solution (The gel was formed within an area delineated by the circular o-ring of an electrochemical SPR cell).

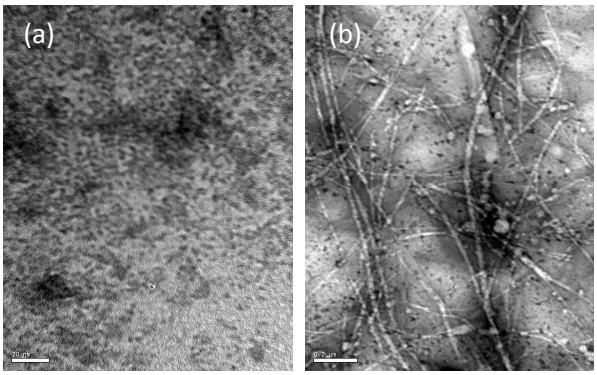

Fig. 2 (a) shows a TEM image of the seeding layer grown electrochemically onto the TEM grid (the grid was used as the electrode); (b) shows the development of fibrous structures after the seeding layer has been left in contact with Fmoc-LG-OH in solution for 48 hours. The scale bar on the left hand image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

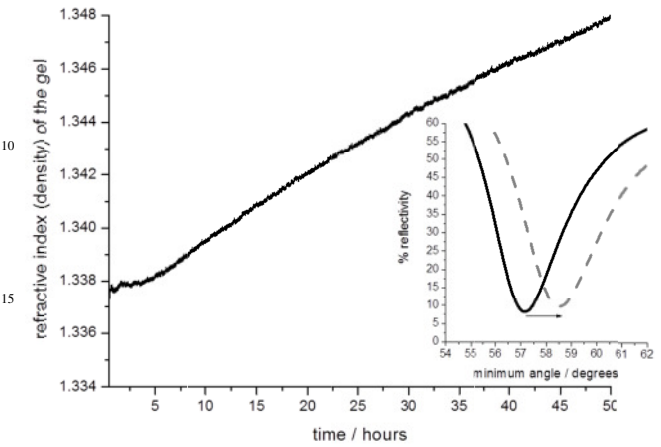

Fig. 3 The real time change in refractive index as the thick gel layer nucleated and grew on the pre-seeded surface. The refractive index shift represents a steady increase in gel density with time. Insert shows the corresponding shift in minimum angle as the thick gel layer formed.

The pH of the growth solution was measured after 48 hours and remained at pH 7. The pH of the 1-2mm thick gel film (measured with both pH paper and by inserting a pH probe designed for gels into the film) was 6.5 after 48 hours. Importantly, control experiments showed that no gel layer grew in the absence of the seeding layer. In a second set of control experiments, all of the experimental steps (electrochemistry, rinsing and 48 hour monitoring) were carried out in the absence of hydroquinone. Hydroquinone is necessary to generate the surface localised pH drop that induces formation of the seeding layer. Without hydroquinone, no seeding layer formed and no thicker gel layer grew on the surface.

Both the growth of the seeding layer and the subsequent spontaneous assembly of a thicker layer were followed in-situ using SPR, a technique which can be used to measures changes in refractive index on top of a metal surface.[24-26] As the evanescent wave of the surface plasmon only extends ~ 200 nm into the material above the metal, SPR cannot be used to measure the thickness of gel layers above 200 nm. SPR can, however, be used to prove the existence of the seeding layer (Fig. 2S). It was also used to measure changes in the gel density as hierarchical structures developed during the 48 hour growth period. The real time change in gel refractive index measured over 48 hours is shown in Figure 3. The increase in refractive index is directly related to an increase in structure density, e.g. the formation of aggregates or fibres at the surface.

Interestingly there was a four-hour time lag during which the gel layer at the metal surface remained unchanged, followed by 44 hours where the amount of structure in the gel increased steadily with time. The refractive index increased from 1.338 to 1.348 which corresponds to a fivefold increase in gel density over 44 hours (ESI). The time lag does not necessarily indicate that the film remained static during this time, just that close to the surface no changes were occurring.

A nucleation and growth mechanism for Fmoc-LG gels has previously been reported in mixed DMSO/water systems.[27] Using a pH trigger, fibre growth was imaged, although the nucleation step was below the temporal and spatial resolution of confocal microscopy.[28] 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.[29, 30] 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.[30] Fibre formation by the miss-folding of silk-like proteins has also been induced by the presence of a mica surface.[31]

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_as considerably higher than the pK_a of the free dipeptide have been reported. [32-35] Surface induced pK_a shifts have also been reported for a range of self-assembled monolayers (SAMs) and surface bound molecules.[36-39] 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.[38] 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.[37] 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.[36] 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 $moldm^{-3}$ 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.[40] Enzymes and bacteria have also been incorporated in Fmoc-F/gelatin films.[41] 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 H_2O_2 (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 H_2O_2 and the colour change in the gel was monitored by UV-Vis in reflectance mode.

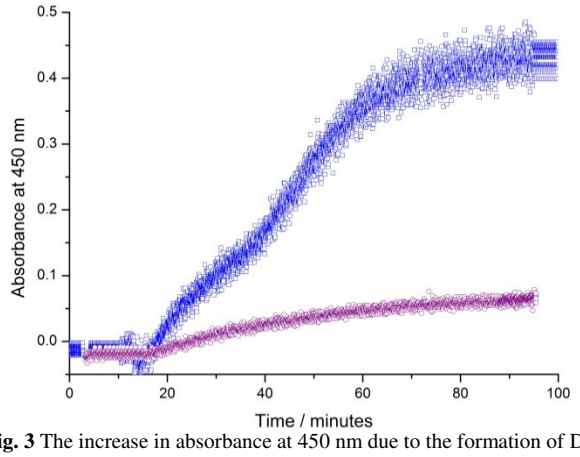

Fig. 3 The increase in absorbance at 450 nm due to the formation of DAP after the addition of OPD and H_2O_2 at 18 minutes. The purple circles show the change for a gel that does not contain HRP (DAP forms in contact with oxygen); the blue squares show the change for a gel incorporating HRP.

The solution containing the OPD and H_2O_2 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 pK_a 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

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† 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/

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