Biomaterials Science

PAPER

View Article Online
View Journal | View Issue

Cite this: *Biomater. Sci.*, 2015, **3**, 298

Dissolution and degradation of Fmoc-diphenylalanine self-assembled gels results in necrosis at high concentrations *in vitro*†

Warren Ty Truong,[a] Yingying Su,[b,c] Danmar Gloria,[a] Filip Braet[b,c] and Pall Thordarson*[a]

Received 15th July 2014,
Accepted 10th October 2014

DOI: 10.1039/c4bm00244j

www.rsc.org/biomaterialsscience

Herein we report an approach to assess *in vitro* cellular responses to the dissolution or degradation products from Fmoc-diphenylalanine (**Fmoc-FF**) self-assembled hydrogels. Three cell lines were used in these studies and two-way ANOVA was used to assess (i) the age of gel dissolution and degradation products and (ii) exposure time on cell fate and state, using viability assays in conjunction with time-lapse fluorescence and high-resolution scanning electron microscopy investigation. The studies show that leaching time but not the exposure time affects the overall cell viability. The cytotoxic effect was only observed once the gel is completely dissolved. Further analysis revealed that the principal mechanism of cell death is necrosis. In addition, the effect of chemotherapeutics (5-fluorouracil and paclitaxel) released from the **Fmoc-FF** gel (with addition before and after gelation) on colorectal cancer cells were investigated using this methodology, demonstrating enhanced activity of these drugs compared to bulk control. This enhanced activity, however, appears to be a combination of the apoptosis caused by the cancer drugs and necrosis caused by gel dissolution and degradation products. Given that *in vivo* studies by others on Fmoc-peptides that this material is not harmful to animals, our work highlights that conventional *in vitro* cellular assays may yield conflicting messages when used for the evaluation of cytotoxicity and drug release from self-assembled gels such as **Fmoc-FF** and that better *in vitro* models, (*e.g.* 3D cell culture systems) need to be developed to evaluate these materials for biomedical applications.

Introduction

Self-assembly is ubiquitous to life with membranes and organelles providing two illustrative examples. Naturally occurring self-assembled systems such as amyloid plaques and prions are also implicated in the pathogenesis of major diseases including Alzheimer's, Parkinson's and Creutzfeldt-Jakob disease.[1] Synthetic self-assembled materials, such as self-assembled gels are of increasing interest in medicine due to their promise in biomedical applications such as a drug delivery vehicle and in tissue engineering and regenerative medicine.[2,3]

The unique properties of self-assembled gels pose some challenges when it comes to assessing their safety. Typical self-assembled gels consists of 0.1%–2% w/w of a low molecular-mass organic gelator (around 1–20 mM). Gels designed for medical applications practically almost always use water as a solvent.[4] Classical cytotoxicity assays are, however, usually not conducted above the concentrations of 0.05–0.5 mM (50–500 μM).[5,6] Previously, we,[7] and others,[8] have reported on the cytotoxicity of the gelator at the sub-millimolar level but cytotoxicity studies on gelators above 1 mM concentration are rare due to practical issues such as trying to homogenise a > 1 mM solution of a material that wants to form a self-assembled gel.[9]

The cytotoxicity of self-assembled gels has also been assessed by seeding cells on top of pre-formed gels but this approach may be highly sensitive to the cell line used and the batch-to-batch rigidity of the gels formed.[10] In early work by Ulijn and co-workers, bovine chondrocytes would proliferate for up to a week after incorporation or when seeded on top of

[a]*School of Chemistry, the Australian Centre for Nanomedicine and the ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, The University of New South Wales, Sydney, NSW 2052, Australia. E-mail: p.thordarson@unsw.edu.au; Tel: +61-2-9385-4478*
[b]*Australian Centre for Microscopy & Microanalysis, Madsen Building F09, The University of Sydney, Sydney, NSW 2006, Australia*
[c]*School of Medical Sciences (Discipline of Anatomy and Histology) — The Bosch Institute, The University of Sydney, Sydney, NSW 2006, Australia*
† Electronic supplementary information (ESI) available: Details on ANOVA analysis, Figures showing the details on drug loading experiments, additional HO/PI and SEM micrographs, details on LC-MS analysis of gel leachate. See DOI: 10.1039/c4bm00244j

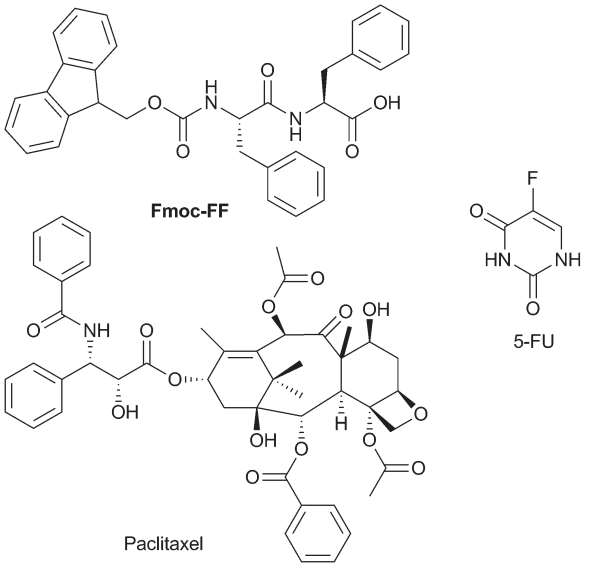

Fig. 1 Chemical structures of **Fmoc-FF**, paclitaxel (Taxol®), a mitotic inhibitor and 5-FU, a thymidylate synthase inhibitor.

gels formed from the peptide-based Fmoc-diphenylalanine (**Fmoc-FF**, Fig. 1).[11] At the same time, Gazit and co-workers seeded Chinese hamster ovary cells (CHO-K1) (ATCC® CCL-61™) on top of pre-formed self-assembled gels from the **Fmoc-FF** gelator. The cytotoxicity of this gelator was assessed with the MTT assay, showing over 90% viability for the CHO-K1 cells.[12] A similar study by Zhou and co-workers[13] using **Fmoc-FF** gels mixed with the transmembrane integrin binding sequence Fmoc-RGD using adult human dermal fibroblasts (ATCC® PCS-201-012™) also indicated good viability of cells in the presence of self-assembled gels.

It is noteworthy here though that the above studies mostly focussed on the "contact toxicity" or two-dimensional (2D) exposure of self-assembled gels to cell lines, rather than how cells respond to global three-dimensional (3D) exposure to the gelators. The biological relevance of the currently used methods for these studies also remains questionable. There is a growing body of evidence that at high concentrations, amphiphilic molecules can be exchanged between different types of self-assembled structures, *e.g.* as in the case of lipid exchange between cubosome and model membranes.[14] This suggests that cytotoxic effects observed in *in vitro* studies using millimolar quantities of amphiphile-like gelators might be due to non-specific lipid exchange in the target cells, resulting in membrane destabilisation and subsequent cell death.[15] However, several *in vivo* studies on amphiphilic peptides[16,17] and Fmoc-peptides[18] self-assembled gels (albeit none to date on **Fmoc-FF**) have indicated that these same amphiphiles (gelators) have relatively benign cellular effects. This is not surprising as metabolic processes *in vivo* could easily clear these amphiphiles away from their delivery site before their soluble concentration reaches millimolar levels. Questions remain, however, concerning the potential degree of cytotoxicity of low-molecular weight gelators that leach out of the self-assembled gel matrix into the surrounding tissue. Additionally, the exact mechanism of gelator cytotoxicity *in vitro* is poorly understood as is how the cytotoxicity of self-assembled gels might influence *in vitro* drug release evaluations; drug release is often highlighted as one of the most promising type of application that these materials have.[2]

Herein, we report on work that was undertaken to address the current lack of knowledge on how different cell lines respond *in vitro* to the exposure of a low-molecular weight gelator leachate (dissolution) and degradation products from a pre-formed self-assembled gel. The methodology reported here is inspired by earlier studies concerning drug delivery from polymer-microspheres.[19] It must be emphasised here that the purpose of this work is not explore if the cytotoxicity of self-assembled gels could be useful as, *e.g.* cancer theurapeutics in their own right, but rather how gel leachate at high concentration may create false positives in typical *in vitro* cytotoxicity screens. This method can also be used to study the effect of drug release from self-assembled gels and, if the mechanism of cell death is taken into account, it is possible to use this method to differentiate drug activity from cytotoxic effects arising from the gel. The **Fmoc-FF** gelator was chosen in this work as it forms stable self-assembled gels under physiological conditions, and has been extensively used since it was first reported to form a gel in 2005,[20] including *in vitro* as mentioned above.[11–13]

Three cell lines were used in these studies; the human cervical cancer cell line HeLa (ATCC® number: CCL-2™), human colorectal cancer cell line Caco-2 (ATCC® number: HTB-37™) and human gingival fibroblasts HGF-1 (ATCC® number: CRL-2014™). The cytotoxicity of the **Fmoc-FF** gelator was tested against two parameters: (i) the age of gel dissolution or degradation products and (ii) exposure time, with results showing that only the former affects cell viability, with necrosis being the main mode of cytotoxicity. The cytotoxicity of gels formed from **Fmoc-FF** in combination with chemotherapeutics towards the colorectal Caco-2 cell line was next assessed with 5-fluorouracil (5-FU) and paclitaxel (Taxol®) shown in Fig. 1, demonstrating enhanced cell killing by both apoptosis and necrosis when 5-FU was directly released from the **Fmoc-FF** self-assembled gels, suggesting that the drug and gelator cytotoxicity is acting in combination under these conditions.

Combined, our detailed analysis on the potential cytotoxicity of self-assembled gels alone and gels in combination with chemotherapeutic drugs underlines that the *in vitro* biological activity of these and other self-assembled materials needs to be evaluated with caution and that conventional assays may not be ideally suited to investigate the scope and limitation of these novel materials in modern medicine.

Experimental

Synthesis of Fmoc-FF

The synthesis of **Fmoc-FF** was based on a previous reported synthesis by Lamothe *et al.*[21]

Cell lines & cell culture models

The human colorectal cancer (CRC) cell line Caco-2[22,23] (ATCC® number: HTB-37™), human cervical cancer cell line HeLa[24] (ATCC® number: CCL-2™) and human gingival fibroblasts[25–27] HGF-1 (ATCC® number: CRL-2014™) were kindly provided by the Australian Centre for Microscopy & Microanalysis (ACMM), University of Sydney. Cell lines were originally obtained from the American Type Culture Collection (ATCC, Manasas VA). Caco-2 is a well-established CRC cell model used in biopharmacological studies. HeLa is one of the most commonly used cancerous human control cell lines and has been widely used in cell biology assays to assess drug responses.[21] HGF-1 is a non-cancerous fibroblast cell line and was used as a control cell line in this study.

All reagents and chemicals used for cell culture were purchased from Invitrogen, Australia unless stated otherwise. Caco-2, HeLa and HGF-1 cells were cultured in Advanced Dulbecco's Modified Eagle Medium (Advanced DMEM, Cat. no. 12491) supplemented with 5% v/v fetal bovine serum (FBS, Cat. no. 10100), 1× antibiotics and antimycotics containing 100 U mL^{-1} penicillin and 100 μg ml^{-1} streptomycin (Cat. no. 15240), and 2 mM L-Glutamine (Cat. no. 35050). Cells were kept at 37 °C in a humidified incubator (Thermo Scientific, USA) with 5% CO_2 and maintained in exponential growth. The media was routinely changed every 2 days. When 70–80% confluence was reached, cells were detached with 0.12% trypsin in PBS/EDTA (Cat. no. 59430C, SAFC Biosciences, USA) and subcultured into 75 cm^2 tissue culture flasks according to the ATCC recommended subcultivation ratio. For the experiments, cells were seeded in cell culture dishes (96-, 24-well plates or 35 mm dishes) at a density of 1.25×10^4 cells cm^{-2}. Cells were cultivated in a humidified incubator at 37 °C with 5% CO_2 for 24 h before incubation with hydrogels.

Hydrogel preparation

Typically, 1.5–3.0 mg of **Fmoc-FF** crystals was weighed into 1 mL glass vials and water (100 μL per 1 mg) would be added (Fig. 2a), followed by addition of aqueous sodium hydroxide (40 μL per mL of water, 1 M) to a final pH ≈ 9. During this time, samples were placed in a sonicator and vortexed interchangeably until the powder dissolved and a clear solution was formed (Fig. 2b). This solution (120 μL) was then pipetted into cell culture inserts (BD Falcon™ Cat. no. 353095) in 24-well cell culture plates. Aqueous hydrochloric acid (HCl, 400 μL, 1 M) was then added (outside the insert) and gelation occurred after 1 h (Fig. 2c). Gelation was considered to have occurred when a homogenous substance was obtained which exhibited no gravitational flow upon inverting the vial. Following gelation, the cell culture insert was transferred to a new well and phosphate buffered saline (PBS) (600 μL, pH 7.4) was added (outside the insert) to rinse the gel (3 × 10 min). The PBS was replaced with advanced DMEM (1 mL) (outside the insert) and hydrogels were allowed to equilibrate overnight at 37 °C (Fig. 2d) with the pH of the gel stabilized at around pH 7.5 (±0.5). Over time the volume of the gel in the cell culture insert decreases which is interpreted as gel dissolution and when the volume has decreased to less than 10% of the original gel volume (by visual inspection), the gel is said to be fully dissolved in the media.

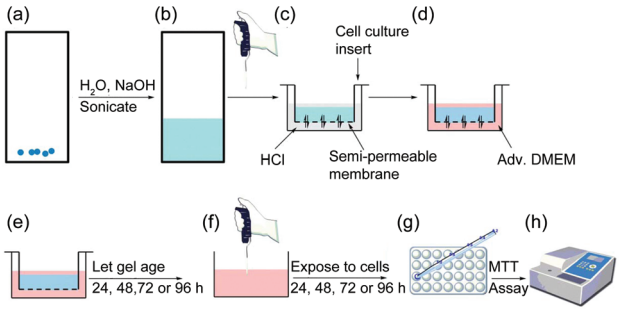

Fig. 2 Methodology for collecting gel degradation products. (a) The gelator (10 mg) is dissolved in water (1 mL) and NaOH (40 μL mL^{-1} of H_2O, 1 M) and vortexed/sonicated interchangeably until homogenous. (b) This solution (120 μL) is then transferred into cell culture inserts, in 24-well cell culture plates. HCl (400 μL, 1 M) is then added in the well (outside the insert). After 1 h, a homogenous gel is formed, confirmed with the inversion test. (d) The gel is rinsed with phosphate buffered saline (3 × 10 min) (outside the well) and equilibrated with Adv. DMEM (1 mL, outside the well) overnight in an incubator (37 °C, 5% CO_2) for 24 h. (e) The gel is then aged for the selected amount of time. (f) The media was not changed during the incubation period, to allow for the accumulation of these products. The leachate (100 μL) is collected from the well after the selected time period (24, 48, 72 or 96 h); denoted as leaching time (h) in Fig. 2. (g) The collected leachate is exposed to cells for the selected amount of time (24, 48, 72 or 96 h). Cytotoxicity is then determined with the MTT assay.

Drug loading for chemotherapeutic and hydrogel experiments

Chemotherapeutics were loaded in the **Fmoc-FF** gels prior to or after gelation (See Fig. S1†). To load chemotherapeutics prior to gelation, **Fmoc-FF** gelator solutions were prepared as described in the hydrogel preparation section. Prior to the addition of aqueous hydrochloric acid outside the inserts, a stock solution of paclitaxel in absolute ethanol (12 μL, 772 μM) or 5-FU (12 μL, 7.7 mM) was added to the gel (120 μL) to give final concentrations of (82 μM 5-FU or 8.2 μM paclitaxel) when incubated with 1.132 mL of DMEM outside the cell culture insert. To load chemotherapeutics after gelation, gels from **Fmoc-FF** were prepared as described in the hydrogel preparation section and after gelation, the stock solutions of paclitaxel or 5-FU in absolute ethanol using the same concentrations and volume as described above, followed by the addition of 1.132 mL of DMEM was added outside the cell culture insert. It was assumed the porous nature of the gels would allow the penetration of the stock solutions into the gels after gelation.

Methodology for collection of gel dissolution and degradation products

The use of cell culture inserts for gel formation was based on a previously reported method by Jayawarna *et al.*[28] **Fmoc-FF** gels were prepared as specified in the hydrogel preparation section.

After preparation and prior to collection, gels in cell culture inserts were transferred to new wells at which point time-lapse experiments would start.

Gels were allowed to age (Fig. 2e) over a specified time period (24, 48, 72 or 96 h) at 37 °C and the dissolution and degradation products were collected in the media (100 μL) outside the well (Fig. 2f). The media was not changed during the incubation period, to allow for the accumulation of these products. These products were then exposed to cells grown in 96-well plates for 24, 48, 72 or 96 h (Fig. 2g).

For LC-MS analysis, aliquots (10 μL) were collected at various time points (24, 48, 72, 96 h) from the gels under the same conditions as above (Fig. 2f), and diluted with water (490 μL) so that the final concentration is 98% water and 2% DMEM. This procedure was repeated four times (n = 4). Samples were then sonicated to ensure any potential fibril formation is disrupted before LC-MS analysis. A blank sample of DMEM (10 μL) was also collect and prepared in the same manner. Standard solutions containing Fmoc-Phenylalanine (**Fmoc-F**) and **Fmoc-FF** solutions were prepared from lyophilised powders in a matrix matched solvent (H_2O–DMEM, 98 : 2 v/v) at concentrations from 0.2–2.0 mM. The 2.0 mM concentration was chosen to match the theoretical maximum concentration (*i.e.* 2.0 mM) of **Fmoc-FF** if it were to leach fully into the system. These samples were then analysed by LC-MS using standard peptide condition on a C18 reverse phase HPLC column (see ESI†).

MTT assay

Typically 1 × 10^4 (Caco-2, HeLa, HGF-1) cells in 100 μL of media were seeded in 96-well plates and incubated at 37 °C with 5% CO_2 atmosphere for 24 h. Attachment of cells was confirmed by light microscopy at 20× magnification. For blank controls, wells with media (*i.e.* no cells) were used. To evaluate the toxicity of the gels, 100 μL of advanced DMEM media (outside the insert) was removed from the wells at 24, 48, 72 and 96 h time points (Fig. 2e). The media (100 μL, Fig. 2f) was then added to cells grown in 96-well plates, replacing the media they were incubated in, for 24, 48, 72 or 96 h (Fig. 2g). At the end of the exposure times, the media was replaced with 100 μL of fresh media and 20 μL of MTT solution (2.5 mg mL^{-1} in PBS) was added to each well and mixed thoroughly. Cells were incubated for 4 h to allow the MTT to be metabolised. After this time, the culture media was removed and DMSO (150 μL) was added into each well to dissolve the resultant formazan crystals. The plates were then shaken for 1 min and the absorbance was read at 600 nm at 37 °C using a Victor^3 V microplate reader (PerkinElmer, Fig. 2h). For control (100% viability) cells were grown in same conditions/time but without any gel degradation products added. The standard deviation for the MTT assay on the controls varied within 10%.

Fluorescence microscopy

Nuclear morphology analysis was performed by double staining with Hoechst 33342 and propidium iodide (PI). After incubation with the hydrogel dissolution and degradation products, cells were stained with 0.5 μg mL^{-1} Hoechst and 1 μg mL^{-1} PI for 10 min. The cells were immediately imaged and nuclei counted using a 10× objective (0.4 N.A.) on an Olympus Cell^R *epi*-fluorescence microscope (Olympus, Japan). Living cells with smooth, intact nuclei stained only with Hoechst 33342. Apoptotic cells showed condensed or fragmented nuclei as observed with Hoechst 33342 staining. Necrotic cells had a compact nuclear morphology similar to living cells but additionally stained with Hoechst 33342 and PI due to disrupted cell membrane integrity (Fig. S†). Late stage apoptotic cells underwent secondary necrosis, and fragmented nuclei stained with Hoechst 33342 and PI. Cell counts were performed on 30 random fields (500 μm × 500 μm) per dish, as previously described in detail.[29]

Scanning electron microscopy

At the endpoints of the HO/PI experimentation, cells were prepared for scanning electron microscopy (SEM) as described in detail in our earlier work.[30] Briefly, cells were fixed with 2% (v/v) glutaraldehyde (Cat. no. C001, ProSciTech, Australia) in 0.1 M sodium cacodylate buffer enriched with 0.1 M sucrose (300–320 mOsmol kg^{-1}, pH 7.4) at room temperature (RT). Next, samples were postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate/sucrose buffer, dehydrated in graded series of ethanol solutions (70%, 80%, 90%, 100%) followed by exposure to 100% hexamethyldisilazane (HMDS) for 3 min then air dried for 1 min as described previously.[31] Samples were sputter coated with 2.5 nm chromium (Xenosput, Dynavac) and imaged with a field emission-scanning electron microscope (FE-SEM, Zeiss Ultraplus, Germany) at 3–5 kV, with an Inlens detector and at a working distance of 3–6 mm.[30]

Results and discussion

Cytotoxicity of gel dissolution and degradation products and the influence of exposure time

The overall procedure for investigating the effect of dissolution and degradation products from the **Fmoc-FF** self-assembled gels is shown in Fig. 2. Detailed statistical analyses of the repeated measurement (n = 3) results indicates that the critical factor affecting cell viability is the time the gel was allowed to dissolve, degrade and leach into the media (Leaching Time (h)), as can be seen by the significant decrease in the viability of the cells (*i.e.*, p-value < 0.0001 for HGF-1 and HeLa cells and 0.0006 for Caco-2 cells) exposed to the leached media collected after 72–96 h (see Fig. 3). It should be noted that the p-values calculated for leaching times take into account data across all exposure times. This drop in viability at later time points could be most likely due to higher levels of gel dissolution and degradation products, inferred from the detachment of the gels within the cell culture inserts from 48 h onwards and the decrease in volume of the gel relative to the initial volume.

The results also show that the length of exposure time of the cell lines to the leached media, only had a significant

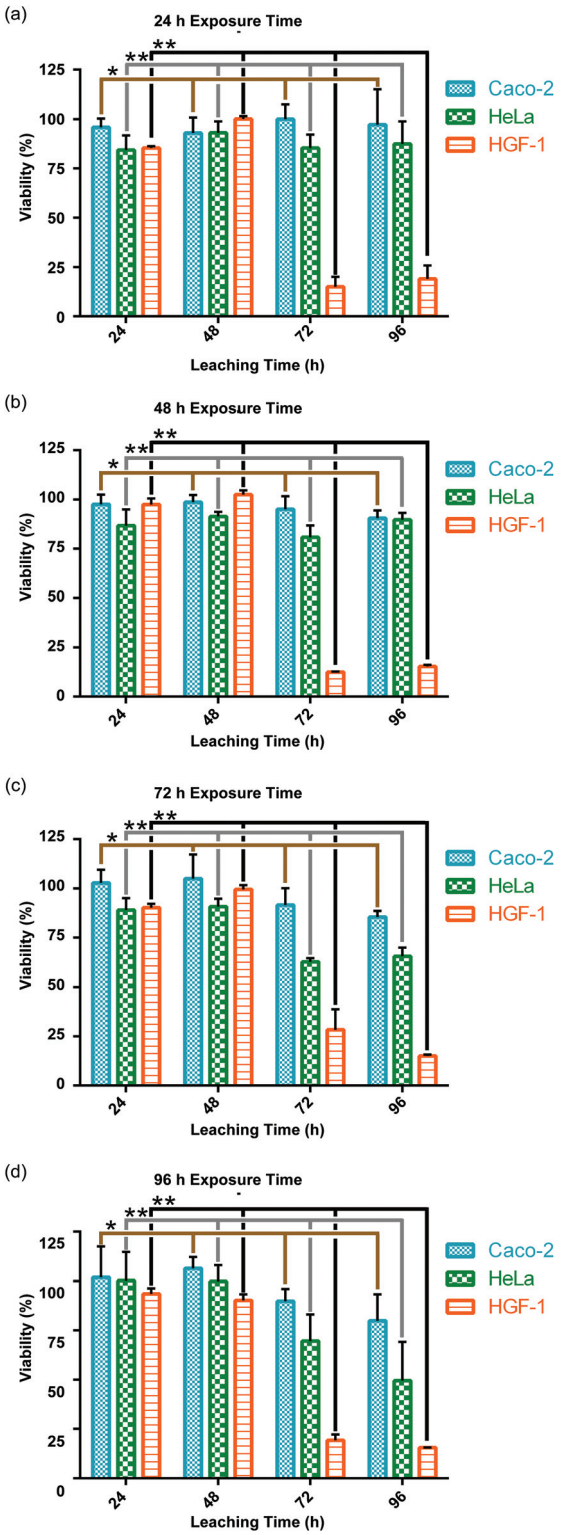

Fig. 3 Effects of **Fmoc-FF** leach times (24–96 h) *vs.* exposure times (24–96 h) on the viability of various cell lines, HeLa, Caco-2 and HGF-1. Leaching time *p-value < 0.0005, **p-value < 0.0001 (these p-values are same for (a)–(d) as they take all the exposure times into account). For the exposure times, p-values were non-significant for Caco-2 and HGF-1 with p-value = 0.0008 for HeLa (*i.e.* data points at equivalent leaching times are compared across the four different exposure time points in (a)–(d)). All experiments were done in at least triplicate. Error bars represent 95% confidence interval.

effect on the viability of HeLa cells whereas it had a negligible effect on the viability of the Caco2 and HGF-1 cells.

The most notable drop in viability could be observed in the HGF-1 cells exposed to gel dissolution and degradation products collected after 72 h and 96 h leaching times. HGF-1 cell viability was least affected by the gel dissolution and degradation products at 24 h and 48 h. Two-way ANOVA analysis (α = 0.05, see ESI†) of the results showed that the leaching time was highly significant (p-value < 0.0001), whereas the exposure time (p-value = 0.0829) and the interaction between leaching time and the exposure time (p-value = 0.0685) were insignificant.

Analysis of the results from the MTT assay of the HeLa cell line revealed that the leaching times (p-value < 0.0001), the exposure time (p-value = 0.0008) and the interaction between these two parameters (p-value < 0.0001) have significant effect on the viability of the HeLa cells. HeLA cells exposed to the gel dissolution and degradation products collected after 24 h and 48 h time points have significantly higher percentage viability compared to the cells exposed to the media collected after 72 h and 96 h time points. The difference in the percentage viability is more pronounced when the cells are exposed to the media for more than 72 h.

Caco-2 cells also tended towards the decline in viability as the cells are exposed to dissolution and degradation products collected at longer leaching times, however the decrease was not as pronounced compared to the two other cell lines. Statistical analysis suggests that the leaching time (p-value = 0.0006) and the interaction between the leaching time and exposure time (p-value = 0.0011) have significant effect on the percentage viability of the Caco-2 cells, whereas the exposure time alone (p-value = 0.6781) does not have a significant effect on the viability of Caco-2 cells.

The decline in viability for all three cell lines when exposed to gel dissolution and degradation products at 72 h onwards could be due to the higher levels of gel dissolution and degradation products accrued over longer leaching times. Attempts to identify and quantify these products using LC-MS were not successful (Fig. S2–S8†). Two unidentified peaks did appear in the LC-MS traces at 72 h and 96 h which had similar but not identical retention times to **Fmoc-FF** but the exact molecular weight of these products could not be obtained from the very noisy mass spectra.

The concentration of gel dissolution and degradation products is inversely related to the viability of the three cell lines. It is to be noted that from about 48 h onwards, the total volume of the gels in the inserts has decreased considerably due to dissolution and degradation with most of the gels dissolving within 72–96 h. To ascertain the mechanisms behind the low viability levels at these time points, fluorescence microscopy studies were carried out, as MTT assays.

Biomolecular imaging studies on Fmoc-FF leachate effects

To verify and gain further qualitative insight into the effect that the **Fmoc-FF** leachate has on the cell line tested, a combination of fluorescence microscopy and scanning electron

microscopy was carried out at a selected combination of leaching and exposure times (see Table S1†). For fluorescence microscopy studies, cells were double stained at these time points with Hoechst 33342/propidium iodide (HO/PI) to assess the type of cell death induced in cells through analysis of their morphologies. Two main forms of cell death, apoptosis and necrosis have been widely studied and characterised,[32–36] The blue fluorescent HO stain can cross the intact membrane of live cells. It stains the condensed chromatin of apoptotic cells more brightly than the looser chromatin of normal cells and enables the monitoring of nuclear changes associated with apoptosis (see Fig. S9†). The red-fluorescent PI is excluded from viable and early apoptotic cells and its uptake is characteristic of late apoptotic and necrotic cells (membrane-altered cells).[37]

Once the fluorescence microscopy analysis had been completed on these samples, they were subsequently processed for further surface morphology studies analysed by scanning electron microscopy (SEM).[30] The same conditions for incubation were used for cells treated with dissolution and degradation products for the experiments described above (Fig. 1) except cells were seeded onto glass coverslips to allow subsequent analysis with fluorescence microscopy and SEM.

The results of the fluorescence microscopy (Fig. S10†) and SEM (Fig. S11†) study, by and large support the quantitative MTT results discussed above (Fig. 3). The fluorescence microscopy results (Fig. S10†) show no discernible change in nuclear or cellular morphology characteristic of apoptosis in the Caco-2 and HeLa cells exposed to dissolution and degradation products from **Fmoc-FF** relative to the control groups. A few necrotic cells were observed in the Caco-2 and HeLa cell lines *via* HO/PI staining but generally the cells looked healthy. In contrast, there was a difference in the morphology of HGF-1 cells exposed to the 24 h and 72 h dissolution and degradation products for 24 h exposures, with higher levels of cell aggregation and also the cells exposed to the 24 h dissolution and degradation products for 72 h exposures, relative to controls. This could be due to the poor adhesion of HGF-1 cells to the glass coverslips.

The SEM analysis (Fig. S10†) also shows that the gel dissolution and degradation products did not alter the cell morphology of Caco-2 and HeLa relative to the controls. At higher magnifications (500–5000×), the high levels of cell aggregation in HGF-1 observed in the HO/PI fluorescence micrographs could be attributed to the low levels of cell spreading on the glass substrates. The poor adhesion explains the aggregation of the cells together, as it has been recognized that cells of multicellular organisms are significantly affected by contacts with other cells or biological substrates. A lack thereof is unfavourable to cell health.[38]

The toxic effects of gels in combination with chemotherapeutics *in vitro*

In addition to assessing the cytotoxicity of **Fmoc-FF** gels as a function of (i) leaching time and (ii) exposure time, the combined effects of chemotherapeutics (paclitaxel (8.2 μM) or 5-FU (82 μM), see Fig. 1) encapsulated within gels formed in the cell culture inserts was evaluated using *epi*-fluorescence microscopy.

The rationale for incorporating chemotherapeutic agents into gels was to develop a test methodology for assessing combined cytotoxicity for future gels; as they are envisaged to be used for localised drug delivery. To minimize possible complication due to cytotoxic effects of the **Fmoc-FF** gelator, these studies were limited to the most robust cell line identified above; Caco-2. In addition, Caco-2 monolayers are widely used across the pharmaceutical industry as an *in vitro* model of the human small intestinal mucosa to predict the absorption of orally administered drugs.[39]

Paclitaxel (Taxol®) (Fig. 1) is an effective anti-cancer drug which was first isolated in 1971 from the bark of the Pacific yew tree, *Taxus brevifolia*[40] Notoriously insoluble in water, paclitaxel is usually administered in a formulation of polyoxyl-35 hydrogenated castor oil and ethanol for the treatment of breast, ovarian, prostate and lung carcinomas, through arresting rapidly dividing cells in mitosis through stabilisation of their spindle microtubules.[40–42] As current delivery methods for paclitaxel could stand to benefit from alternative strategies, and due to its broad spectrum activity, it was chosen as one of the chemotherapeutics to be tested in this study.

As Caco-2 cells were found to be the most robust cell line, the chemotherapeutic 5-FU (Fig. 1), the gold standard for treating colorectal cancers, was also chosen for these studies.[43,44] An analogue of uracil, 5-FU has a fluorine atom at the C-5 position in place of hydrogen. It works through inhibiting essential biosynthetic processes, through incorporation into macromolecules such as DNA or RNA and is widely used to treat a range of cancers, including colorectal and breast cancers.[45]

Gels were prepared in cell culture inserts and chemotherapeutics were added to the gel either: (i) prior to gelation or (ii) after gelation (see Fig. S1†). The bulk IC_{50} value for 5-FU has been reported to be 25 μM for Caco-2 cells[46] but experimentally determined to be 82 μM in our system (results not shown). The bulk IC_{50} value of paclitaxel for Caco-2 has been previously reported to be 3.23–10 μM[47] – experimentally it was determined as 8.2 μM in our system (results not shown).

Caco-2 cells were exposed to the combination of monomer dissolution and degradation products and chemotherapeutics within the cell culture inserts for 24, 48 and 96 h. The 72 h time point was omitted as the results from the earlier toxicity studies showed that it would be similar to 96 h. Because leaching time was found to be the only significant factor affecting the viability of Caco-2 cells, cells were exposed directly to gels with chemotherapeutics within the cell culture inserts, rather than collecting the media outside the cell culture inserts and incubating the cells with the collected media (Fig. S1†).

The addition of chemotherapeutics before and after gelation was performed to assess if the method applied to load the gel with the drug had a significant effect. The cell fate and state of cells was next quantitatively and qualitatively assessed by time-lapse live cell imaging using the Hoechst 33342/propidium iodide (HO/PI) assay. Of special note, compelling

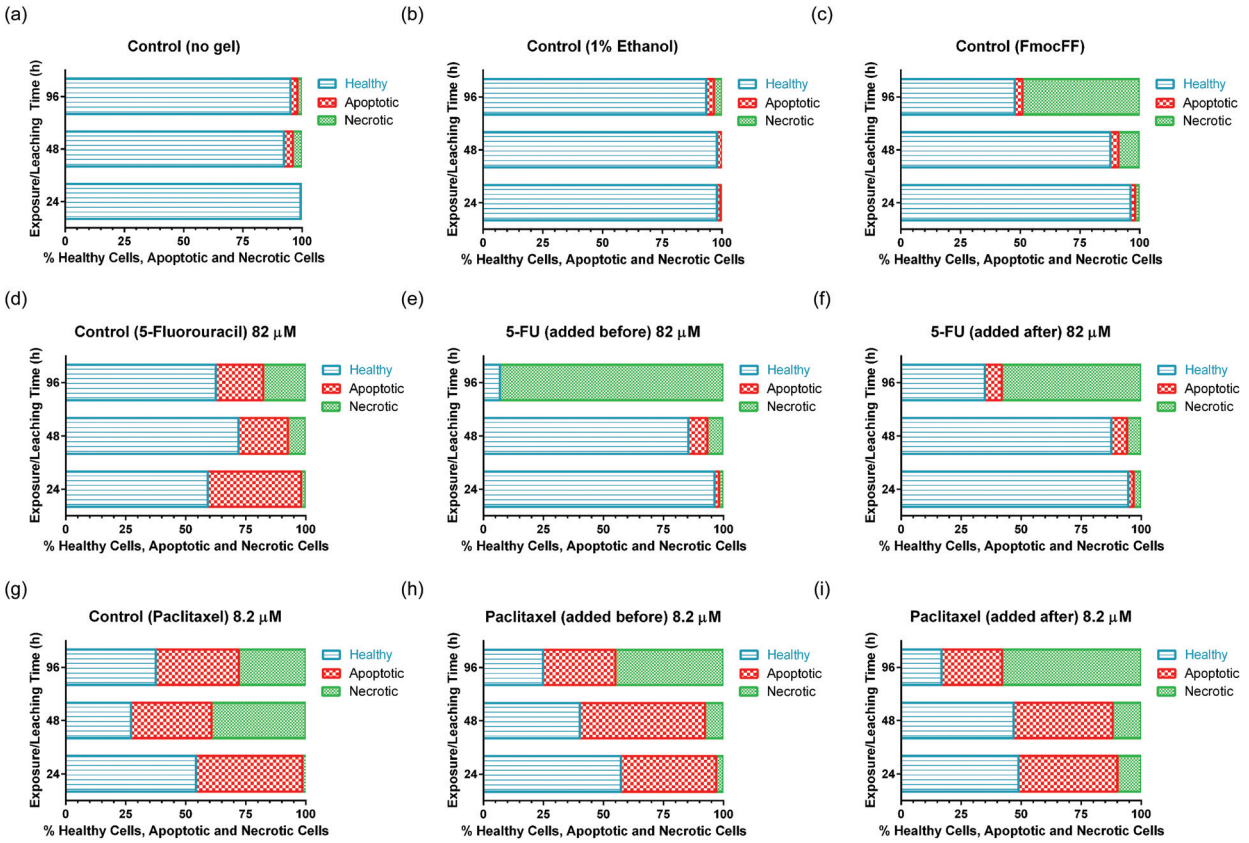

Fig. 4 Caco-2 cells exposed to: (a) Adv. DMEM (b) Ethanol 1% (v/v) (c) **Fmoc-FF** (1% (w/v), 18.7 mM) (d) 5-Fluorouracil (5-FU, 82 μM) (e) 5-FU (82 μM) released from **Fmoc-FF** (added before gelation) (f) 5-FU (82 μM) released from **Fmoc-FF** (added after gelation) (g) Paclitaxel (8.2 μM) and (h) Paclitaxel (8.2 μM) released from **Fmoc-FF** (added before gelation) (i) Paclitaxel (8.2 μM) (added after gelation) released from **Fmoc-FF**.

evidence indicates that both paclitaxel and 5-FU kills cancer cells *via* apoptosis.[44,48] Quantification of live (blue fluorescence), apoptotic cells (intense bright-blue and blue-violet fluorescence) and necrotic (red fluorescence) cells was performed by counting 30 random fields (500 μm × 500 μm) per dish as previously described.[29]

The results of the cell counts are summarised in Fig. 4 and shown in more detail in the micrographs, (Fig. S12, S13 and S14†) for control, 5-FU and paclitaxel, respectively, with representative examples shown in Fig. 5. Since paclitaxel is insoluble in water, ethanol was used as solvent and hence 1% ethanol was included as a control. The blank (Fig. 4a) and 1% ethanol (Fig. 4b) controls exhibited negligible amounts of cytotoxicity while the **Fmoc-FF** gel control (Fig. 4c) showed a decrease in the viability of Caco-2 cells as the leaching time increases. These observations conform with the MTT assay results discussed above (Fig. 2).

From Fig. 4, after 96 h leaching time, Caco-2 cells exposed to 5-FU released from within the **Fmoc-FF** gel (Fig. 4e–f) showed a significant increase in the amount of necrotic cell death compared to the control experiment, where Caco-2 cells were exposed to plain 5-FU, not released from a gel (Fig. 4d). The control experiment had 63% healthy cells remaining, whereas cells exposed to 5-FU (added before gelation) had 3.4% (Fig. 4e) and cells exposed to 5-FU (added after gelation) had 39% healthy cells remaining (Fig. 4f). Statistical analyses indicates that there is a significant difference in the number of healthy cells (p-value = 0.0049) and necrotic cells (p-value = 0.00699) between the three sets of experiments while the difference in the number of apoptotic cells between the sets of experiments was not found to be significant different (p-value = 0.145). The apparent increase in 5-FU activity with **Fmoc-FF** gel-mediated release is not as pronounced at earlier time points (24 and 48 h) however the 5-FU control experiments exhibited significantly greater levels of apoptosis relative to **Fmoc-FF**-mediated release of 5-FU both for the 24 h (p-value < 0.0001) and 48 h (p-value < 0.0065) time points (Table 1).

Interestingly, for the control 5-FU at the 24 h exposure time point, there are significantly more apoptotic cells (40% compared to (Fig. 4e) 2.0% and (Fig. 4f) 2.6%) (p-value < 0.0001) and less healthy cells (58% compared to (Fig. 4e) 96% and (Fig. 4f) 95%) (p-value < 0.0001), than the cells exposed to **Fmoc-FF** gel-mediated release of 5-FU. This could most likely be due to a delayed effect of 5-FU, as it was slowly released from within **Fmoc-FF** gel.

For the cells exposed to the **Fmoc-FF** gel and paclitaxel, there was a decreased amount of healthy cells at the 96 h exposure/leaching time relative to the control, with 27% (Fig. 4h) and 17% (Fig. 4i) remaining healthy when the Fmoc-FF gel was loaded with paclitaxel before or after gelation

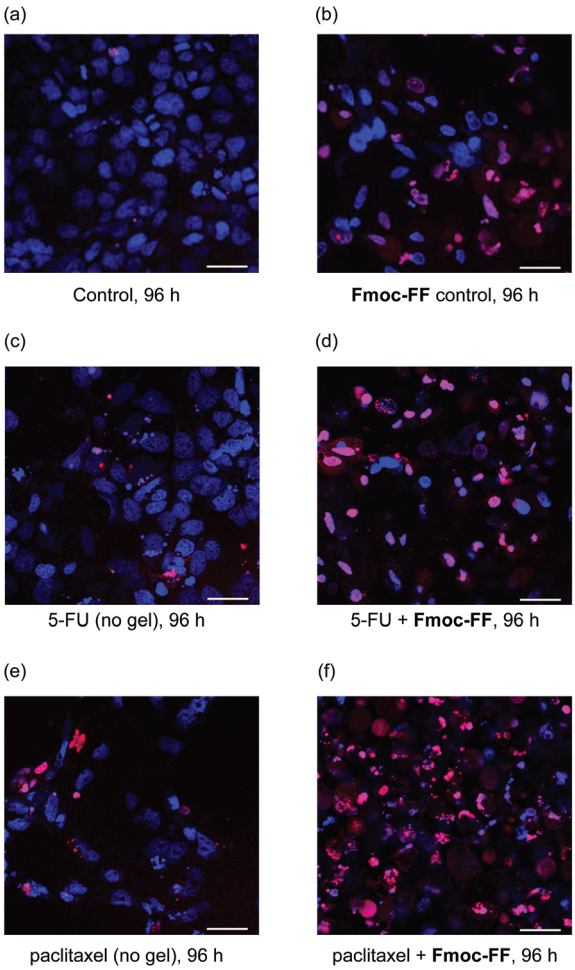

Fig. 5 Selected HO/PI fluorescence micrographs of Caco-2 cells after 96 h exposure to various **Fmoc-FF** (leachate from 1% gel – see Fig. 2 and S1†), 5-Fluorouracil (5-FU, 82 μM) and Paclitaxel (8.2 μM) combinations. Scale bar, 50 μm. Live cells (blue fluorescence), early apoptotic cells (intense bright-blue fluorescence), late apoptotic cells (blue-violet fluorescence) and necrotic cells (pink fluorescence). (a) Control (no additive). (b) **Fmoc-FF** (no drug). (c) 5-FU (no gel). (d) 5-FU from **Fmoc-FF** gel. (e) Paclitaxel (no gel). (f) Paclitaxel from **Fmoc-FF**. For the drug release studies from **Fmoc-FF** – (d, f), the drug was added prior to gelation (Fig. S1†). See text and Fig. S12–S14† for details.

Table 1 *P*-values calculated from one-way ANOVA analysis conducted on the 5-FU data set (Fig. 4d–f)

	Time		
	24 h	48 h	96 h
Live	<0.0001	0.0018	0.0049
Apoptotic	<0.0001	0.0065	0.1459
Necrotic	0.6889	0.8929	0.0069

respectively, compared with 38% for the control (Fig. 4g). Statistical analysis of these results inferred that the levels of healthy cells were not significantly different (p-value = 0.3525). This decrease is mostly attributed to the similar high levels of necrosis (Fig. 4h) 41% and (Fig. 4i) 57% relative respectively

Table 2 *P*-values calculated from one-way ANOVA analysis conducted on the paclitaxel data set (Fig. 4g–h)

	Time		
	24 h	48 h	96 h
Live	0.4063	0.1293	0.3525
Apoptotic	0.8760	0.1361	0.8370
Necrotic	0.0698	0.0955	0.5266

when compared to the control (Fig. 4g) 28%) (p-value = 0.5266) – see also Table 2.

In terms of the level of apoptotic cells induced by **Fmoc-FF**-mediated delivery of paclitaxel, the cell viabilities of the gel-mediated paclitaxel were similar to the control (p-value = 0.876, 0.136, 0.837 for 24, 48 and 96 h exposure/leaching times). Evidence suggests paclitaxel has been shown to kill cancer cells through the induction of apoptosis.[48]

Interestingly, at the 24 h time point, the percentage of healthy cells is similar across all three conditions (Fig. 4g–h) (p-value = 0.406). This is in contrast to the results for 5-FU (Fig. 4d–f) which show significantly less healthy cells and more apoptotic cells in the control (Fig. 4d) relative to Fig. 4e and f (p-value < 0.0001).

HO/PI fluorescence micrographs of the controls used (see Fig. 5a and S12†) show the cells exposed to ethanol in the micrographs exhibit no morphological changes indicative of apoptosis or negligible levels of necrosis and looked similar to the controls. The HO/PI fluorescence micrographs of Caco-2 exposed to the **Fmoc-FF** gel (see Fig. 5b and S11,† bottom row) supports the observations in the initial MTT experiments which showed that gel dissolution and degradation products older than 72 h, in this case 96 h, showed an increase in cytotoxicity over time.

The HO/PI stained fluorescence micrographs for 5-FU released from within the **Fmoc-FF gel** added either before gelation (Fig. 5d and S13†) or after gelation (Fig. S12†) showed significantly more Caco-2 cells died compared to just plain 5-FU (Fig. 5c and S13†), after 96 h exposure. This difference was supported by statistical analysis of the percentage of necrotic cells at 96 h with one-way ANOVA (p-value = 0.00699). This could be due to the combined or synergistic effects of the **Fmoc-FF** gel and 5-FU and/or the sustained release of the drug over time.

Examination of HO/PI stained Caco-2 cells exposed to bulk or gel-mediated delivery of paclitaxel (Fig. 5f and S12†) revealed an apparent increase in the level of necrotic cells for **Fmoc-FF**-mediated delivery of paclitaxel at the 96 h time point compared to paclitaxel on its own (Fig. 5e and S14†), similar to the trend observed for **Fmoc-FF**-mediated delivery of 5-FU However, one-way ANOVA of the levels of necrotic cells suggested that this was not the case (p-value = 0.527), unlike the clear statistical difference seen in **Fmoc-FF**-mediated delivery of 5-FU when quantified (Fig. 4, p-value = 0.0069). As elaborated earlier, this could be due to the larger molecular weight of paclitaxel relative to 5-FU and therefore potential slower release kinetics. Preliminary analysis of the data suggests that

adding chemotherapeutics 5-FU or paclitaxel prior to gelation has a slightly more potent effect on decreasing the viability of Caco-2 cells. This is more pronounced for 5-FU added before gelation, as seen the significantly higher levels of necrotic cells (p-value = 0.0069); paclitaxel was less affected by the order of addition (p-value = 0.527).

Conclusions

In this work, a new approach to assessing cellular response to dissolution and degradation products from self-assembled **Fmoc-FF** gels is described. Two-way ANOVA revealed the critical factor affecting cell viability is the time a gel is allowed to degrade and leach into the media, with gel leachate times >72 h found to be cytotoxic. On the other hand, the duration of cell exposure to the leachate, the exposure time (24–96 h), had a significant effect on only HeLa and not the Caco-2 and HGF-1 cell lines. These results stress that a consideration of the parameters used in cytotoxicity studies and even cell lines is warranted to reduce the possibility of obtaining a false negative result (*i.e.* the gelator is non-toxic). Conversely, it also needs to be stressed when using self-assembled gels, either on their own or combined with other therapeutics, if cytotoxicity is observed in *in vitro* assays for drug activities, such results could be misinterpreted as false positives if our results are not taken into account. Although it was shown that accumulation of gelator dissolution and degradation products over time were detrimental to cell health, this result might not be as relevant *in vivo* as they would be cleared by *in vivo* processes. It also needs to be stressed here that the nature of these degradation products remains unclear and will require further investigation. Attempts to analyse these products by LC-MS did reveal some new peaks appearing after 72–96 h but these could not be identified and did not match **Fmoc-FF** or any reasonable derivatives of **Fmoc-FF**.

These studies also assessed the potential synergies of chemotherapeutics released from within the **Fmoc-FF** gel on the most robust cell line identified, Caco-2. With the aid of HO/PI staining and *epi*-fluorescence microscopy, necrosis was revealed as the mechanism of cell death from gelator dissolution and degradation products >72 h and not through apoptosis which are the known mechanisms of cell death induced by 5-FU and paclitaxel.

As gelator dissolution and degradation products <72 h were established within these studies to be not cytotoxic, it was assumed that the levels of apoptosis quantified were induced solely by 5-FU diffused from **Fmoc-FF**. These levels were found to be significantly low at 24 and 48 h compared to addition of neat 5-FU for the same concentrations and time points. It is postulated that this was due to the slow diffusion of 5-FU from within the gel. This was surprisingly not the case for paclitaxel leached from the **Fmoc-FF** gel; the levels of apoptosis induced were similar to plain paclitaxel at all the time points investigated.

This suggests that while 5-FU is preferentially retained with the **Fmoc-FF** gel, paclitaxel is not. At the 96 h time point, the cytotoxic contributions from the gelator become evident for both 5-FU and paclitaxel as a significant proportion of cells died with morphologies characteristic of necrosis. Consequently, a synergistic cytotoxic effect was observed in both chemotherapeutics released from within hydrogel **Fmoc-FF** at the 96 h time point.

Combined, these results suggest that stability of self-assembled gels plays a key role in determining their potential usefulness for medical application. More stable gels are likely to leach their monomers more slowly into the surrounding biological environment, reducing the overall concentration of these monomers and hence any potential adverse effected. Better understanding of how self-assembled gels are formed[49] and which factors control their stability are therefore critical to advance this field. Equally importantly, these results also highlight that it is crucial to investigate the mechanism of (potential) cytotoxicity of self-assembled gels at high concentrations *in vitro* as they may influence the outcome of studies aimed at evaluating their usefulness for applications such as drug delivery and tissue engineering.

Acknowledgements

The authors acknowledge the use of NCRIS-funded Australian Centre for Microscopy and Microanalysis for microscopy studies as well as the facilities of the Mark Wainwright Analytical Centre of the University of New South Wales. We acknowledge the Australian Research Council for a Discovery Project Grants DP09855059 to P. T. and F. B., a DP130101512 and ARC Centre of Excellence CE140100036 to P. T., a Future Fellowship (FT120100101) to P. T. and a Scholarship to Y. S. as well as the NSW Cancer Institute (08/RFG/1-29) for supporting our work and the University of New South Wales for a Scholarship to W.T.T.

Notes and references

1 I. W. Hamley, *Angew. Chem., Int. Ed.*, 2007, **46**, 8128–8147.
2 W. T. Truong, Y. Su, J. T. Meijer, P. Thordarson and F. Braet, *Chem. – Asian. J.*, 2011, **6**, 30–42.
3 W. T. Truong, L. Lewis and P. Thordarson, in *Functional Molecular Gels*, ed. J. F. Miravet and B. Escuder Gillier, Royal Society of Chemistry, Cambridge, UK, 2014, pp. 156–194.
4 Z. Yang and B. Xu, *J. Mater. Chem.*, 2007, **17**, 2385–2393.
5 S. M. Guichard, J. S. Macpherson, I. Mayer, E. Reid, M. Muir, M. Dodds, S. Alexander and D. I. Jodrell, *Eur. J. Cancer*, 2008, **44**, 310–317.
6 M. Miwa, M. Ura, M. Nishida, N. Sawada, T. Ishikawa, K. Mori, N. Shimma, I. Umeda and H. Ishitsuka, *Eur. J. Cancer*, 1998, **34**, 1274–1281.
7 L. Y. G. Lim, Y. Su, F. Braet and P. Thordarson, *Aust. J. Chem.*, 2009, **62**, 653–656.
8 Z. Yang, G. Liang, M. Ma, A. S. Abbah, W. W. Lu and B. Xu, *Chem. Commun.*, 2007, 843–845.

9 M. Ma, Y. Kuang, Y. Gao, Y. Zhang, P. Gao and B. Xu, *J. Am. Chem. Soc.*, 2010, **132**, 2719–2728.
10 D. J. Adams, M. F. Butler, W. J. Frith, M. Kirkland, L. Mullen and P. Sanderson, *Soft Matter*, 2009, **5**, 1856–1862.
11 V. Jayawarna, M. Ali, T. A. Jowitt, A. F. Miller, A. Saiani, J. E. Gough and R. V. Ulijn, *Adv. Mater.*, 2006, **18**, 611–614.
12 A. Mahler, M. Reches, M. Rechter, S. Cohen and E. Gazit, *Adv. Mater.*, 2006, **18**, 1365–1370.
13 M. Zhou, A. M. Smith, A. K. Das, N. W. Hodson, R. F. Collins, R. W. Ulijn and J. E. Gough, *Biomaterials*, 2009, **30**, 2523–2530.
14 H.-H. Shen, P. G. Hartley, M. James, A. Nelson, H. Defendi and K. M. McLean, *Soft Matter*, 2011, **7**, 8041–8049.
15 S. M. Sagnella, C. E. Conn, I. Krodkiewska, M. Moghaddam and C. J. Drummond, *J. Phys. Chem. B*, 2010, **114**, 1729–1737.
16 V. M. Tysseling-Mattiace, V. Sahni, K. L. Niece, D. Birch, C. Czeisler, M. G. Fehlings, S. I. Stupp and J. A. Kessler, *J. Neurosci.*, 2008, **28**, 3814–3823.
17 R. Solaro, M. Alderighi, M. C. Barsotti, A. Battisti, M. Cifelli, P. Losi, R. Di Stefano, L. Ghezzi and M. R. Tine, *J. Bioact. Compat. Polym.*, 2013, **28**, 3–15.
18 H. Wang, J. Wei, C. Yang, H. Zhao, D. Li, Z. Yin and Z. Yang, *Biomaterials*, 2012, **33**, 5848–5853.
19 C. Wang, G. N. Adrianus, N. Sheng, S. Toh, Y. Gong and D.-A. Wang, *Biomaterials*, 2009, **30**, 6986–6995.
20 M. Reches and E. Gazit, *Isr. J. Chem.*, 2005, **45**, 363–371.
21 M. Lamothe, M. Lannuzel and M. Perez, *J. Comb. Chem.*, 2002, **4**, 73–78.
22 J. Fogh, W. C. Wright and J. D. Loveless, *J. Natl. Cancer Inst.*, 1977, **58**, 209–214.
23 J. Fogh, J. M. Fogh and T. Orfeo, *J. Natl. Cancer Inst.*, 1977, **59**, 221–226.
24 H. Landecker, in *Biotechnology and culture: bodies, anxieties, ethics*, ed. P. Brodwin, Indiana Univ. Press, Bloomington, Ind., 2000, pp. 53–72.
25 A. Johansson, A. Bergenholtz and S. E. Holm, *J. Periodontal Res.*, 1996, **31**, 477–482.
26 S. Y. Rawal, M. K. Dabbous and D. A. Tipton, *J. Periodontal Res.*, 2011, **47**, 320–329.
27 F. Guo, D. E. Carter, A. Mukhopadhyay and A. Leask, *PLoS One*, 2011, **6**, e27097.
28 V. Jayawarna, S. M. Richardson, A. R. Hirst, N. W. Hodson, A. Saiani, J. E. Gough and R. V. Ulijn, *Acta Biomater.*, 2009, **5**, 934–943.
29 K. Vekemans and F. Braet, in *Current Issues on Multidisciplinary Microscopy Research and Education*, ed. A. Méndez-Vilas and L. Labajos-Broncanos, Formatex Press, Extremadura, Spain, 2004, pp. 81–86.
30 Y. Su, M. Nykanen, K. Jahn, R. Whan, L. Cantrill, L. L. Soon, K. R. Ratinac and F. Braet, *Biophys. Rev.*, 2010, **2**, 121–135.
31 F. Braet, R. De Zanger and E. Wisse, *J. Microsc.*, 1997, **186**, 84–87.
32 L. F. Lincz, *Immunol. Cell Biol.*, 1998, **76**, 1–19.
33 Z. Darzynkiewicz, G. Juan, X. Li, W. Gorczyca, T. Murakami and F. Traganos, *Cytometry*, 1997, **27**, 1–20.
34 R. T. Allen, W. J. Hunter and D. K. Agrawal, *J. Pharmacol. Toxicol. Methods*, 1997, **37**, 215–228.
35 G. Kroemer, P. Petit, N. Zamzami, J. L. Vayssière and B. Mignotte, *FASEB J.*, 1995, **9**, 1277–1287.
36 G. Majno and I. Joris, *Am. J. Pathol.*, 1995, **146**, 3–15.
37 A. Koceva-Chyła, M. Jedrzejczak, J. Skierski, K. Kania and Z. Jóźwiak, *Apoptosis*, 2005, **10**, 1497–1514.
38 G. Bell, *Science*, 1978, **200**, 618–627.
39 P. Artursson and J. Karlsson, *Biochem. Biophys. Res. Commun.*, 1991, **175**, 880–885.
40 M. C. Wani, H. L. Taylor, M. E. Wall, P. Coggon and A. T. McPhail, *J. Am. Chem. Soc.*, 1971, **93**, 2325–2327.
41 D. M. Vyas and J. F. Kadow, *Prog. Med. Chem.*, 1995, **32**, 289–337.
42 M. E. Wall and M. C. Wan, *Cancer Res.*, 1995, **55**, 753–760.
43 M. Malet-Martino and R. Martino, *Oncologist*, 2002, **7**, 288–323.
44 P. M. Hwang, F. Bunz, J. Yu, C. Rago, T. A. Chan, M. P. Murphy, G. F. Kelso, R. A. J. Smith, K. W. Kinzler and B. Vogelstein, *Nat. Med.*, 2001, **7**, 1111–1117.
45 D. B. Longley, D. P. Harkin and P. G. Johnston, *Nat. Rev. Cancer*, 2003, **3**, 330–338.
46 S. Carnesecchi, K. Langley, F. Exinger, F. Gosse and F. Raul, *J. Pharmacol. Exp. Ther.*, 2002, **301**, 625–630.
47 W. Bouquet, T. Boterberg, W. Ceelen, P. Pattyn, M. Peeters, M. Bracke, J. P. Remon and C. Vervaet, *Int. J. Pharm.*, 2009, **367**, 148–154.
48 T.-H. Wang, H.-S. Wang and Y. K. Soong, *Cancer*, 2000, **88**, 2619–2628.
49 K. W. K. Tong, S. Dehn, J. E. A. Webb, K. Nakamura, F. Braet and P. Thordarson, *Langmuir*, 2009, **25**, 8586–8592.