Supplementary information

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

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

aSchool 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; Fax: +61-2-9385-6141; Tel: +61-2-9385-4478.
bAustralian Centre for Microscopy & Microanalysis, Madsen Building F09, The University of Sydney, Sydney, NSW 2006, Australia.
cSchool of Medical Sciences (Discipline of Anatomy and Histology) — The Bosch Institute, The University of Sydney, Sydney, NSW 2006, Australia.

Contents:

Statistical Approach used for ANOVA S2
Table S1 Matrix representing the time points sampled S3
Fig. S1 Schematic showing the protocol used to evaluate the combined effects of Fmoc-FF with chemotherapeutics added. LC-MS method for gel leachate analysis S3 S4
Fig. S2 LC-MS analysis of Fmoc-FF in DMEM/Water matrix S5
Fig. S3 LC-MS analysis of Fmoc-F in DMEM/Water matrix S6
Fig. S4 LC-MS analysis of DMEM (blank) in DMEM/Water matrix S7
Fig. S5 LC-MS analysis of Gel leachate after 24 h S8
Fig. S6 LC-MS analysis of Gel leachate after 48 h S9
Fig. S7 LC-MS analysis of Gel leachate after 72 h S10
Fig. S8 LC-MS analysis of Gel leachate after 96 h S11
Fig. S9 Stages of cell death as visualised by Hoechst 33342/propidium iodide (HO/PI) S12
Fig. S10 Fluorescent micrographs of Hoescht/propidium iodide stained HGF-1, Caco-2 and HeLa cell lines exposed to Fmoc-FF degradation products. S13
Fig. S11 SEM of cell lines. S14
Fig. S12 HO/PI fluorescence micrographs of controls. S15
Fig. S13 HO/PI fluorescence micrographs of Caco-2 cells exposed to 5-FU. S16
Fig. S14 HO/PI fluorescence micrographs of Caco-2 cells exposed to paclitaxel (Taxol®). S17
Statistical Approach used for ANOVA

Two-way ANOVA (repeated measures with both factors) was performed using GraphPad Prism version 6.03 for Windows, GraphPad Software, San Diego California USA, (www.graphpad.com) for the exposure time vs. leaching time experiments. One-way ANOVA was performed using the Analysis ToolPak in Microsoft Excel version 14.0.7109.5000 for Windows for the chemotherapeutic and Fmoc-FF.

When an ANOVA (Analysis of Variance) test gave a significant result, to determine which group differs, the least significant difference test (LSD) was employed. The LSD calculates the smallest significant difference between two means as if a t-test had been run on those two means. Hence, direct comparisons between two means from two groups are possible. Any difference larger than the LSD calculated is considered a significant result.

\[
LSD = t \sqrt{\frac{2MSE/n}{n^*}}
\]

Where t is the critical tabled value of the t-distribution with the degrees of freedom (df) associated with mean square error (MSE), the denominator of the F statistic and n* is the number of scores used to calculate the means of interest. Further details on the LSD test can be found in the Encyclopedia of Research Design.¹

Reference:

Supplementary Information:

Table S1

Matrix representing the time points sampled and evaluated for Hoechst 33342/propidium iodide (HO/PI) fluorescence microscopy and SEM.

<table>
<thead>
<tr>
<th>Leaching time (h)</th>
<th>Exposure time to cells (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>X</td>
</tr>
<tr>
<td>48</td>
<td>X</td>
</tr>
<tr>
<td>72</td>
<td>X, X</td>
</tr>
<tr>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

Fig. S1. Schematic showing the protocol used to evaluate the combined effects of Fmoc-FF with chemotherapeutics added: (i) before gelation or (ii) after gelation. Stock solutions (12 µL) of 5-Fluorouracil (7.7 mM) or paclitaxel (772 µM) were added to gels (120 µL volume in insert). In the final stage of exposing cells to the gel and drug mixtures, cells were incubated in Adv. DMEM (1.132 mL) to give final concentrations of 82 µM and 8.2 µM for 5-fluorouracil and paclitaxel respectively.
High-performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS)

Methods for analysis gel lechate

All the samples were analysed using reverse phase liquid chromatography-mass spectrometry (LC-MS) used a Shimadzu Prominence UFLC HPLC system with a LC-20AD pump and associated SIL-20A autosampler. Detection was made using a SPD-M20A PDA detector at 254 nm, the stationary phase used was a XBridge™ C18 (5 μm, 150 × 4.60 mm) analytical column using a 50 μL injection volume on an autosampler. It should be noted that the samples collected were not filtered but just sonicated after 50-fold dilution in water. Additionally, a guard column was not used, however, there was no noticeable pressure building during the course of this analysis (4 time points × n = 4 + blanks and controls > 20 samples). The mobile phase consisted of eluents A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) run at gradient with 5% B from 0-2.5 min and then from 5-90% B from 2.5-20 min and then 90% B from 20-22.5 min. The flow rate was 1 mL/min and the detector wavelength range was set at 200-800 nm. Mass spectra were collected both in positive (+ESI) and negative (-ESI) mode. The analysis on the gel leachate was repeated four times (n = 4) and representative results from these LC-MS measurements shown in Fig. S5-S8.
**Fig. S2.** LC-MS trace of a standard 2.0 mM solution of Fmoc-FF, diluted 50 fold prior to injection on the LC-MS column. Top panel: UV-Vis chromatograph (254 nm) with the **Fmoc-FF** peak eluting at $R_t = 17.78$ min and a minor peak from Fmoc-phenylalanine (**Fmoc-F**) eluting at $R_t = 16.93$ min (see also Fig. S3). Middle panel: Total Ion-count (TIC) Chromatographs; black = +ESI, pink = -ESI, Bottom panel: Mass spectra (+ESI) of the peak eluting at $R_t = 17.78$ min.
Fig. S3. LC-MS trace of a standard 2.0 mM solution of Fmoc-F, diluted 50 fold prior to injection on the LC-MS column. Top panel: UV-Vis chromatograph (254 nm) with the Fmoc-F eluting at $R_t = 16.93$ min. Middle panel: Total Ion-count (TIC) Chromatographs; black = +ESI, pink = -ESI, Bottom panel: Mass spectra (+ESI) of the peak eluting at $R_t = 16.93$ min.
Fig. S4. LC-MS trace of a blank DMEM sample (10 μL) diluted 50 fold prior to injection on the LC-MS column. Top panel: UV-Vis chromatograph (254 nm). An unidentified peak at $R_t = 16.61$ min has been highlighted. Middle panel: Total Ion-count (TIC) Chromatographs; black = +ESI, pink = -ESI. Bottom panel: Mass spectra (+ESI) of the peak eluting at $R_t = 16.61$ min.
Fig. S5. LC-MS trace of a gel leachate sample (10 μL) taken after 24 h and diluted 50 fold prior to injection on the LC-MS column. Top panel: UV-Vis chromatograph (254 nm). An unidentified peak at $R_t = 16.47$ min has been highlighted. Middle panel: Total Ion-count (TIC) Chromatographs; black = +ESI, pink = -ESI. Bottom panel: Mass spectra (+ESI) of the peak eluting at $R_t = 16.47$ min. The ions identified do not match Fmoc-FF or any obvious derivatives of Fmoc-FF.
Fig. S6. LC-MS trace of a gel leachate sample (10 µL) taken after 48 h and diluted 50 fold prior to injection on the LC-MS column. Top panel: UV-Vis chromatograph (254 nm). An unidentified peak at $R_t = 16.47$ min has been highlighted. Middle panel: Total Ion-count (TIC) Chromatographs; black = +ESI, pink = -ESI. Bottom panel: Mass spectra (+ESI) of the peak eluting at $R_t = 16.47$ min. The ions identified do not match Fmoc-FF or any obvious derivatives of Fmoc-FF.
**Fig. S7.** LC-MS trace of a gel leachate sample (10 µL) taken after 72 h and diluted 50 fold prior to injection on the LC-MS column. Top panel: UV-Vis chromatograph (254 nm). Unidentified peaks at $R_t = 16.08$ and $R_t = 16.89$ min have been highlighted. Middle panel: Total Ion-count (TIC) Chromatographs; black = +ESI, pink = -ESI. Bottom panel: Mass spectra (+ESI) of the peak eluting at $R_t = 16.89$ min. The ions identified do not match Fmoc-FF or any obvious derivatives of Fmoc-FF.
Fig. S8. LC-MS trace of a gel leachate sample (10 μL) taken after 96 h and diluted 50 fold prior to injection on the LC-MS column. Top panel: UV-Vis chromatograph (254 nm). An unidentified peak at $R_t = 16.61$ min have been highlighted. Middle panel: Total Ion-count (TIC) Chromatographs; black = +ESI, pink = -ESI. Bottom panel: Mass spectra (+ESI) of the peak eluting at $R_t = 16.69$ min. The ions identified do not match Fmoc-FF or any obvious derivatives of Fmoc-FF.
Fig. S9. Stages of cell death as visualised by Hoechst 33342/propidium iodide (HO/PI) staining on a fluorescence microscope.
Fig. S10. Fluorescent micrographs of Hoescht/propidium iodide stained HGF-1, Caco-2 and HeLa cell lines exposed to Fmoc-FF degradation products. L denotes leaching time. E denotes exposure time. Scale bar, 100 μm. Live cells (blue fluorescence), apoptotic cells (intense bright-blue and blue-violet fluorescence) and necrotic cells (pink fluorescence).
**Fig. S11.** SEM of cell lines. (L = leaching time), (E = exposure time). Scale bar, 20 µm.
Fig. S12. HO/PI fluorescence micrographs of controls. Scale bar, 50 µm. Live cells (blue fluorescence), apoptotic cells (intense bright-blue and blue-violet fluorescence) and necrotic cells (pink fluorescence).
Fig. S13. HO/PI fluorescence micrographs of Caco-2 cells exposed to 5-FU. 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).
Fig. S14. HO/PI fluorescence micrographs of Caco-2 cells exposed to paclitaxel (Taxol®). Scale bar, 50 μm. Live cells (blue fluorescence), apoptotic cells (intense bright-blue and blue-violet fluorescence) and necrotic cells (pink fluorescence).