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The Effect of Unsaturation on the Formation of Self-Assembled Gels from Fatty Acid L-Serine Amides and their Cytotoxicity Towards Caco-2 Cancer Cells

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A series of saturated and unsaturated fatty acid L-serines **3** were synthesized and their ability to form self-assembled gels was investigated. The saturated (lauroyl **3a** and steraoyl **3b**) and monounsaturated (oleoyl **3c**) fatty acid L-serines form gels in both water and organic solvent, whereas the diunsaturated linoleyl-L-serine **3d** does not form gels in these solvents, indicating that unsaturation adversely affects the gelation process. Cytotoxicity studies on these compounds with Caco-2 cancer cells in vitro show that these gels are only moderately cytotoxic at concentrations up to 0.5 mM, making them a promising candidate for applications such as drug delivery.

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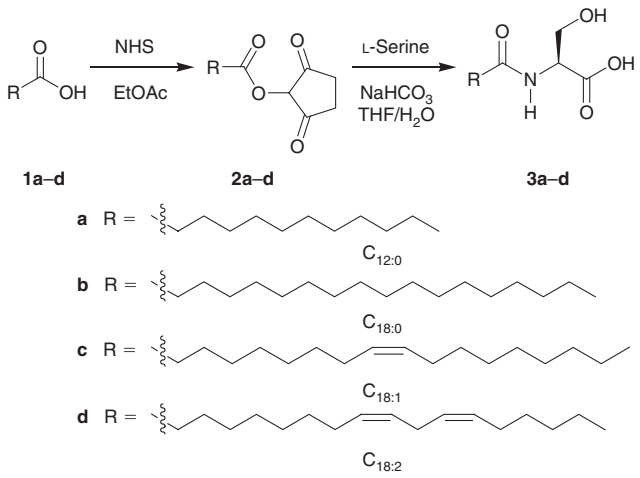
Introduction

Self-assembled or molecular gels are formed by small low-molecular weight organic gelators (LMOG) that self-assemble into a supramolecular polymer network that encapsulates the solvent in a solid-like state.[1] Self-assembled gels can be classified as smart materials owing to the reversible nature of the bonds that hold them together, as shown in our recent work on a novel series of anion-sensitive pyromellitamide-based self-assembled gels.[2,3] As the related and more commonly known (covalent) polymeric gels, self-assembled gels can be further subdivided into organogels or hydrogels depending on the solvent used. Both polymeric and self-assembled gels are showing great promise for various medical applications, and their reversible nature makes them a particularly attractive target for tissue regeneration[4,5] and targeted drug delivery.[6,7] Leroux and coworkers have shown that fatty acid amide methyl ester-based organogels can be used for the controlled delivery of rivastigmine, which is used to treat Alzheimer's disease.[8] The fatty acid–amino acid is a well-known motif in LMOG, including the dodecyl-L-serine hydrogelator **3a**[9] but little is known about the effect of unsaturation on the gelation properties of these compounds. To this end, we synthesized a series of C_{18}-fatty acid amides and compared their gelation properties with the known hydrogelator **3a**. Additionally, the toxicity of these compounds was tested, but biological compatibility issues are often overlooked in papers describing developments in this field.

The fatty acid amides described here are derived from lauroyl ($C_{12:0}$) **1a**, stearoyl ($C_{18:0}$) **1b**, oleoyl ($C_{18:1}$) **1c**, and linoleyl ($C_{18:2}$) **1d** fatty acids, the latter two mono- and di-unsaturated, respectively. Using the method described by Lapidot and coworkers,[10] these were converted to the corresponding *N*-hydroxysuccinimide (NHS) esters **2a**–**d**, followed by a reaction with L-serine under Schotten–Baumann conditions[11,12] to yield the fatty acid L-serines **3a**–**d** (Scheme 1).



Scheme 1. Synthesis of fatty acid L-serine amides.

The gelation of the synthesized fatty acid L-serine amides **3a**–**d** was studied in pure water (pH 5–6), chloroform, and hexane (Table 1).

The dodecanoyl-L-serine **3a** ($C_{12:0}$) has been reported to gel water (pH 4.9–6.4) and toluene,[9] a finding that was confirmed in the present work. The saturated **3b** ($C_{18:0}$) and the monounsaturated **3c** ($C_{18:1}$) fatty acid-L-serines synthesized here were also able to form opaque white gels in water. However, the diunsaturated linoleyl-L-serine **3d** ($C_{18:2}$) remained insoluble even after heating to the boiling point of the solvent. It was also noted

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Table 1. Gelation properties of fatty acid L-serine amides 3 in various solvents

OG, opaque gel; TG, transparent gel; PG, partial gel (opaque); IS, insoluble

Gelator	Water[A]	$CHCl_3$	Hexane
3a ($C_{12:0}$)	OG	TG	PG
3b ($C_{18:0}$)	OG	IS	PG
3c ($C_{18:1}$)	OG	TG	TG
3d ($C_{18:2}$)	IS	IS	IS

[A] Unbuffered deionized water, final pH = 5–6.

that the gels formed from oleoyl-L-serine **3c** in water started to collapse within 30 min. These results, and the inability of the diunsaturated **3d** to form gels, indicate that restrictions on the flexibility of the chains of these fatty acid L-serine amides compared with the saturated steraoyl derivative **3b** significantly impact on their ability to form gels owing to their diminished solubility in water.

A similar but slightly more complex trend is observed for the ability of these fatty acid L-serines **3** to form gels in chloroform and hexane. The diunsaturated linoleyl-L-serine **3d** is insoluble in these solvents, whereas the monounsaturated oleoyl-L-serine **3c** forms transparent gels. The two saturated gelators are a little different; the shorter dodecanoyl-L-serine **3a** ($C_{12:0}$) forms a gel in chloroform and partial gels hexane, whereas the stearoyl-L-serine **3b** ($C_{18:0}$) is not soluble enough in chloroform to form gels while it does form partial gels in hexane. These results suggest that, as for water, the solubility of these gelators is the key factor in determining their ability to form gels in organic solvents.

In order to access the suitability of these fatty acid L-serine amides **3** for applications in biological systems, including drug delivery, cytotoxicity studies were undertaken on Caco-2 cells. The viability of these human colorectal adenocarcinoma cells was assessed using a colourimetric assay based on the capacity of cells to bind to the trophenylmethane dye crystal violet (CV), which is used to directly quantify the cell numbers in vitro in response to various experimental conditions. The results from this assay are therefore proportional to the absorbance of the dye taken up by the cells.[13] As it was essential that the hydrogelators were in a solution in the assay, they were tested for dissolution in various solvents. Dodecanoyl-L-serine **3a**, oleoyl-L-serine **3c**, and linoleoyl-L-serine **3d** could be dissolved up to 1 mM in 4% ethanol with 96% aqueous phosphate buffer saline (PBS), whereas to dissolve stearoyl-L-serine **3b**, it was found necessary to use 50% ethanol in 50% PBS, making the results obtained for this gelator difficult to compare with the others. The results obtained from these assays show that cells in both dodecanoyl-L-serine **3a** and oleoyl-L-serine **3c** had similar viabilities of close to 95% after 24 h at 0.1 mM concentration, which gradually declined to 86–92% for 0.2–0.5 mM concentrations of **3a** and **3c**, with the results for the less reliable stearoyl-L-serine **3b** (in 50% ethanol) following a similar trend (Fig. 1).

Of the four compounds tested, the linoleyl-L-serine **3d** seems to be slightly more toxic than the others, with observed cell viabilities of 93% after 24 h at 0.1 mM concentration, declining to less than 80% at 0.5 mM. It is noteworthy, though, that when this compound was tested at 1 mM, cells did also show ~80% viability (not shown), indicating that although it does adversely affect Caco-2 cells, they remain viable even in the presence of a relatively high concentration of this compound. The results in Fig. 1 also indicate that stearoyl-L-serine **3b** was the least toxic of these gelators; however, given the unusual conditions used for this compound (50% ethanol), which may cause the Caco-2 cells to be fixed to the 96-well plate and therefore give false-positive results in the CV assays, these results should be interpreted with caution.[14]

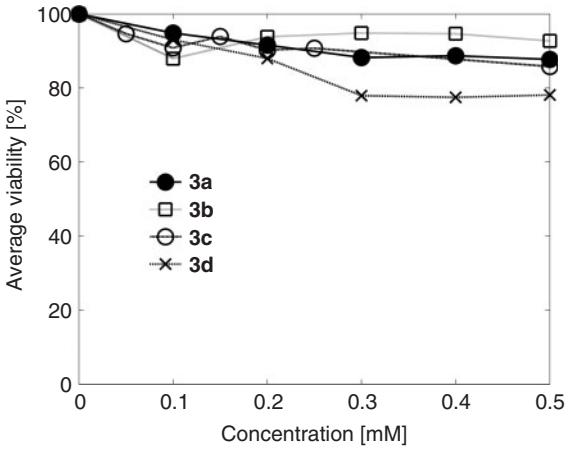

Fig. 1. The average percentage viability of Caco-2 cells in dodecanoyl-L-serine **3a**, stearoyl-L-serine **3b**, oleoyl-L-serine **3c**, and linoleyl-L-serine **3d**.

In conclusion, we have shown that introducing unsaturation in the family of fatty acid L-serine gelators **3** does seem to adversely affect their ability to aggregate into gel structures in both water and organic solvents. The solubility of these compounds seems to be the dominating factor in determining their ability to form stable gels. We have also shown that this family of gelators shows only mild toxic effects (<15–20% reduction in viability) towards Caco-2 even at a fairly high concentration (0.5 mM). These results indicate that these compounds could be useful as carriers in localized drug delivery.

Experimental Details

Materials

The syntheses of the *N*-succinimidyl dodecanoate **2a**,[9,10,15] dodecanoyl-L-serine **3a**,[10,16] *N*-succinimidyl octadecanoate **2b**,[10,17] and octadecanoyl-L-serine **3b**[10,17] have been reported previously. All synthetic reactions were carried out in an inert environment containing nitrogen, unless otherwise specified. All reagents used in all reactions were commercially available reagent-grade chemicals. Dichloromethane, hexane, methanol were purified by distillation before use. Dry solvents including tetrahydrofuran and ethyl acetate were distilled over appropriate drying agents or obtained from a Pure Solv dry solvent system (Innovative Technology Inc., model PS-MD-7). Deuterated solvents for NMR were purchased from Merck and Aldrich.

Equipment

Melting points were measured with a melting point apparatus and are uncorrected. Infrared spectra were recorded on a Nicolet Avatar 360 Fourier-transform (FT)-IR or Nicolet Avatar 370 FTIR using *EZ OMNIC ESP 5.1* software. ^{1}H Nuclear magnetic resonance spectra were recorded on a Bruker DPX 300 spectrometer at a frequency of 300.17 MHz. ^{13}C NMR spectra were recorded on a Bruker DPX 300 spectrometer at a frequency of 75.48 MHz at 300 K. Low-resolution electrospray ionization (ESI) was carried out on a Finnigan LCQ-DECA electrospray instrument at the University of Sydney or a Waters Micromass ZQ electrospray instrument at the University of

New South Wales. High-resolution ESI (HR-ESI) was carried out on a Thermo LTG FT instrument at the Bioanalytical Mass Spectrometry Facilities (BMSF), Analytical Centre, University of New South Wales.

Synthesis of N-*Succinimidyl* cis-*9-Octadecenoate (NHS Ester of Oleic Acid)* ***2c***

Oleic acid **1c** (2.82 g, 3.17 mL, 10.0 mmol) was added to a solution of NHS (1.17 g, 10.2 mmol) in dry ethyl acetate (40 mL). A solution of dicyclohexylcarbodiimide (2.07 g, 10.0 mmol) in dry ethyl acetate (10 mL) was added and the reaction mixture was stirred overnight at room temperature and under nitrogen. The side product dicyclohexylurea was removed by filtration and the filtrate was evaporated. It was left overnight under nitrogen to precipitate out and dried to give *N*-succinimidyl *cis*-9-octadecenoate (NHS ester of oleic acid) **2c** as pale yellow gel-like paste (3.43 g, 90%). ν_{max}(MeOH)/cm^{-1} 2924s, 2853s, 1781s, 1741s, 1464s. δ_H (300 MHz, [D$_6$]DMSO) 0.85 (t, *J* 6.8, 3H, –C*H*$_3$), 1.24–1.47 (m, 20H, –(C*H*$_2$)$_{10}$–), 1.61 (m, 2H, –C*H*$_2$CH$_2$C=O(ON)–), 1.97–1.98 (m, 4H, –C*H*$_2$C=C–C*H*$_2$), 2.14–2.19 (t, 2H, *J* 7.2, –C*H*$_2$C=O(NO)–), 2.61–2.67 (t, *J* 7.1, 4H, –C*H*$_2$C(N)=O), 5.27–5.37 (m, 2H, –*H*C=C*H*–). δ_C (75 MHz, [D$_6$]DMSO) 13.81, 21.96, 24.14, 24.38, 25.30, 26.44, 27.87, 28.46, 28.56, 28.70, 28.89, 28.97, 30.05, 31.14, 33.57, 129.48, 129.53, 168.83, 170.10 (2C), 174.35. *m*/*z* (ESI) 402.21; [M + Na]$^+$ requires $C_{22}H_{37}NO_4Na$ 402.52.

Synthesis of cis-*9-Octadecenoyl-*L*-serine (Oleoyl-*L*-serine)* ***3c***

Based on the method reported by Lapidot,[10] a solution of *N*-hydroxysuccinimide ester of oleic acid **2c** (0.50 g, 1.33 mmol) in distilled tetrahydrofuran (10 mL) was added to a solution of L-serine (0.14 g, 1.31 mmol) and sodium bicarbonate (0.13 g, 1.52 mmol) in water (10 mL). It was stirred under nitrogen for 43 h and acidified to pH 2 with aqueous hydrochloric acid (5 M, 1–2 mL). Tetrahydrofuran was then removed under vacuum. Water (50 mL) was then added to the remaining suspension which was then filtered and the precipitate dried to give a white solid. This crude product was dissolved in a minimum of ether (10 mL) and precipitated into cold hexane (150 mL). The solution was centrifuged and precipitate was dried to give *cis*-9-octadecenoyl-L-serine (oleoyl-L-serine) **3c** as white powder (0.09 g, 19%). Mp 50–60°C. ν_{max}(KBr)/cm^{-1} 3339s, 3000s, 2919s, 2852s, 1739s, 1614s. δ_H (300 MHz, [D$_6$]DMSO) 0.85 (t, *J* 6.8, 3H, –C*H*$_3$), 1.24 (m, 20H, –(C*H*$_2$)$_{10}$–), 1.44–1.49 (t, *J* 6.0, 2H, –C*H*$_2$CH$_2$C=O), 1.97–1.99 (m, 4H, –C*H*$_2$C=C–C*H*$_2$), 2.10–2.15 (t, *J* 7.5, 2H, –C*H*$_2$C=O(N)–), 3.53–3.67 (m, 2H, ^{2}C*H*$_2$), 4.18–4.24 (m, 1H, ^{1}C*H*), 5.27–5.37 (m, 2H, –*H*C=C*H*–), 7.80–7.83 (d, *J* 7.9, 1H, –N*H*–). δ_C (75 MHz, [D$_6$]DMSO) 13.93, 22.20, 26.55, 26.60, 28.56, 28.62, 28.65, 28.70, 28.80, 29.07, 29.11, 30.67 (2C), 31.25, 35.01, 54.51, 61.42, 129.63 (2), 172.13, 172.27. *m*/*z* (ESI) 368.43; [M]$^-$ requires 368.52. *m*/*z* (HR-MS ESI) 368.2803; [M – H]$^-$ $C_{21}H_{38}NO_4$ requires 368.2801.

Synthesis of N-*Succinimidyl* cis,cis-*9,12-Octadecadienoate (NHS Ester of Linoleic Acid)* ***2d***

Linoleic acid **1d** (2.80 g, 3.10 mL, 10 mmol) was added to a solution of NHS (1.16 g, 10.1 mmol) in dry ethyl acetate (40 mL). A solution of dicyclohexylcarbodiimide (2.09 g, 10.1 mmol) in dry ethyl acetate (10 mL) was added and the reaction mixture was stirred overnight at room temperature and under nitrogen. The side product dicyclohexylurea was removed by filtration and the filtrate was evaporated. It was left overnight under nitrogen to precipitate out and further cooled in an ice bath to give *N*-succinimidyl *cis*,*cis*-9,12-octadecadienoate (NHS ester of linoleic acid) **2d** as colourless liquid with sediments (3.00 g, 79%). ν_{max}(MeOH)/cm^{-1} 3363w, 2856–3008s, 1785–1815m, 1740s, 1465m. δ_H (300 MHz, [D$_6$]DMSO) 0.86 (t, *J* 7.14, 3H, –C*H*$_3$), 1.15–1.64 (m, 14H, –(C*H*$_2$)$_7$–), 1.59–1.64 (m, 2H, –C*H*$_2$CH$_2$C=O(ON)), 1.98–2.03 (m, 4H, –C*H*$_2$C=C–C*H*$_2$), 2.51–2.61 (t, *J* 7.1, 2H, –C*H*$_2$C=O(ON)), 2.66–2.75 (t, *J* 7.1, 4H, –C*H*$_2$C=O(ON)–), 5.26–5.39 (m, 4H, –*H*C=C*H*–). *m*/*z* (ESI) 376.12; [M]$^+$ requires 376.53.

Synthesis of cis,cis-*9,12-Octadecadienoyl-*L*-serine (Linoleyl-*L*-serine)* ***3d***

Based on the method reported by Lapidot,[10] a solution of NHS ester of linoleic acid **2d** (0.76 g, 2.02 mmol) in distilled tetrahydrofuran (10 mL) was added to a solution of L-serine (0.23 g, 2.17 mmol) and sodium bicarbonate (0.18 g, 12.17 mmol) in water (10 mL). It was stirred under nitrogen for 24 h and acidified to pH 2 with aqueous hydrochloric acid (5 M, 1–2 mL). Tetrahydrofuran was then removed under vacuum. Water (50 mL) was then added to the remaining suspension which was then filtered to give a sticky colourless crude product. This crude was dissolved in a minimum of diethyl ether (20 mL) and precipitated into cold hexane (150 mL). The solution was centrifuged and dried to give *cis*,*cis*-9,12-octadecadienoyl-L-serine (linoleyl-L-serine) **3d** as pale yellow gel-like substance (0.33 g, 44%). ν_{max}(MeOH)/cm^{-1} 3327w, 3011w, 2856–2928s, 2364w, 1727m, 1648m, 1544m. δ_H (300 MHz, [D$_6$]DMSO) 0.94 (t, *J* 7.1, 3H, –C*H*$_3$), 1.33 (m, 14H, –(C*H*$_2$)$_7$–), 1.55 (s, 2H, –C*H*$_2$CH$_2$C=O), 2.06–2.11 (m, 2H, –C*H*$_2$C=O(N)–), 2.18–2.23 (t, *J* 6.7, 4H, –C*H*$_2$C=C–C*H*$_2$), 3.63–3.76 (m, 2H, ^{2}C*H*$_2$), 4.29–4.34 (m, 1H, ^{1}C*H*), 5.34–5.47 (m, 4H, –*H*C=C*H*–), 7.94–7.97 (d, *J* 7.5, 1H, –N*H*–). δ_C (75 MHz, [D$_6$]DMSO) 13.88, 21.93 (2C), 25.19 (2C), 26.56, 26.61, 28.56, 28.60, 28.68, 29.01, 30.86, 35.01, 54.49, 61.43, 127.72 (2C), 129.72 (2C), 172.12, 172.24. *m*/*z* (ESI) 366.30; [M]$^-$ requires 366.53. *m*/*z* (HR-MS ESI) 366.2667; [M – H]$^-$ $C_{21}H_{36}NO_4$ requires 366.2644.

Gelation

In a typical experiment, 1% gel/water (w/v) was made by mixing gelator **3** (5 mg) and MilliQ water (0.5 mL) in a sealed vial. The vial was heated until a clear solution was obtained before the pH was adjusted to 5–6 using dilute hydrochloric acid or sodium hydroxide. The solution was allowed to cool to room temperature before the gelation was checked by the inversion test. If there was no solvent flow, gelation was achieved.

Cell Culture

Caco-2 cells were cultured to 80% confluency in 75-cm^2 cell culture flasks with an aliquot of Advanced Dulbecco's medium from Dulbecco's Modified Eagle Medium (40 mL; DMEM from GIBCO BRL 12491), supplemented with 5% (2 mL) heat-inactivated foetal calf serum (Gibco 10100–147), 0.5 mL of antibiotic-antimycotic (Gibco 15240) and 0.5 mL of Glutamax (Gibco 35050, 200 mM).

The used medium was then removed and the cells were rinsed in PBS (5 mL; Gibco 10010) before trypsin–EDTA (2 mL; SAFC Biosciences 59430C) was added to the cell monolayer at 37°C for 5–10 min for trypsinization, which detaches the cells from the culture flask. The enzymatic reaction was inactivated by the addition of fresh serum-containing DMEM media

(5 mL) and the cells were centrifuged in a Labofuge 300 Heraeus for 2 at 1800 rpm. The cells were then resuspended in fresh DMEM media (2 mL, 37°C) to produce a suspension of cells suitable for testing. All cell culture reagents were obtained from Invitrogen Australia, except trypsin, which was obtained from Sigma–Aldrich.

Crystal Violet Assay

Typically, 10 000 cells in DMEM (100 μL) were seeded per 96-multiwell plate and allowed to adhere to the bottom of the well for 24 h at 37°C. The cell cultures were then exposed to the toxic agent (100 μL) for a further of 24 h at 37°C. The supernatant was removed and crystal violet solution (100 μL; CVS) was added. The cells were then incubated at 37°C for 10 min CVS before it was removed and washed thoroughly with distilled water. The stained cells were then air-dried and 33.3% acetic acid solution (100 μL) was added to lyse the cells. The multiwall plate was gently shaken for 10 min before the absorbance was recorded on a Perkin–Elmer 1420 Victor^3 V Multilabel Counter at 531 nm. The data were processed with *Wallac 1420 Manager* software.

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