Supporting Information

Injectable small molecule hydrogel as a potential nanocarrier for localized and sustained in vivo delivery of Doxorubicin

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Scheme 1a

\[
\text{1} \xrightarrow{(i)} \text{ALA-HYD} \\
\text{2} \xrightarrow{(ii)} \text{ALA-CAM} \\
\text{2} \xrightarrow{(iii)} \text{3} \xrightarrow{(iv)} \text{ALA-HYD+}
\]

Reagents, Reactions Conditions, and Yields: (i) \( \text{N}_2\text{H}_4\cdot\text{H}_2\text{O}, \text{MeOH}, 18\text{h}, \text{RT}, 67\% \); (ii) CDI, Dry THF, \( \text{NH}_3 \) in MeOH, \( \text{N}_2 \), 1h, RT, 81\%; (iii) CDI, tert-butyl carbazate, Dry THF, \( \text{N}_2 \), 1h, RT, 86\%; (iv) HCl gas, Et\(_2\)O, 4h, RT, 89\%. 

**Figure S1.** Toxicities of gelators (Normalized) at different concentrations in 4T1 cells after 48h showing the non-toxic nature the gelator molecules up to 250 μg/mL concentration.
Figure S2. SEM images of xerogels formed by a) ALA-HYD, and b) ALA-HYD+ showing the nanofiber and globular microstructures, respectively.
Figure S3. Rheological studies on pristine hydrogels. a) Amplitude sweep and b) Frequency sweep studies performed at 25 °C. SM stands for storage modulus while LM refers to loss modulus; c) Time-dependent changes in the rheological behavior of gels at 37 °C subjected to three consecutive cycles of deformation (from 0.01-200 Pa) followed by recovery; d) Amplitude sweep study to prove the existence of gel-phase at 37 °C; e) Amplitude sweep experiments performed at pH = 7.4 and pH = 5.8 to show the stability of the gel-phase at pH 7.4 and 5.8.
Figure S4. a-b) SEM images of DOX-Gel having doxorubicin (a) (40 µg/mL) and (b) (80 µg/mL); c) Rheology studies of ALA-HYD gelator doped with doxorubicin (40 µg/mL) and (80 µg/mL). SM stands for storage modulus while LM refers to loss modulus; d) Fluorescence titration of doxorubicin (20 µg/mL) with gelator molecules showing quenching of doxorubicin fluorescence; e) MALDI-TOF spectra showing formation of imine adduct between the gelator and doxorubicin. Peaks at 819 and 835 show the Na⁺ and K⁺ adducts of imine respectively.
Figure S5. Orthogonal sections of confocal images showing the co-localization of doxorubicin with DAPI in nucleus.
Figure S6. Graph showing treatment of Free Gel, DOX (IV), and DOX-Gel causing negligible change in body weight of mice.
Materials: 1-Naphthylacetic acid (1-NAA) and Hydrazine hydrate were purchased from Spectrochem, 1,1-Carbonyl diimidazole (CDI) was purchased from Alfa Aesar, Sodium hydroxide, Hydrochloric acid, Triethylamine were obtained from Merck. 1-Butylcarbazate (tBC), Doxorubicin, Transwells, matrigel were purchased from Sigma Aldrich. Sodium sulphate was obtained from Rankem. All solvents used in the synthesis were dried, or distilled, as required. NMR (1H, 13C) spectra were recorded by using Bruker Ultra shield (400 MHz) spectrometer. Mass spectra were recorded in ESI-MS mode on MicroTOF-Q-II instrument manufactured by Bruker Daltonics; rheological experiments were performed using Rheoplus MCR102 (Anton paar) rheometer.

Syntheses and characterizations

We have reported the synthesis and characterization of compounds 1 and 2 shown in Scheme 1 previously (RSC Advances 2013, 3, 18900-18907).

(S)-N-(1-hydrazinyl-1-oxopropan-2-yl)-2-(naphthalene-1-yl)acetamide (ALA-HYD)

To dry methanolic solution of (S)-Methyl 2-(2-(naphthalene-1-yl)acetamido)propanoate (1) (2 g, 7.35 mmol), hydrazine hydrate (536 µL, 11.02 mmol) was added drop wise at 0 °C. The reaction mixture was further stirred for 18 h at RT. The complete consumption of starting material was not observed even after prolonged reaction time. Methanol was removed under vacuum and the product was further purified by column chromatography (stationary phase was silica 100-200 mesh and eluent phase was CHCl₃/MeOH) which resulted a white solid in 67% isolated yield. m.p=205 °C. ¹H NMR (400 MHz, DMSO-d6) δ=9.07(1H, N-H), 7.39-8.33 (7H, Nap-H), 7.38(1H, NH), 4.19-4.26 (1H, C-H), 4.17 (2H, NH₂), 3.87-3.96 (2H, Nap-CH₂), 1.16-1.18 (3H, CH₃). ¹³C NMR (100 MHz, DMSO-d6) δ=171.99, 170.10, 133.77, 133.32, 132.48, 128.76, 128.16, 127.44, 126.35, 126.05, 125.94, 124.85, 47.33, 39.87, 19.14. C₁₅H₁₇N₃O₂+H cacl.: 272.1394, found: 272.1401.

(S)-2-(2-(naphthalene-1-yl)acetamido)propanamide (ALA-CAM)
Compound 2 (1 g, 3.87 mmol) and CDI (0.69 g, 4.26 mmol) were dissolved in dry THF (5 mL) and stirred together for 5 min at RT. Methanolic solution of ammonia (7 N) (0.61 mL, 4.26 mmol) was added drop wise to the activated acid at 0 °C. This reaction mixture was stirred for 1 h at RT. At the end of this period, THF was removed from the reaction mixture to yield crude compound, which was purified by column chromatography on silica (100-200 mesh) using CHCl₃/MeOH. Compound was obtained as white solid (0.807 g, 81%). FTIR (KBr pellet, cm⁻¹): 3393, 3287, 3200, 1673, 1644, 1537. ¹H NMR (400 MHz, DMSO-d₆, ppm): δ=8.26-8.28 (1H, NH₂), 7.44-8.11 (7H, Nap-H), 6.98 (1H, NH₂), 7.34 (1H, NH₂), 4.20-4.27 (1H, C-H), 3.92-4.00 (2H, Nap-CH₂), 1.22-1.23 (3H, CH₃) ¹³C NMR (100 MHz, DMSO-d₆, ppm) δ= 174.71, 170.15, 133.78, 133.34, 132.48, 128.77, 128.19, 127.45, 126.35, 126.00, 124.84, 48.46, 39.96, 18.98. C₁₅H₁₆N₂O₂ calcd.: 257.1285, found: 257.1292.

(tert-butyl 2-(2-(2-(naphthalene-1-yl)acetamido)propanoyl)hydrazinecarboxylate (3)

Compound 2 (1g, 3.87 mmol) and CDI (0.69 g, 4.26 mmol) were dissolved in dry THF (5 mL) and stirred for 5 min. Tertiary butyl carbazate (0.56 g, 4.26 mmol) was dissolved in dry THF and added to activated acid at 0 °C. This mixture was stirred for 1 h at RT. THF was removed from the reaction mixture by rotary evaporator and 70 mL of CHCl₃ was added to it. The CHCl₃ layer was washed with 1 N HCl and brine, dried with sodium sulphate. CHCl₃ was removed by rotary evaporator to obtain crude compound, which was purified by column chromatography on silica (100-200 mesh) using CHCl₃/MeOH to obtain compound as white colored foam like solid (1.25 g, 86%). FTIR (KBr pellet, cm⁻¹): 3250, 1688, 1652, 1539, 1512. ¹H NMR (400 MHz, CDCl₃, ppm): δ=8.47(1H, N-H), 7.29-7.85(7H, Nap-H), 6.50(1H, N-H), 6.30-6.29(1H, N-H), 4.45-4.48(1H, C-H), 3.85-3.94(2H, Nap-CH₂), 1.35(9H, C(CH₃)₃). 1.13-1.14(3H, CH₃) ¹³C NMR (100 MHz, CDCl₃) δ= 171.91, 171.51, 155.14, 133.92, 132.00, 130.61, 128.84, 128.37, 126.69, 126.05, 125.63, 123.63, 81.64, 47.27, 41.09, 28.15, 17.48. C₂₀H₂₃N₃O₄⁺NH₄ calcd: 389.2183, found: 389.2177.
(S)-N-(1-hydrazinyl-1-oxopropan-2-yl)-2-(naphthalene-1-yl)acetamide salt (ALA-HYD+)

Compound 3 (1 g, 2.8 mmol) was dissolved in diethyl ether and dry HCl gas was passed into the solution at RT and purging was continued till the carboxylate was completely consumed (monitored by TLC). The product was separated out as a precipitate, which was filtered and washed with diethyl ether (2 x 10 mL) to give pale yellow colored solid. The compound was dissolved in methanol and re-precipitated using diethyl ether to obtain a white colored solid.

FTIR (KBr pellet, cm\(^{-1}\)): 3468, 3416, 3274, 1693, 1644, 1535. \(^1\)H NMR (400 MHz, D\(_2\)O, ppm): \(\delta=7.40-7.93(7H, \text{Nap-H}), 4.28-4.34(1H, \text{C-H}), 4.03-4.12(\text{Nap-CH}_2), 1.34-1.35(3H, \text{CH}_3)\). \(^1\)C NMR (100 MHz, D\(_2\)O) \(\delta=174.63, 173.17, 133.52, 131.69, 130.73, 128.79, 128.53, 128.14, 126.72, 126.21, 125.85, 123.44, 48.33, 39.23, 16.14\). \([\text{C}_{15}\text{H}_{18}\text{N}_3\text{O}_2]^+\)calcld: 272.1394, found: 272.1404

**Protocol for gel formation:** Desired amount of gelator (ALA-CAM or ALA-HYD) was suspended in 1 mL distilled water and heated until a clear solution was obtained. This solution was bath-sonicated for 1 min and kept for gelation at room temperature. A turbid gel was observed upon undisturbed cooling of the mixture. Similarly, desired amount of ALA-HYD\(^+\) was dissolved in 0.5 mL of distilled water and 0.5 mL of 1 M Tris buffer (pH=7.5) (1:1 v/v) was added to it. This suspension was heated and resulting clear solution was bath-sonicated for 60 sec, and kept for gelation at room temperature. Formation of a turbid gel was observed with in 30 min. If the resultant gel was stable to inversion, 200 µL of water was added to it, and the above process repeated till excess solution started flowing. Above studies was performed to determine minimum gelation concentration.
Table S1: Minimum gelator concentration (MGC) and melting temperature ($T_m$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MGC (wt. %)</th>
<th>$T_m^a$ (°C)</th>
</tr>
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<tbody>
<tr>
<td>Ala-CAM</td>
<td>0.5</td>
<td>85</td>
</tr>
<tr>
<td>Ala-HYD$^+$</td>
<td>1.0</td>
<td>57</td>
</tr>
<tr>
<td>Ala-HYD</td>
<td>0.8</td>
<td>60</td>
</tr>
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$^a$ Measured at 2 x MGC.

**Doxorubicin encapsulation and Injectability studies:** Doxorubicin encapsulation was studied by adding desired amount of doxorubicin in warm soluble solution of hydrogel, and allowed to cool at room temperature to test its gelation ability. Injectability of the gelators was studied by casting the gels in syringe, and passing it through 20 gauage needle.

**Gel-melting temperature ($T_m$):** Gels were made in 2 mL polypropylene centrifuge tubes and tube was closed and attached to the thermometer near the bulb. This system was immersed in stirred water bath and temperature was raised from room temperature at 5 °C·min$^{-1}$ using a hot plate. The temperature at which gel mass fell from the top was recorded as melting temperature. Melting temperature of the gels were found at twice the MGC.

**Microscopy:** For SEM, doxorubicin entrapped gels (40 or 80 µg DOX in 15 mg/mL gelator) were dried inside vacuum desicator for 48 h. These samples were scooped and spread on carbon tape and gold coated for 120 s. The images were taken on Carl Zeiss (Ultraplus) FE-SEM used at 5 kV accelerating voltage.

For AFM, 10 µL of gel (1 mg/mL of ALA-CAM, ALA-HYD and ALA-HYD+) samples were dropped on freshly-cleaved mica surface. Samples were air dried for 1 h before AFM imaging. The images were obtained by scanning the mica surface in non-contact mode using NSC 19/AIBN cantilever (Micromash), length =125 ± 5 nm, width = 35 ± 3 nm, thickness = 1.0 ± 0.5 nm, tip radius <10 nm, resonant frequency = 80 kHz, force constant=0.6 N·m$^{-1}$. 
AFM scans were recorded at 256 x 256 pixels resolution and topographic, amplitude and phase images were taken. Scan rate in AFM studies was 1.3 lines/s for all samples.

**Confocal microscopy:** 4T1 cells were grown on cover slips in 24 well plates at a density of 6x10^3 cells per well for 24 hours. Cells were treated with DOX-Gel as described above in transwells. After 24 h of treatment, media was aspirated from each well, and cells were washed with 1x PBS twice. Cells were later fixed with 4% paraformaldehyde for 10 min at RT, then again washed with 1x PBS. The fixed coverslips were stained with DAPI and mounted with 70% glycerol on microscopic slides. The cells were then examined by Confocal laser scanning microscopy (Leica Confocal Microscope) at excitation wavelength 458 nm for doxorubicin and 405 nm for DAPI nuclear counter staining. Images were then processed using IMARIS software.

**Fluorescence Titration Studies:** Fluorescence titration studies were performed using Hitachi fluorescence spectrometer. Doxorubicin (20 µg/mL) was titrated with ALA-HYD gelator molecules from a heated stock solution of 1.5% (15 mg/mL), and fluorescence spectra were recorded at 25 °C with excitation wavelength of 480 nm and slit width of 5 nm.

**MALDI:** Aqueous solution of DOX (1 mg, 0.0017 mmol) was added to a methanolic solution of ALA-HYD (10 mg, 0.0036 mmol) and stirred for 30 min at RT. Resulting mixture was analyzed on Matrix Assisted Laser Desorption /ionisation (MALDI) by using 2,5-dihydroxy benzoic acid (DHB) matrix.

**Rheology:** Rheological experiments were performed on hydrogelators at 1 mg above the MGC of respective gelators using cone and plate geometry at 25 °C. For the Strain Sweep experiments, 25 mm diameter and 1 degree angle cone plate was used at top and parallel plate was used at bottom. Samples were placed on the bottom plate. 50 µm gap distance were maintained between top and bottom plate. Strain-sweep test was performed from 0.01 to 100% at constant frequency = 1 rad·s⁻¹. Frequency sweep experiment was carried out from
0.1 to 100 at constant strain 0.1%. At 37 °C, strain sweep experiment of ALA-HYD was performed from 0.01 to 100% at constant frequency = 1 rad·s⁻¹ using 15 mg/mL gelator. pH dependent strain sweep experiment of ALA-HYD (8 mg/mL) was performed at 25 °C.

Thixotropic behavior of ALA-HYD hydrogel (8 mg/mL) was examined by rheometer under the application of shear stress and release. Frequency (1 Hz) and temperature (37 °C) were kept constant when deformation and recovery of gel was observed under the shear stress. ALA-HYD underwent deformation from 0.01 to 200 Pa shear stress for 8 min. Recovery of gel from sol was observed at constant (0.01 Pa) shear stress for 12 min. We applied and released shear stress for four successive times to compare the gel recovery.

Strain sweep experiment of doxorubicin-loaded ALA-HYD was performed from 0.01 to 100% strain at constant frequency = 1 rad·s⁻¹. Experiment was performed at 25 °C using 15 mg/mL gelator.

**Pharmacokinetic studies:** Pharmacokinetic studies were performed on female Balb/c mice bearing tumor by having four animals per group treated with DOX-Gel, DOX-Local, and DOX-IV. The blood samples were collected from the retro-orbital plexus under mild ketamine anesthesia at 2, 6, 24, and 72 h in micro-centrifuge tubes (having 40 µL of 3.8% sodium citrate solution). Blood samples were centrifuged at 2,500 rpm for separation of plasma. To 150 µL blood plasma, 200 µL (pH 9.2) alkaline PBS buffer was added, and vortex-mixed for 2 min. The drug was extracted using 0.5 µL Chloroform:Isopropanol (2:1 v/v) solution. Fluorescence of extracted drug was recorded at excitation of 480 nm and emission at 560 nm.