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Supporting Information

Supramolecular hydrogel as carrier to deliver microRNA into the encapsulated cells

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Materials and Methods

Materials and methods

1. General materials and methods

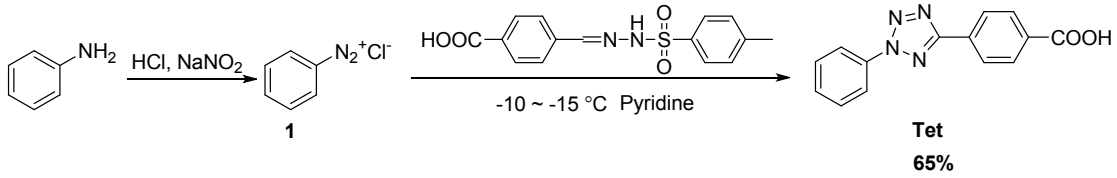
All chemicals and solvents were purchased from J&K chemicals or Sigma-Aldrich. MiR-122 mimics, Cy3-labeled miR-122, live/dead staining kit and Lipo-fectamine 2000 were purchased from Invitrogen. Luciferase assay kits were purchased from Promega.

^{1}HNMR and ^{13}CNMR spectra were obtained on a 400 MHz Bruker AVANCE III–400 spectrometer. Chemical shifts are reported in δ (ppm) relative to the solvent residual peak. Coupling constants are reported in Hz with multiplicities denoted as s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). MS were done on a SHIMADZU LC-MS 2020. Transmission electron microscopy (TEM) images were taken on JEM-2100 LaB6 Transmission Electron Microscope (JEOL). Fluorescent images were taken under confocal microscopy (Leica). MiRNA expression was quantified using Taqman miRNA detection assay (Applied Biosystems).

2. Synthesis and characterization of Tet-GA

The tetrazole (Tet) was synthesized according to the literature procedure.[1]

Scheme S1. Synthesis of Tet.



4-(2-phenyl-2*H*-tetrazol-5-yl)benzoic acid (Tet):

Benzenediazonium chloride **1** was prepared by adding a cooled solution of sodium nitrite (5 mmol) in 2 mL of water to a solution of aniline (5 mmol) and 1.3 mL of concentrated hydrochloric acid in 8 mL of 50% ethanol below 5 °C. The resulted solution of **1** was directly added dropwise over a period of 30 minutes into a solution of 4-((2-tosylhydrazono)methyl)benzoic acid (5 mmol) in 30 mL pyridine at -10 ~ -15 °C. The reaction mixture was extracted with chloroform and water. The chloroform layer was washed with dilute hydrochloric acid and dried with Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (petroleum ether/ethyl acetate = 1:1) to give a white powder (0.86 g, 65%), m.p. 221 – 222 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.30 (s, 1H), 8.33 – 8.26 (m, 2H), 8.21 – 8.14 (m, 4H), 7.77 – 7.69 (m, 2H), 7.69 – 7.61 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.6, 163.7, 136.0, 132.7, 130.3, 130.2, 130.1, 130.1, 126.7, 119.9. MS (ESI) calcd. for $C_{14}H_9N_4O_2$ 265.07 $[M–H]^-$; found 265.00.

Solid-phase peptide synthesis (SPPS): Tet-GA was synthesized using standard solid phase peptide synthesis (SPPS) protocol using 2-chlorotrityl chloride resin (100~200 mesh and ~1.0 mmol/g) and *N*-Fmoc-protected amino acids. The resin was swelled in dry dichloromethane (DCM) for 5 minutes, then alanine (Ala) was loaded onto resin at its C-terminal with Fmoc-Ala-OH (1.1 equiv.) and *N,N*-diisopropylethylamine (DIPEA) in DCM for 1 hour. After washing with *N*,*N*-Dimethylmethanamide (DMF) (3 × 3 mL), the resin was agitated with the blocking solution (16:3:1 of DCM/MeOH/DIPEA) for 20 minutes to deactivate the unreacted sites. Then the resins were treated with 20% piperidine (in DMF) for 20 minutes to remove the protecting group,

followed by coupling Fmoc-Gly-OH (3 equiv.) to the free amino group on the resin using HOBt/TBTU as the coupling reagent. At the final step, the N-terminus of the glycine was coupled with Tet (3 equiv.) by the same protocol. The resin was washed with DMF for 3~5 times after each step. After the last coupling step, the tetrazole linked peptide was cleaved with TFA (5 mL) for 2 hours and the resulted crude products were purified by chromatography.

Tet-GA ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.61 (s, 1H), 8.91 (t, J = 5.8 Hz, 1H), 8.36 – 8.24 (m, 3H), 8.18 (d, J = 7.8 Hz, 2H), 8.13 (d, J = 8.3 Hz, 2H), 7.72 (t, J = 7.3 Hz, 2H), 7.65 (t, J = 7.3 Hz, 1H), 4.34 – 4.24 (m, 1H), 4.02 (dd, J = 16.5, 5.9 Hz, 1H), 3.96 (dd, J = 16.5, 5.9 Hz, 1H), 1.32 (d, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.0, 168.5, 165.7, 163.9, 136.1, 136.0, 130.3, 130.1, 128.9, 128.4, 126.5, 120.0, 47.5, 42.3, 17.3. MS (ESI) calcd. for $C_{19}H_{18}N_6NaO_4$ 417.13 $[M+Na]^+$; found 417.10.

3. Hydrogelation test

The hydrogel was prepared by dissolving Tet-GA in phosphate buffered saline (PBS) or Dulbecco's Modified Eagle Medium (DMEM) by gentle heating. The gel formed gradually after cooling to room temperature, which was confirmed by inverting the vial. The minimum gelation concentration was determined by heating the gel of a determined concentration of Tet-GA and then diluting it with the appropriate solution until it could not form stable hydrogel after cooling down to room temperature.

4. TEM

Tet-GA gel was prepared by dissolving Tet-GA in PBS. TEM samples were prepared by directly dipping the samples onto copper omentum, which was further dried in the air for 30 minutes.

5. CD

A quartz cuvette with sample thickness of 1 mm was used and CD spectra were acquired on a Jasco J-810 spectropolarimeter.

6. Rheology

Rheology tests were performed on an HAAKE Rheostress 6000 rheometer (Thermo Scientific) and tests were done with a cone and a plate (19.992 mm diameter plate and 1^{o} cone angle). The gap between the cone and the plate was 0.051 mm. The dynamic frequency sweep of the Tet-GA gel was measured at 1% strain and dynamic strain sweep was measured at 1% frequency.

7. Stability test

The resistance of miRNA to degradation by serum was performed by preparing samples of 0.8 μM miR-122 in PBS with or without 3 mg/mL Tet-GA gel. The gel was left to form for 30 min after which fetal bovine serum (FBS) (Invitrogen) was added on top of the samples at the specified concentrations. Samples were than taken at different time points and the amount of miRNA was determined by running an agarose DNA electrophoresis stained with ethidium bromide. Pictures of the bands were taken under a UV trans-illuminator. For every sample, the intensity of the band was compared with imageJ (NIH) to the same 4 standard of miR-122 in PBS of concentration 0, 0.25, 0.5, 1 μM. The remaining fraction of miRNA was calculated with the value obtained.

Histograms show the normalized value with the error bar representing the variance.

8. Cell culture

HepG2 cells were cultured in high glucose DMEM (Gibco) containing 10% fetal bovine serum and 1% penicillin/streptomycin, and maintained in 5% CO_2 at 37 ^oC.

For 3D culture of the cells, HepG2 cells were added to a viscous Tet-GA solution (3 mg/mL in DMEM), cell density is $6 * 10^5$/mL. The Tet-GA gel formed within 5 minutes and was allowed to stand at room temperature for another 30 minutes before adding DMEM containing 2% fetal bovine serum onto the top of gel.

9. MTT

HepG2 cells were seeded into a 96 well plate with a concentration of 5000 cells per well in 100 μL culture medium. After 24 hours, medium was changed to 100 μL culture medium containing different concentrations of Tet-GA. MTT assay was then performed at different time points to assess the cytotoxic effects of Tet-GA. Relative cell viability was calculated by following equation: Relative cell viability = $(A_{sample}-A_{blank})/(A_{control}-A_{blank})$, where A is the absorbance at 490 nm.

10. Live/Dead assay

Viability of the cells inside the Tet-GA gel was determined by using a live/dead staining kit (Invitrogen) according to the manufacturer's instruction. Briefly, after 24 hours of incubation, the gel and cell complexes were washed with PBS twice. Then, staining reagent containing 2 μM calcein AM and 4 μM EthD-1 was added. After incubation in the incubator for 30 minutes, HepG2 cells were then observed directly under confocal microscopy.

11. Confocal imaging

4 μL Cy3-miR-122 (20 μM) was mixed with $3* 10^5$ HepG2 cells and encapsulated in 3 mg/mL Tet-GA gel. As a control, same amount of Cy3-miR-122 was directly added onto the cells cultured in dishes. Fluorescent images of HepG2 cells were taken directly after incubation for 4 hours.

12. RNA isolation and qRT-PCR

2 and 4 μL miR-122 (20 μM) were mixed with $3 * 10^5$ HepG2 cells respectively and encapsulated in 3 mg/mL Tet-GA gel. As a control, the same amount of miR-122 was directly added into the cells cultured in dishes, and $3 * 10^5$ HepG2 cells were encapsulated in Tet-GA gel directly. After incubation for 24 hours, cells were isolated by diluting with PBS and centrifuged, isolated cells were then further washed with PBS for three times.

Total RNA was isolated using TRIZOL reagent (Invitrogen) according to manufacturer's protocol. Mature hsa-miR-122 was quantified using Taqman miRNA detection assay. The reactions were carried out using 2 μg of RNA extracted from cells and U6 was used as internal control.

13. Luciferase assay

HepG2 cells at 80% confluency in 10 cm dish were co-transfected with Luc-miR-122 plasmid (15 μg) and β-galactosidase expressing plasmid (9 μg) by using Lipo-fectamine 2000 according

to manufacturer's protocol. β-galactosidase was used as an internal control. After 6 hours, HepG2 cells were collected. Samples were then prepared same as decribed above.

HepG2 cells encapsulated in the gel were further incubated for 48 hours at 37 ^{o}C and cells were then collected by washing with PBS and centrifuge. Cells were then further washed with PBS for three times and assayed using luciferase assay kits.

Reference:

1. Ito, S.; Tanaka, Y.; Kakehi, A.; Kondo, K. *Bull. Chem. Soc. Jpn.* **1976,** *49,* 1920-1923.

Supporting Table:

Table 1. Hydrogelation properties of several Tet substituted dipeptides

Tet-peptide	pH	c(mg/mL)[a]
Tet-GG	6.0	5.5
Tet-GF	6.5	4.2
Tet-GA	7.4	1.5
Tet-AG	-	-
Tet-AA	-	-
Tet-AF	-	-

[a]Lowest concentration for hydrogelation

Supporting Figures

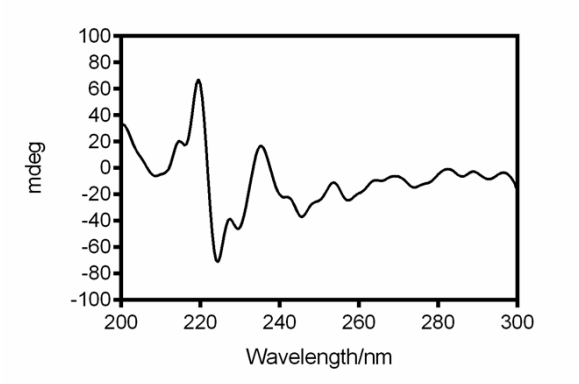


Figure S1. CD spectrum of Tet-GA gel.

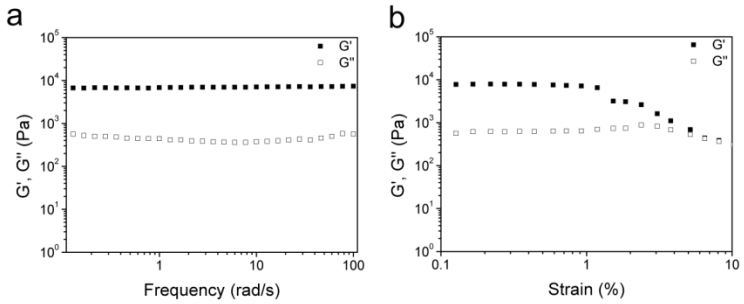


Figure S2. (a) Dynamic frequency sweep of the Tet-GA gel (3 mg/mL) at 1% strain. **(b)** Dynamic strain sweep of the Tet-GA gel (3 mg/mL) at 1% frequency.

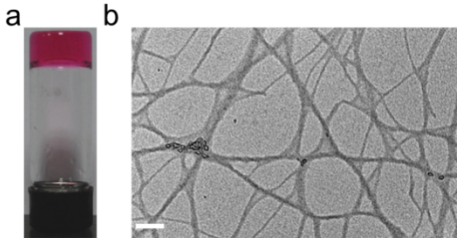


Figure S3. (**a**) Pictures and (**b**) TEM image of Tet-GA gel (3 mg/mL) in DMEM containing 80 pmole/mL miR-122. Scale bar: 100 nm.

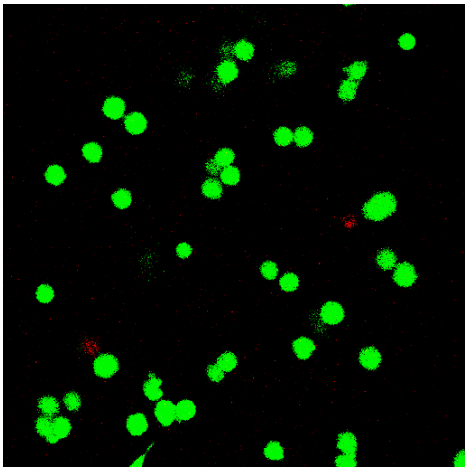


Figure S4. Confocal images of HepG2 cells encapsulated in 3 mg/mL Tet-GA gel for 24 hours. Calcein AM and EthD-1 were used to stain living (green) and dead (red) cells respectively. Over 95% of the HepG2 cells were alive after encapsulation in Tet-GA gel for 24 hours.

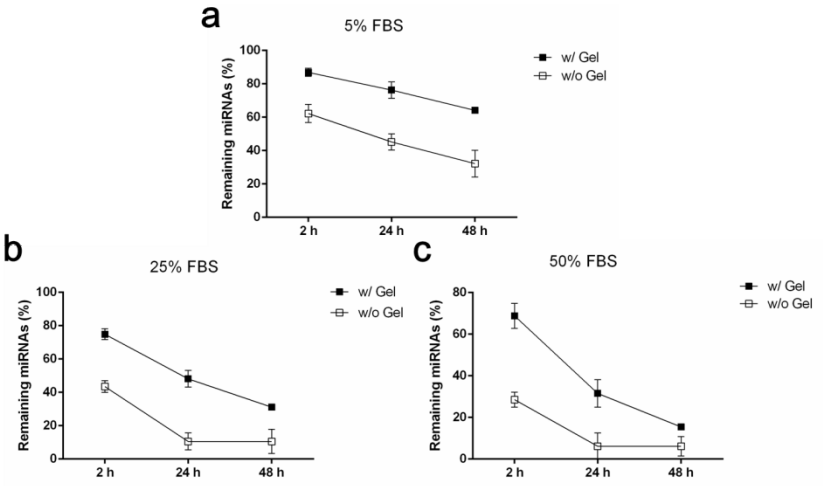


Figure S5. Relative stability of miR-122 embedded in Tet-GA gel (3 mg/mL) and in PBS buffer after treatment with various concentrations of FBS. Data are shown as mean ± s.d. (n=3).

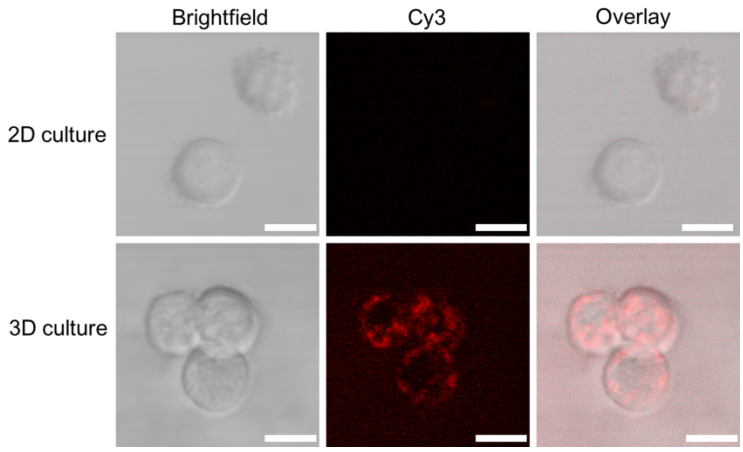


Figure S6. Brightfield, fluorescent and overlaid images of HepG2 cells **(a)** 2D cultured in dishes after incubation with 80 pmole Cy3-miR-122 for 24 hours; (**b**) 3D cultured in Tet-GA gel containing 80 pmole Cy3-miR-122 for 24 hours. Scale bar: 10 μm.

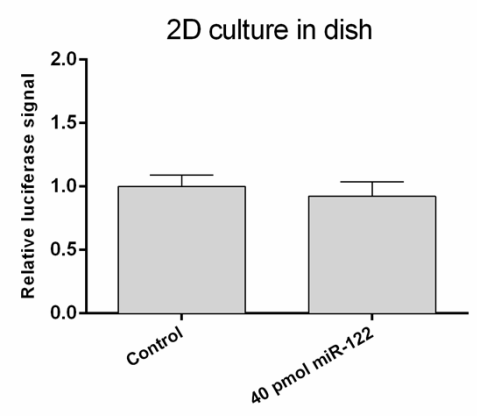


Figure S7. Relative luciferase signals from reporter HepG2 cells after cells were incubated with 40 pmol miR-122 in dishes for 48 hours. Data are shown as mean ± s.d. (n=3).

NMR Spectra