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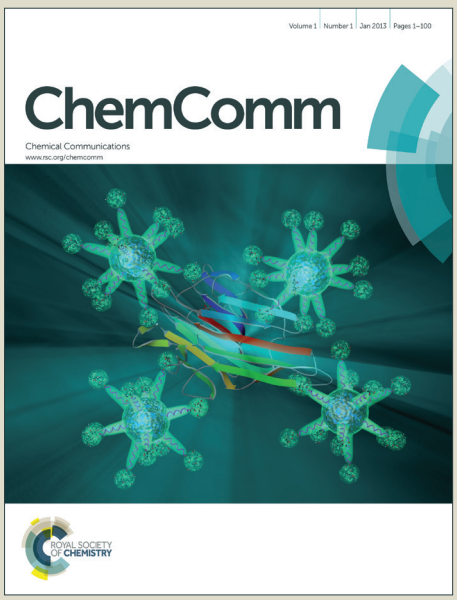





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COMMUNICATION

# Supramolecular hydrogel as carrier to deliver microRNA into the encapsulated cells

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**Supramolecular hydrogel formed by dipeptide Gly-Ala linked with biphenyl-substituted tetrazole serves not only as 3D matrix for live cells, but also as carrier to deliver microRNA into the encapsulated cells.**

Supramolecular hydrogels formed by the self-assembling of biocompatible small-molecule hydrogelators have emerged as "smart" biomaterials.[1] Recent examples using hydrogels formed by self-assembled peptides have demonstrated diverse biomedical applications of these supramolecular hydrogels in drug delivery,[2] controlled release,[3] tissue engineering[4] and regenerative medicine.[5] With concise structural modification of synthetic peptides, supramolecular hydrogels that respond to ligand-receptor interaction,[6] enzymes[7] and other bio-mimetic stimuli[8] have been constructed. Exploration of the bio-medical applications of these new types of supramolecular hydrogels is of current research interest to us and others.[9]

MicroRNAs (miRNAs) are endogenous small non-coding RNAs of ~22 nucleotides that regulate target gene expression at post-transcriptional level.[10] Chemical-biological studies with miRNAs as biological targets are emerging, with a focus on the detection, delivery and regulation of disease-related miRNAs.[11] It is important to develop various delivery systems for the efficient delivery of miRNA mimics into cells to study the biological functions of specific miRNAs as well as to realize therapeutic purpose.[12] Due to poor cell permeability of oligonucleotides and their strong tendency to be degraded in biofluids, most miRNA delivery strategies have involved the association of miRNA with a carrier such as virus vectors, liposomes and nanoparticles.[13] While polymeric hydrogels have been demonstrated to be effective carriers enabling miRNAs to enter cells,[14] the application of supramolecular hydrogels to the delivery of miRNAs into cells has not been explored. Here we report a supramolecular hydrogel that acts not only as 3D culture media for live cells, but also as an effective carrier to deliver miRNAs into living cells encapsulated inside the gel matrix (Fig. 1).

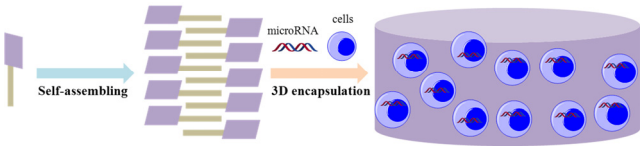


**Fig. 1** Schematic illustration of the dual-functional supramolecular hydrogel.

The chemical structure of the small-molecular hydrogelator Tet-GA is shown in Fig. 2A. It contains a dipeptide Gly-Ala with the N-terminal linked with a biphenyl-substituted tetrazole moiety. In our previous work, we have used a biaryl-substituted tetrazole with *o*-allyloxy group at the N-phenyl ring to link with the N-terminal of synthetic short peptides to construct photo-degradable supramolecular hydrogels.[9a] The biaryl tetrazole moiety when linked with short peptides has proved to be able to provide intramolecular π-π stacking and upon balance with the hydrophilic interaction of the peptide backbone, it also promoted the self-assembling process.[9a] Therefore we synthesized 4-(2-phenyl-2H-tetrazol-5-yl)benzoic acid and linked it to the N-terminal of dipeptides with different sequences. Hydrogelation tests showed that only Tet-GA was able to form stable hydrogel under neutral pH (ESI).

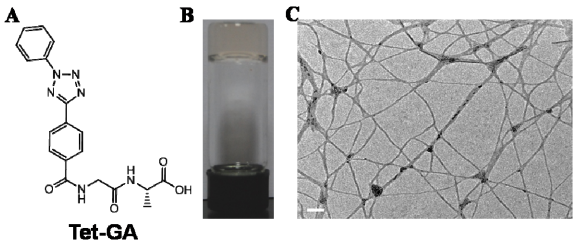


**Fig. 2** (**A**) Chemical structure of Tet-GA. (**B**) Picture of Tet-GA gel (1.5 mg/mL in PBS). (**C**) TEM image of Tet-GA gel. Scale bar: 100 nm.

In phosphate buffered saline (PBS), Tet-GA was able to form a transparent and stable hydrogel at concentrations higher than 1.5 mg/mL (Fig. 2B). Transmission electron microscopy (TEM) images of the gel showed a fibrous network in the gel matrix (Fig. 2C). Circular dichroism (CD) spectrum of the gel indicated a β-sheet secondary structure of the self-assembled nanofibers (Fig. S1). Dynamic frequency and strain sweep of the gel formed by Tet-GA at 3 mg/mL showed that the storage modulus (G') of the gel was at kilopascal level (Figure S2), indicating the gel was mechanically strong enough to encapsulate live cell for 3D culture. Hydrogelation properties of Tet-GA in Dulbecco's Modified Eagle Medium (DMEM) with the presence of miRNA mimics were similar to that in PBS (Figure S3).

The biocompatibility of the hydrogelator was then examined. According to MTT assay, the compound Tet-GA at different concentrations ranging from 10 to 500 μM did not show obvious

cytotoxicity on HepG2 cells even after 3 days' culture (Fig. 3A). The results indicated that the hydrogelator itself was highly biocompatible with live cells. Next we tried to culture live cells in a 3D manner with the Tet-GA gel as the 3D culture media. A viscous solution of Tet-GA was first prepared by dissolving Tet-GA in DMEM aided by gentle heating. Upon cooling down to room temperature, HepG2 cells were mixed with the viscous solution before it formed hydrogel, which allowed the encapsulation of the cells inside the matrix upon gel formation. The three dimensionally encapsulated cells were stained with calcein AM and imaged with confocal microscopy (Fig. 3B). The cells were 3D cultured for 24 hours and a live/dead assay was performed using Calcein AM (green) and EthD-1(red) to stain living and dead cells respectively (Fig. S4). The results indicated that over 95% of the cells encapsulated in the Tet-GA gel were alive after 24 hours' 3D culture, which made it possible for us to study the delivery of miRNA inside the gel matrix into the encapsulated living cells.

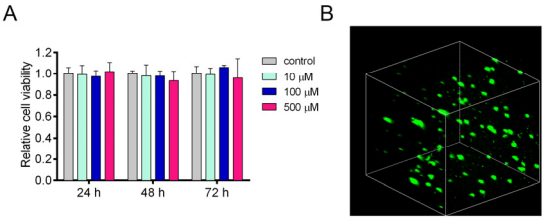


**Fig. 3** (**A**) MTT assays of the viability of HepG2 cells after treatment with Tet-GA at various concentrations for 1-3 days. Data are shown as mean ± s.d. (n=3). (**B**) 3D fluorescent images of HepG2 cells, which were encapsulated inside 3 mg/mL Tet-GA gel for 24 hours and stained with calcein AM (the cube size is 450*450*450 $\mu m^3$). Images were taken every 0.2 μm in the Z direction with a frame size of 450*450 $\mu m^2$ in the XY plane, and a 3D projection was performed to get 3D image.

Effective delivery of synthetic oligonucleotides into cells has proved to be able to regulate endogenous miRNAs and realize important bio-functions.[11,12] The use of low-molecular-weight hydrogels (LMWGs) has been reported to mediate the delivery of oligomers and siRNAs into cells cultured in dishes.[15] Besides, peptides have also demonstrated the ability to mediate the intracellular delivery of miRNA-29b for osteogenic stem cell differentiation.[16] Since most of the cells in living creatures grow in 3D microenvironment, we hope to investigate the delivery of miRNA into cells encapsulated in peptide-based hydrogel that mimic the 3D microenvironment of normal cells. Therefore we explored the possibility to realize dual function of the Tet-GA gel for 3D culture and for miRNA delivery into the 3D cultured cells.

We used miR-122 that is a liver-specific miRNA and a tumor-suppressor in liver cancers[17] to test the delivery efficiency of Tet-GA gel. Given that a major problem of miRNA delivery arises from the instability of miRNA in biofluids, we examined whether the miR-122 embedded in the Tet-GA gel was protected against the degradation by fetal bovine serum (FBS) added on top of the gel. The results indicated that the remaining miR-122 embedded in the Tet-GA gel upon exposure to 5% FBS was comparable to that after 2 hours' incubation with 5% FBS in DMEM medium (Fig. S5). This made it possible for us to study the delivery of miR-122 into the cells 3D cultured for several days with FBS-containing medium on top of the gel. Since nanofibers in supramolecular hydrogel were able to mediate the delivery of oligomers and siRNAs into cells,[15] it is also possible for Tet-GA gel matrix to mediate the intracellular delivery of miRNA into cells.

We then embedded miR-122 and HepG2 cells simultaneously inside the Tet-GA gel to study the intracellular delivery of miRNA in 3D manner. HepG2 cells were 3D cultured in the miR-122 containing gel matrix for 24 hours. Then the gel matrix was degraded and the released cells were collected. The expression level of miR-122 inside the cells was quantified by qRT-PCR. HepG2 cells cultured in Tet-GA gel without miR-122 were used as a control to exclude the possible changes in endogenous miRNA expression level in response to gel matrix alone. Fig. 4 showed the relative miR-122 level in HepG2 cells cultured in different microenvironment for 24 hours. While addition of 40 pmol miR-122 mimic into the medium for 2D HepG2 cell culture in dishes did not change the miR-122 level inside the HepG2 cells after 24 hours (Fig. 4A), the presence of same amount of miR-122 in the 3D culture gel matrix promoted the endogenous miR-122 level by more than 4 fold (Fig. 4B). Dose-dependence was also observed on the delivery of miR-122 in the hydrogel matrix. The presence of 80 pmol miR-122 mimic in the hydrogel matrix was able to promote the miR-122 level in the encapsulated cell by ~10 fold. Using miR-122 mimics labeled with a Cy3 tag (Cy3-miR-122) to incubate HepG2 cells embedded in the gel matrix, we were able to image the presence of the Cy3-miR-122 delivered inside the HepG2 cells even after a short culture time of 4 hours (Fig. S6).

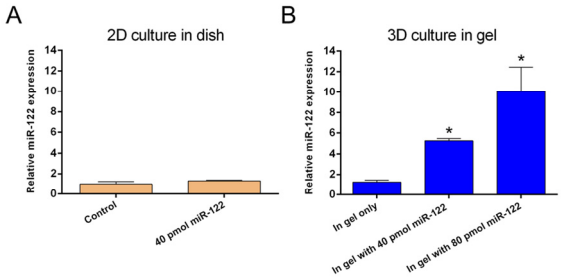


**Fig. 4** RT-PCR quantification of miR-122 expression levels after HepG2 cells were cultured in dish (**A**) or 3 mg/mL Tet-GA gel (**B**) with 40 pmol or 80 pmol miR-122 for 24 hours. Data are shown as mean ± s.d. (n=3). $^{*}P < 0.05$.

Upon confirmation on the dual function of the Tet-GA gel both as 3D culture media and as carrier to deliver miRNA, we next tried to assess whether the intracellular delivery of miR-122 was able to repress protein expression inside the cells. We used HepG2 cells transiently transfected with a luciferase reporter gene containing a complementary miR-122 binding site at its 3' untranslated regions (3'-UTR) for the assay of functional miR-122 inside the cells. As illustrated in Fig. 5A, the binding of functional miR-122 to its complementary sequence at the 3'-UTR of the luciferase gene will repress the expression of luciferase and further decrease the bioluminescence signal from the transfected cell. The HepG2 cells transfected with the luciferase reporter were encapsulated in the gel containing 0, 40 pmol or 80 pmol miR-122 and cultured for 48 hours. Then the cells were isolated

to measure relative luciferase signal using protocol as we described before.[18] Fig. 5B showed relative luciferase signals from the reporter cells cultured in different microenvironment. For cells cultured in the gel containing 40 pmol or 80 pmol miR-122 mimic, relative luciferase expression showed a decrease of ~65% and ~80% respectively. No obvious change was observed on the reporter cells incubated with 40 pmol miR-122 in dishes (Fig. S7). The results indicated that the miRNAs delivered inside the cells with the aid of the supramolecular gel were able to exert their biological function to repress target gene expression.

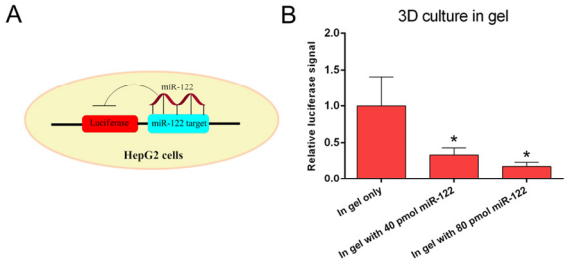


**Fig. 5** (**A**) Schematic illustration of the luciferase assay system for monitoring the activity of delivered miR-122. (**B**) Relative luciferase signals from reporter HepG2 cells after cells were cultured in 3 mg/mL Tet-GA gel with 40 or 80 pmol miR-122 for 48 hours. Data are shown as mean ± s.d. (n=3). $^{*}P < 0.05$.

In summary, we demonstrated the first example of 3D miRNA delivery into live cells mediated by supramolecular hydrogel using a dual-functional Tet-GA gel. The superior biocompatibility as well as mechanical strength of the supramolecular hydrogel made it possible to culture cells inside the 3D gel matrix. At the same time, the miRNA encapsulated together with cells inside the short-peptide based hydrogel matrix were delivered into the encapsulated cells and subsequently repressed target gene expression. This short-peptide based hydrogel thus provided a unique platform to study miRNA delivery and function in live cells under biomimetic 3D microenvironment. Further exploration of the biological functions of specific miRNA delivered into the 3D encapsulated cells is underway in our group.

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## Notes and references

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† Electronic Supplementary Information (ESI) available: [Experimental procedures and supporting figures]. See DOI: 10.1039/b000000x/

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