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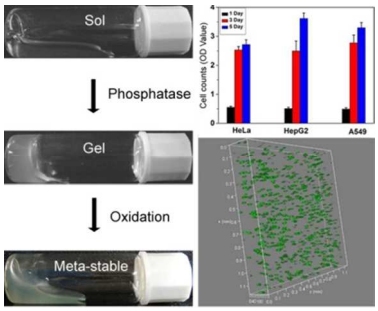

A meta-stable supramolecular hydrogel triggered by phosphatase that allowing separation of cells post culture by simply pipetting and centrifugation.

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ARTICLE TYPE

Enzymatic Formation of a Meta-stable Supramolecular Hydrogel for 3D Cell Culture

Guoqin Chen,[#,a] Jiaxin Chen,[#,b] Qicai Liu,[b] Caiwen Ou*[c] and Jie Gao*[d]

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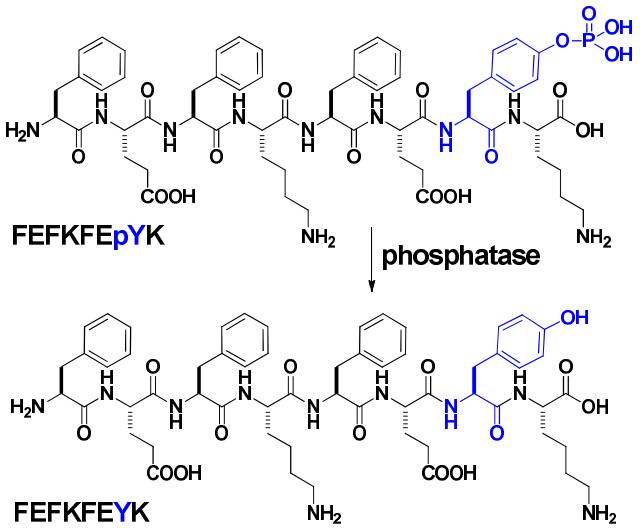
We reported on phosphatase-triggered formation of a meta-stable peptide-based supramolecular hydrogel that can easily change to a clear solution by mechanically forces. Cells cultured in the hydrogel can therefore be separated by pipetting and then centrifugation.

Peptide-based supramolecular hydrogels[1] are promising biomaterials because they mimic extra-cellular matrix (ECM) and have been widely used for cell culture[2], drug delivery[3], sensing[4], and regenerative medicine[5]. Up to now, cell culture is probably the most successful one among those applications[6, 7], and peptide-based supramolecular hydrogels of RADARADARADARADA (RADA16), FEFKFEFK (EFK8) and Fmoc-FF are already commercially available. In order to facilitating their application in cell culture, especially in three dimensional (3D) cell culture, biocompatible methods allowing homogeneously encapsulation of cells hold advantages[8]. Extensive research efforts have been paid to develop such methods including enzymatic triggerations[9], ionic strength increase[10], photo irradiation[11], redox control[12], disulfide bond reduction[13, 14], ligand-receptor interactions[15], etc. These pioneering works provide useful methods to prepare hydrogels for 3D cell culture.

Supramolecular hydrogels made from peptides of EFK8 and RADA16 are firstly developed by Zhang and co-workers and are promising for protein delivery and cell culture due to their excellent biocompatibility and degradability[6]. However, these two peptides can only dissolve in acidic aqueous solution and form hydrogels after mixing with an equal volume of alkali buffer solution or cell culture medium. To overcome this shortcoming, derivatives of EFK8 and RADA16 that can form homogeneous solutions in neutral conditions have been developed[16]. These peptides can form hydrogels in neutral conditions after mixing with buffer solutions. Recently, the method of disulfide bond reduction has also been applied to prepare the EFK8 peptide hydrogels directly in cell culture medium[13]. Phosphatase has been used to catalyze the formation of supramolecular hydrogels for many biomedical applications[17]. Stimulated by these pioneering works, we opt to develop an phosphatase catalyzed supramolecular hydrogel based on EFK8 for 3D cell culture.

As shown in Scheme 1, we designed the peptide of FEFKFEpYK. We believed that it could dissolve in neutral buffer solutions to form homogeneous solutions and then be converted to FEFKFEYK by phosphatase. The resulting peptide of FEFKFEYK might form supramolecular hydrogels because of its chemical similarity to the EFK8 peptide. We then used standard Fmoc- solid phase peptide synthesis (SPPS) to prepare the designed peptide directly. The pure peptide was obtained by high performance liquid chromatography (HPLC).



Scheme 1. Chemical structures of the peptides and schematic illustration of the transformation catalyzed by the enzyme of phosphatase

After the synthesis, we firstly tested the solubility of FEFKFEpYK in aqueous solutions and its gelling ability after enzymatic conversion. The peptide could form homogeneous solutions in phosphate buffer solution (PBS, pH = 7.4) at concentration up to 2.0 wt% (20 mg/mL). Adding the enzyme of phosphatase to the solutions resulted in rapid hydrogelations. For instance, the addition of 15 U/mL of phosphatase to the PBS solution of the peptide (0.8 wt%) led to a slightly opaque hydrogel formation within 5 minutes (Fig. 1A-II, 20-25 ^{o}C). The LC-MS trace indicated a rapid conversion from FEFKFEpYK to the FEFKFEYK (Fig. 1B), and about 83% of the peptide has been converted after 2h (70.2% has been converted at the gelling point). If using less amounts of the enzyme, it took longer time for hydrogel formation. For example, the gelation time was 20, 40, 120, and 270 minutes when using 10, 5, 2, and 1 U/mL of the

enzyme, respectively. The hydrogelations could also happen in cell culture medium, suggesting its possible application in cell culture. The transmission electron microscopy (TEM) image revealed a dense network of nanofibers with the diameter of about 25 nm in the resulting hydrogel (Fig. 1C). As shown in Fig. 1D, the scanning electron microscopy (SEM) image indicated that these nanofibers entangled with each other and formed bundles of nanofibers with diameter of 30-40 μm in the dry gel.

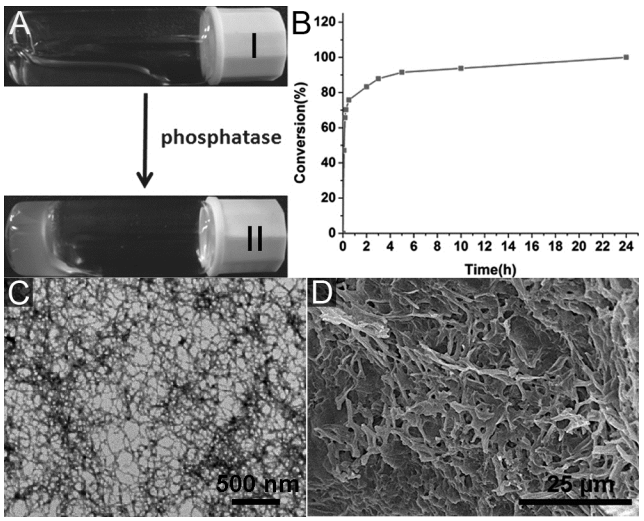


Fig. 1. A) Optical images of I) the PBS buffer solution (pH = 7.4) of FEFKFEpYK (0.8 wt%, 8 mg/mL) and II) the resulting gel formed by adding 15 U/mL of phosphatase to the solution in I), B) the percentage of conversion from FEFKFEpYK to FEFKFEYK in solution of A-II at different time points, and C) a TEM image and D) a SEM image of the gel in A-II at 24h time point

Interestingly, we found that the gels in both PBS and cell culture medium were meta-stable and would shrink after their formation when the concentration was lower than 1.2 wt%. As shown in Fig. S-4, large amount of water was excluded from the gel matrix and the colour changed to slightly yellowish after staying at room temperature for five days. Our recent study indicated that the phenol group on tyrosine (Y) could be oxidized to quinone by tyrosinase or oxygen in atmosphere[18]. Such oxidation would led to the dissociation of supramolecular network and the shrinkage of the gel. If adding equal volume of PBS to the gel at day 5 and then pipetting for several times, the gel would change to a homogeneous solution. Since the gel was meta-stable, we were unable to characterize its mechanical property by the rheometer. However, such unusual property suggested its potential in cell culture because cells could be easily separated from the gels by simply adding culture medium and then pipetting.

We then tested the application of the gel in 3D cell culture. The cell-gel constructs with low concentration of peptide would be easily destroyed when replacing culture medium, while the one with high concentration of peptide would increase the difficulty of microscopic observation of cells within them. Taking into account both stability and clarity of the cell-gel constructs, we choose the peptide concentration to be 0.8 wt%. The hydrogelation could also happen within 5 minutes in Dulbecco's Modified Eagle's Medium (DMEM) cell culture (cell density = 1,400,000/mL and concentration of phosphatase was 15 U/mL). As shown in Fig. 2A, the confocal image revealed that cells evenly distributed in the gels and most of them were alive (green and red dots showed live and dead cells, respectively), suggesting that the rapid enzymatic hydrogelation ensured the homogeneous encapsulation of the cells in gels and kept them alive. We used three kinds of cells (HeLa, HepG2, and A549) and the results obtained by the CCK-8 assay indicated that three kinds of cells kept proliferating within the cell-gel constructs during five-days of culture (Fig. 2B). Similar to the gels formed in PBS solution, the cell-gel constructs kept shrinking during this time(Fig. 2C). We also found that the cells could be easily separated from the cell-gel constructs by pipetting and then centrifuge. These observations indicated that our meta-stable hydrogel was suitable for the 3D cell culture.

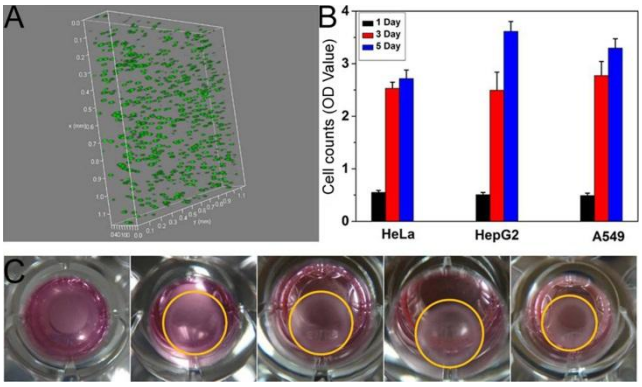


Fig. 2. A) confocal image of the cell-gel construct at 4h time point (green dots reveal live cells), B) cell proliferation curve measured by the CCK-8 assay, and C) optical images of the cell-gel constructs in the incubator at different time points (from left to right: day 1, day 2, day 3, day 4, and day 5, respectively)

In summary, we have developed a peptide-based supramolecular hydrogel formed in a biocompatible and homogeneous way (enzymatic reaction). The resulting gel was meta-stable most probably due to the oxidation of the tyrosine residue on the peptide. Therefore, a gel-sol phase transition could be easily obtained by dilution and mechanical forces. Such property rendered its application in 3D cell culture because cells could be homogeneously encapsulated in the gel and could also be easily separated post culture. However, what we need to point out one shortcoming of our system, that is the oxidation of tyrosine maybe affected by many factors especially the components in the system. We envisioned that the property of meta-stable supramolecular hydrogels might be controlled by using different amounts of tyrosine residues or adding tyrosine-related enzymes such as tyrosinase, and then be applied in controlled delivery and tissue engineering.

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Notes and references

[‡]*The authors pay equal contributions to this work.*
[a]*Cardiovascular Medicine Department of Central Hospital of Panyu District and Cardiovascular Institute of Panyu District, Guangzhou 511400, P. R. China*
[b]*Experimental Medical Research Center, Guangzhou Medical University, Guangzhou 510182, China*
[c]*Key Laboratory of Construction and Detection of Guangdong Province, Southern Medical University, Guangzhou 510280, P.R. China; E-mail: 1284572007@qq.com*
[d]*State Key Laboratory of Medicinal Chemical Biology, Nankai University, Tianjin 300071, P. R. China; E-mail: chemgaojie@nankai.edu.cn*
†Electronic Supplementary Information (ESI) available: [synthesis and characterization of the compounds, optical images of the hydrogel being kept for 5 days, details experimental procedure of CCK-8 assay]. See DOI: 10.1039/b000000x/

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