ARTICLES

Tetrapeptide-Based Hydrogels: for Encapsulation and Slow Release of an Anticancer Drug at Physiological pH

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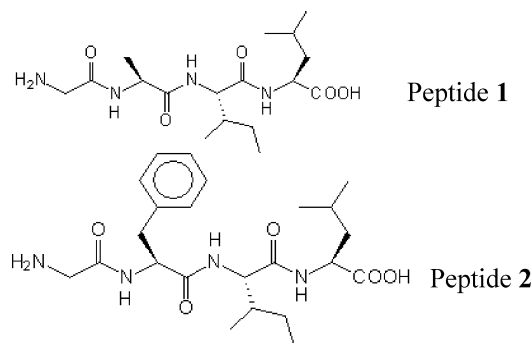
Here, we report two synthetic oligopeptide-based, thermoreversible, pH-sensitive hydrogels. In gel phase, these self-assembling tetrapeptides form a long interconnected nanofibrilar network structure, as is evident from various microscopic techniques, including field emission scanning electron microscopy (FE-SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM). FTIR, circular dichroism, and wide angle X-ray diffraction (WAXD) favor an antiparallel β-sheet structure of these gelators in the gel state. Finally, these hydrogels have been utilized for entrapment and slow release of an anticancer drug doxorubicin at physiological pH, promising their future application as a drug delivery vehicle.

Introduction

Hydrogels belong to an important class of soft materials with a wide range of applications in biomaterials, biosensors, tissue engineering, and drug delivery.[1] There are numerous examples of synthetic and natural polymer-based hydrogels. Synthetic polymers such as poly(ethylene oxide), poly(vinyl alcohol), poly[fumarate-*co*-(ethylene glycol)] can form hydrogel under suitable conditions. Natural polymers, including agarose, collagen, fibrin, gelatin, and hyaluronic acid, can also form hydrogels. Research related to low molecular weight hydrogelators has been rapidly expanding in recent years. Construction of self-assembling small molecular hydrogels has received considerable attention in soft-material research due to their potential uses in cosmetics, toiletries, and pharmaceutical formulations. There are many recent examples of low molecular weight organic molecules, such as amino acid derivatives,[2] cholic acid derivatives,[3] carbohydrate systems,[4] and peptides,[5,8–10] that can form hydrogels under suitable conditions. Two com-

ponent hydrogelators based on small organic compounds are also known.[6] Peptide-based scaffolds are interesting candidates for hydrogelation because they can be self-assembled using various noncovalent interactions in water, including hydrogen bonding, electrostatic, or $\pi-\pi$ interactions. These interactions lead to the formation of organized supramolecular assemblies that can entrap and immobilize many water molecules under appropriate conditions. Moreover, they are easy to manufacture in large quantities, and they can also be easily modified chemically and biologically. Such modification gives the ability to construct an ultra structure that promotes cell adhesion and growth.[7] Xu and his co-workers have made a pioneering contribution in the field of self-assembling, short-peptide-based hydrogels. These hydrogel-based biomaterials have many applications. They can be used for treating simulated uranium wounds, immobilizing enzymes to carry out catalytic reaction in organic solvent with high reactivity and stability, as an anti-inflammatory agent, enzyme inhibitor detection, and for other applications.[8] A series of fluorenyl methoxycarbonyl (Fmoc)-protected dipeptides were thoroughly investigated by Ulijn et

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Figure 1. Chemical structure of hydrogelators **1** and **2**.

al., and they have shown spontaneous self-assembly of these peptides under physiological conditions into fibrous hydrogel. These hydrogels are very similar to the fibrous component of the extra cellular matrix,[9] and this makes them capable of supporting cell culture of chondrocytes in two and three dimensions.[9a] Gazit and his co-workers have shown that Fmoc-protected diphenylalanine can be self-assembled in water to form a hydrogel with remarkable mechanical properties. This hydrogel can be utilized for tissue engineering and regeneration owing to its biocompatibility and also has been utilized for delivering drugs because a small drug molecule can be confined in its hollow cavities and the drug can be released slowly.[10] However, all these above-mentioned hydrogels are based on N-terminally protected self-assembling short peptides. Examples of hydrogels formed from self-assembling, water-soluble, synthetic short oligopeptides having no protecting group are rare in the literature.[5a,7c,11] Our research efforts are directed to search for the oligopeptide and amino acid-based molecules[12] that can form gel phase materials by their self-assembly. In the course of our continuing investigation to study self-assembly of water-soluble oligopeptides, we found that two peptides, namely, $H_2N-Gly-Ala-Ile-Leu-COOH$ (peptide **1**) and $H_2N-Gly-Phe-Ile-Leu-COOH$ (peptide **2**), without having any protecting group can form hydrogels at physiological pH. These hydrogels are pH-responsive and thermoreversible. They are stable in the pH range 6−8.5. Increasing the pH value above 8.5 triggers a gel-to-sol transition, whereas at a pH below 6.0, precipitation of these gelator peptides sets in. Peptide **1** can form a hydrogel at high concentration, and its minimum gelation concentration was 17% (w/v). We are interested in making a variant of peptide **1** by replacing the alanine (Ala) residue with phenylalanine (Phe) and addressing the question of whether this molecule can be self-assembled into an ordered manner to form a hydrogel. Our main intention of this substitution is to introduce π−π packing interaction in self-assembly. It has been found that peptide **2** turns out to be a more efficient gelator than peptide **1**, and it forms a thermoreversible pH-sensitive hydrogel with a minimum gelation concentration of 2.65% (w/v). The gelation has been confirmed by the inverted test tube method, and both of these two gels displayed very good stability over time.

Experimental Section

General Methods and Materials. Amino acids, DCC (dicyclohexyl carbodiimide), HOBt (1-hydroxybenzotriazole), and doxorubicin were purchased from Sigma Chemicals.

Synthesis. These two reported tetrapeptides have been prepared using solution phase racemization free fragment condensation strategy. A tertiary butylcarbonate (Boc) group has been used for protection of the N-terminal end of the amino acid, and C-terminal protection has been done by esterification.

Finally, the deprotection has been done by saponification (for the C-terminal end) and by treating trifluoroacetic acid (for the N-terminal end). The compound was characterized by mass, ^{1}H NMR, and ^{13}C spectroscopy. A detailed synthetic procedure and characterization of the reported gelator peptides have been mentioned in the Supporting Information.

NMR Experiment. NMR studies were carried out on a Brüker DPX 300 MHz spectrometer at 300 K. Compound concentrations were in the range 1−10 mM in CDCl$_3$ and $(CD_3)_2SO$.

Mass Spectroscopy. Mass spectra were recorded on a Hewlett Packard series 1100MSD and Micromass Qtof Micro YA263 mass spectrometer by positive mode electrospray ionization.

Polarimeter. The polarimeter was a Perkin-Elmer instruments, model 341 LC, polarimeter.

FTIR Study. The gels were frozen in liquid N$_2$ and then lyophilized. The solid obtained (xerogel) was studied using FT-IR. All reported FT-IR spectra were taken using a Shimadzu (Japan) model FT-IR spectrophotometer. A Nicolet FT-IR instrument (Magna IR-750 spectrometer (series II)) was used to obtain solid state FT-IR spectra. For the solid-state measurements, the KBr disk technique was used, and for the wet gel state, a CaF$_2$ cell was used.

DSC Experiment. For thermal studies, gels obtained from peptides were taken in Perkin-Elmer LVC capsules, and then they were heated in a differential scanning calorimeter (DSC-7, Perkin-Elmer) with a heating rate of 5 °C/min. Melting points and enthalpy changes were calculated from thermograms using a computer attached to the instrument. The DSC instrument was calibrated with indium before each set of experiments.

Circular Dichroism (CD) Study. Gel samples were taken on a quartz glass, and CD measurement was performed on a JASCO J-815−150S instrument at a temperature of 25 °C.
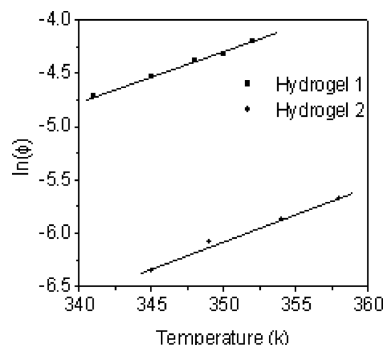
X-ray Diffraction Study. X-ray diffraction study of both wet gel and xerogel samples were done on a Seifert X-ray diffractometer (C3000). Xerogel was prepared as in the FTIR study, and wet gels used for analysis were 18% w/v for peptide **1**, 5% w/v for peptide **2**.

Doxorubicin Release Study. Both gelators (**1** and **2**) were allowed to form a hydrogel in the absence and in the presence of 6.89 × 10^{-3} M doxorubicin solution. Then each set of gels was separately immersed into 1 mL of PBS buffer (10 mM). At different times, the respective solution was removed from the gels of each set and UV/vis absorption (λ_{max} 490 nm) of the solution (containing doxorubicin) was recorded using a blank solution as reference. After recording, previously removed water (1 mL) was added to the respective gels. This cyclic process was continued for several hours. This approach allows us to quantify the amount of doxorubicin released from the gel.

Results and Discussion

Thermal Study. The tube inversion method showed that the gel-to-sol transition temperature appeared at 68 and 72 °C for hydrogels **1** and **2**, respectively. Both gels exhibit thermoreversible behavior. After the gel-to-sol transition, it takes a long time (72 h) to get back gel state materials. Thermodynamic parameters of gelation have been obtained using the following equations:

$$\Delta G^0 = RT \ln(\phi) \qquad (1)$$

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Figure 2. Concentration−temperature phase diagram ln (ϕ) vs temp (K) of hydrogels **1** and **2**, where ϕ is the mole fraction and K is the temperature.

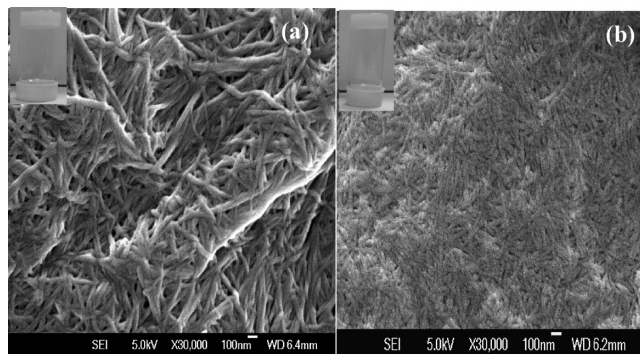**TABLE 1: The Calculated Thermodynamic Parameters of Gelation**

compound	$\delta \ln (\phi)/\delta T$	ΔH, (kJmol^{-1})	ΔG, (kJmol^{-1}K^{-1})	ΔS, (kJmol^{-1}K^{-1})
hydrogel of peptide 1	0.0466	−45.05	−13.36	−92.94
hydrogel of peptide 2	0.0507	−50.17	−18.21	−92.64

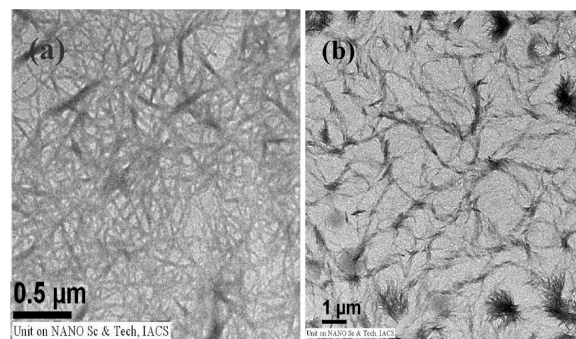$$\Delta H^0 = -RT^2[\delta \ln(\phi)/\delta T] \qquad (2)$$

$$\Delta S^0 = -R \ln(\phi) - RT[\delta \ln(\phi)/\delta T] \qquad (3)$$

Here $\phi = (a/a + b)$, where a is the number of moles of gelator, b is the number of moles of solvent, and T is the corresponding gel melting temperature. This specific representation (Figure 2) is convenient when thermodynamic parameters of the aggregates will be determined,[13] since they are proportional to the slope [$\delta \ln (\phi)/\delta T$]. The calculated enthalpies of gelation were −45.07 and −50.17 kJ/mol (Table 1) for gels obtained from self-assembling peptides **1** and **2**, respectively. These enthalpy values are matched with multiples H-bond formation energy (15 kJ/mol^{-1}). A DSC experiment was also carried out for both hydrogels **1** and **2** to obtain the change in enthalpy values during their gel-to-sol transition. Figure S15 of the Supporting Information shows the DSC thermogram of the corresponding hydrogels. It has been observed that T_{gel} (gel-to-sol transition temperature) values are 68.24 and 72.83 °C for the hydrogels obtained from peptides **1** and **2**, respectively. These T_{gel} values are comparable with the values obtained from tube inversion method. Enthalpy values were also calculated from DSC experiment, and these were found to be 46.22 (for hydrogel **1**) and 53.18 kJ/mol (for hydrogel **2**), and they are also in good agreement with the data obtained from Figure 2.
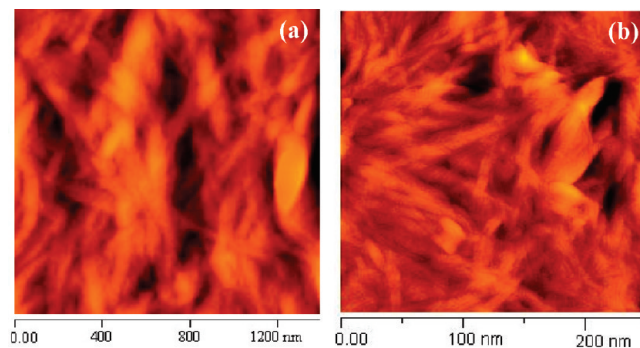
Morphological Study. To study the morphological features of this gel phase, materials field emission scanning electron microscopic (FE-SEM), atomic force microscopic (AFM), and transmission electron microscopic (TEM) studies have been performed. The FE-SEM images of the xerogel (Figure 3) revealed a close-packed nanofibrous network with fiber diameters ranging from 40 to 60 nm and from 40 to 50 nm for gels obtained from peptides **1** and **2**, respectively. TEM (Figure 4) studies of the diluted solution of gel material obtained from both peptides **1** and **2** also exhibit the nanofibrilar network structure at their self-assembling state. The calculated diameters of the nanofibers obtained from self-assembling peptides **1** and **2** in TEM are 15−30 nm and 10−25 nm, respectively. Here, it is important to note that the fibrils obtained from TEM experiments are thinner in nature than the fibrils obtained from FE-SEM. This is due to the fact that the method of sample

Figure 3. FE-SEM images of hydrogels (a) **1** and (b) **2**. Optical images of the gels are shown in inset.

Figure 4. TEM images of hydrogels obtained from peptides (a) **1** and (b) **2**.

Figure 5. AFM images of hydrogels (a) **1** and (b) **2**.

preparation is different for different microscopic experiments. Fibrils observed in TEM experiments are bundled up to give the wider fibrils observed in the FE-SEM experiment. The AFM study also provides (Figure 5) a support of fibril formation by the gelator peptides in their gel state, and the height profile diagram (Figures S13 and S14 of the Supporting Information) shows that the widths of the gel fibers are in the range of 32−56 nm and 30−50 nm for gels obtained from peptides **1** and **2**, respectively.

FTIR Study. To access the structural insight of these self-assembled materials, FT-IR was used to study the three different states: solid amorphous, xerogel, and wet gel. In the solid amorphous state, gelator peptides **1** and **2** show (Figures S9, S10) a strong C=O stretching peak at 1641 cm^{-1} and corresponding N−H bending and N−H stretching frequencies appearing at 1550, 1529 and 3288, 3305 cm^{-1} for peptides **1** and **2**, respectively (Table 2). Interestingly, in the xerogel and wet gel states (Table 2 and Figure 6), this C=O stretching frequency shifted to 1635, 1633 cm^{-1} (for peptide **1**) and 1639 cm^{-1} (for peptide **2**). This indicates the formation of a hydrogen bonded supramolecular β-sheet structure in the respective gel
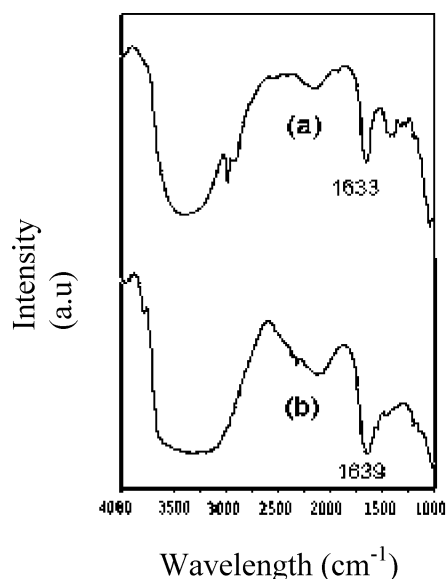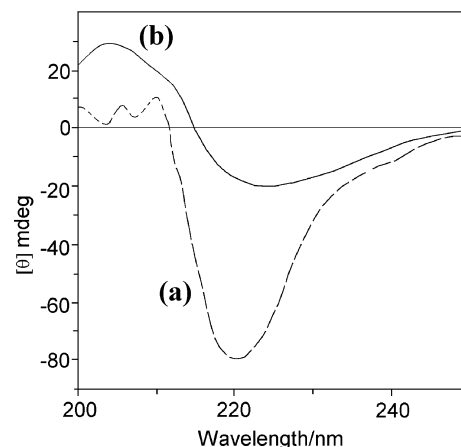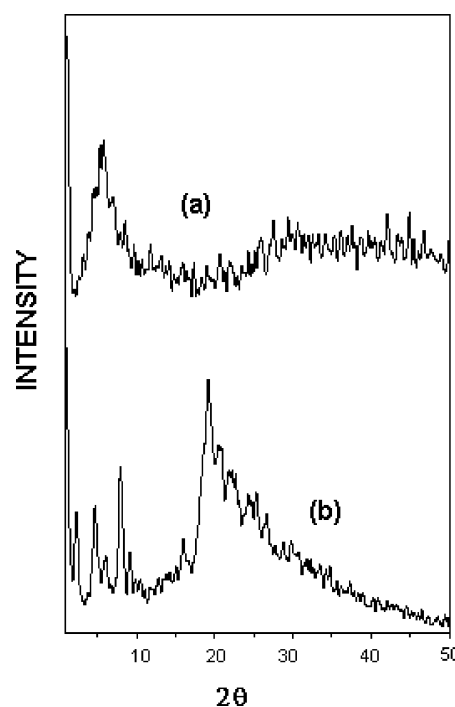
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TABLE 2: Significant Peaks in FTIR Spectra of Xerogels 1 and 2

samples	C=O stretching frequency (cm^{-1})	N−H bending frequency (cm^{-1})	N−H stretching frequency (cm^{-1})
peptide **1** (solid amorphous state)	1641 (strong)	1550	3288
	1672 (weak)		
peptide **1** (wet gel state)	1633 (strong)		
peptide **1** (xerogel state)	1635 (strong)	1542	3265, 3334
	1683 (weak)		
peptide **2** (solid amorphous state)	1641 (strong)	1550, 1529	3305
	1674 (weak)		
peptide **2** (wet gel state)	1639 (strong)		
peptide **2** (xerogel state)	1639 (strong)	1552	3301
	1674 (weak)		

Figure 7. CD measurement of the hydrogels obtained from peptides (a) **1** and (b) **2**.

states.[14a,b,15] Moreover, in the xerogel state, a weak band appeared at 1683 and 1674 cm^{-1} for peptides **1** and **2**, respectively. This is in support of an antiparallel β-sheet arrangement in the gel state.[14c]

Circular Dichroism Measurement. CD studies in the gel state were performed to get structural insight for the preference of secondary structural information of these gelators in gel state. From the CD spectra, it was found (Figure 7) that each of these two gelators showed a negative band at 220 and 222 nm.

X-ray Diffraction Study. Wide angle X-ray diffraction (WAXD) patterns of these gelator peptides were studied in the gel state to access the molecular packing in their respective self-assembled state. Hydrogel (wet gel) obtained from peptide **1** shows a peak (Figure 8 and Table 3) corresponding to *d*-spacing 4.66 Å (2θ = 19.01°) accompanied by the other peak at 10.53 Å (2θ = 8.38°). This indicates the cross β-structure of the peptide chain with an antiparallel alignment present in the wet gel state.[12a,16] The peak at 4.66 Å represents the spacing between the β-strands, and the peak at 10.53 Å can be explained as the distance between two stacked β-sheets.[17a] the diffraction pattern of the hydrogel of peptide **2** also shows

Figure 8. XRD pattern of the wet gels obtained from peptides **1** and **2**.

TABLE 3: Significant WAXRPD Peaks of Xerogels 1 and 2

compound	peak position
wet gel 1	d = 4.66 (2θ = 19.01), 10.53 (2θ = 8.38)
xerogel 1	d = 4.71 Å (2θ = 18.79), 10.63 Å (2θ = 8.30°)
wet gel 2	d = 4.62 (2θ = 19.15), 11.11 (2θ = 7.94), 3.52 (2θ = 25.27)
xerogel 2	d = 4.84 Å (2θ = 18.27), 11.41 Å (2θ = 7.73°), 3.53 (2θ = 25.15°)

peaks at 4.62 Å (2θ = 19.15°) and 11.11 Å (2θ = 7.94°). This also suggests its antiparallel cross-β-packing arrangement in the gel state. Moreover, a peak at 2θ = 25.27° (3.52 Å) was observed for peptide **2** gel, indicating the presence of $\pi−\pi$ interactions[17b,c] in the wet gel state, which is absent in wet gel obtained from peptide **1**. This is due to the absence of any aromatic ring in peptide **1**. Similar data have also been obtained using the xerogels obtained from peptides **1** and **2** (Table 3). Here, it should be noted that a similar type

Figure 6. FTIR spectra of wet gel derived from peptides (a) **1** and (b) **2**.

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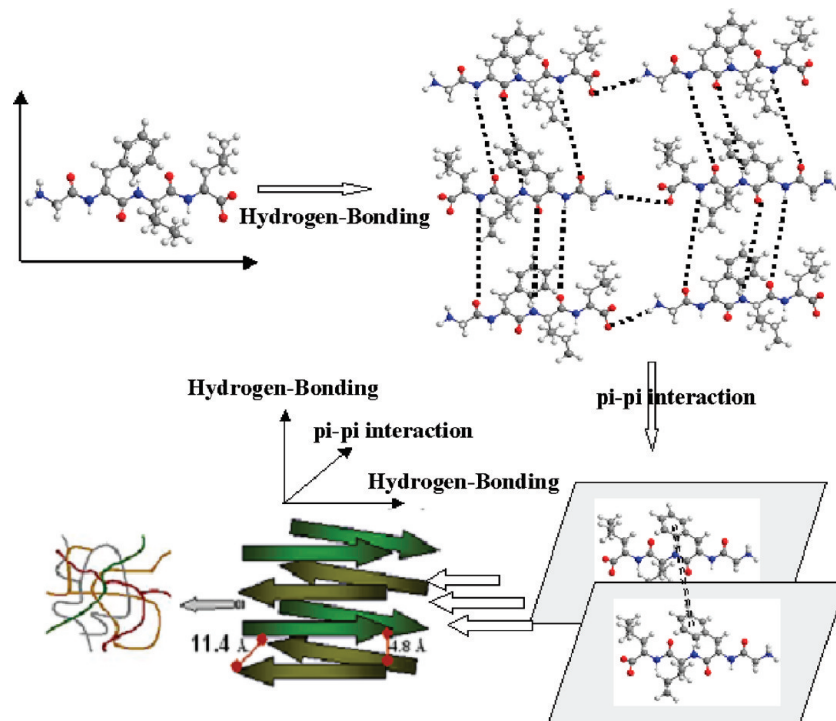*J. Phys. Chem. B, Vol. 113, No. 35, 2009* **11791**

Figure 9. Model highlighting the molecular arrangement in gel state.

of cross-β-arrangement is present in both the wet and dried gel states. On the basis of these observations, we have proposed a model (Figure 9) highlighting the probable packing in gel state for peptide **2**.

Drug Encapsulation and Release Study. Generally, polymer-based hydrogels have been used for delivering drugs. However, in some cases, these polymeric compounds exhibit toxicity.[18a,b] So searching for low molecular weight hydrogels responsive as drug delivery vehicles has been afforded considerable attention.[1c,2b,19] In this study, we want to explore the capacity of these two hydrogels for encapsulation and slow release of an anticancer drug to study whether it can be used as a drug delivery vehicle. Doxorubicin as a single agent is known to provide high response against advanced breast cancer and a favorable result in gastric carcinoma, a tumor against which only a few drugs are believed to be potentially active.[20] Interestingly, these reported peptides **1** and **2** can be self-assembled into hydrogels in an aqueous solution containing doxorubicin. These hydrogels are able to entrap 8.62×10^{-3} (M) and 13.79×10^{-3} (M) drug solutions (for hydrogels **1** and **2**, respectively) at their minimum gelation concentrations. Now it is interesting to examine whether these entrapped drug molecules can be released into the solution at physiological pH. In this regard, the drug-loaded hydrogels were covered with 1 mL of PBS buffer (10 mmol, pH 7.46) to determine the diffusion rate of drug molecules from the respective hydrogels into the surrounding water. The diffusion coefficient of the drug molecule was determined on the basis of a nonsteady state diffusion model equation,[21]

$$(M_t/M_\alpha) = 4(Dt/\pi\lambda^2)^{1/2}$$

where M_t is the total amount of drug released during the measurement, M_∞ is the total amount of drug that was kept within the gel matrix, λ represents the hydrogel thickness, t is the time of measurement, and D is the diffusion constant of the
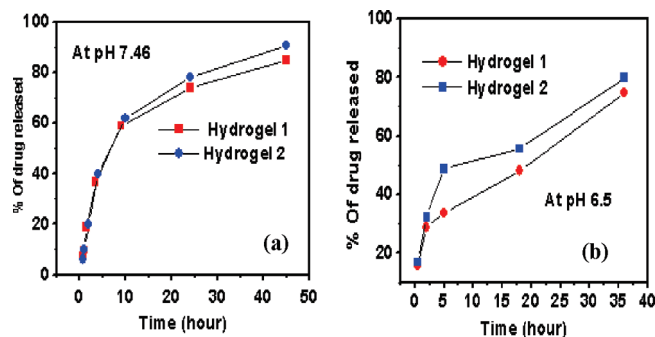

Figure 10. Drug release profile of hydrogel **1** and hydrogel **2** at different pH's: (a) 7.46 and (b) 6.5.

drug molecule. From the release curve (Figure 10a), it has been observed that almost 85% (for peptide gel **1**) and 90% (for peptide gel **2**) of the drug molecules were diffused from the respective gel matrix after 45 h. The diffusion coefficients calculated from the release curve were 2.078×10^{-10} m^2 s^{-1} for hydrogel obtained from peptide **1** and 2.737×10^{-10} m^2 s^{-1} for hydrogel obtained from peptide **2**. Similarly, we have also tested the release of doxorubicin into the solution at acidic pH (pH 6.5). This is due to the existence of many drug delivery targets, including tumors, inflammatory tissues, and phagolysosomes of antigen-presenting cells at acidic pHs.[22] After 35 h, it was observed that hydrogels obtained from peptides **1** and **2** can release 75% and 80%, respectively, of entrapped drug molecules into the solution at pH 6.5 (Figure 10b).

Conclusion

Two synthetic self-assembling oligopeptides, **1** and **2**, form thermoreversible pH-sensitive hydrogels. In the gel phase, these self-assembling tetrapeptides exhibit long, interconnected nanofibrilar network structures, as is evident from various microscopic techniques, including FE-SEM, TEM, and AFM. FTIR, CD,

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and WAXD favor an antiparallel β-sheet structure of these gelators in their respective gel state. Moreover, these hydrogels can entrap a potent anticancer drug, doxorubicin, which also can be released slowly at physiological pH. This may hold future promise for using these water-soluble short peptide based hydrogels as a drug delivery vehicle.

Supporting Information Available: The Supporting Information contains the synthetic procedure of reported peptides **1** and **2**, ^{1}H NMR, ^{13}C NMR, mass spectra, FTIR of solid amorphous compounds and xerogels, WAXPD of xerogels, AFM (height profile diagram and topographic view), m and DSC thermograms. This information is available free of charge via the Internet at http://pubs.acs.org.

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