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Enzyme Catalysis: Tool to Make and Break Amygdalin Hydrogelators from Renewable Resources: A Delivery Model for Hydrophobic Drugs

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Abstract: We report a novel approach for the controlled delivery of an antiinflammatory, chemopreventive drug by an enzyme-triggered drug release mechanism via the degradation of encapsulated hydrogels. The hydro- and organogelators are synthesized in high yields from renewable resources by using regioselective enzyme catalysis, and a known chemopreventive and antiinflammatory drug, i.e., curcumin, is used for the model study. The release of the drug occurred at physiological temperature, and control of the drug release rate is achieved by manipulating the enzyme concentration and/or temperature. The byproducts formed after the gel degradation were characterized and clearly demonstrated the site specificity of degradation of the gelator by enzyme catalysis. The present approach could have applications in developing cost-effective controlled drug delivery vehicles from renewable resources, with a potential impact on pharmaceutical research and molecular design and delivery strategies.

Introduction

The use of renewable resources for production of valuable chemical commodities is becoming a topic of great interest and fueling objectives of promoting the industrial biorefinery concept in which a wide array of valuable chemicals, fuel, food, nutraceuticals, and animal feed products all result from the integrated processing of grains, oil seeds, and other biomass materials.[1,2] Industrial or "white" biotechnology[3,4] is making an increasingly important contribution to the development of a sustainable, biobased economy by an environmental benign approach.[5−7] It uses enzymes and microorganisms to make products in sectors such as chemistry, food and feed, paper, textiles, and medicine. As opposed to the chemical synthesis, enzyme catalysis is highly selective and has been used to generate various specialty chemicals[8,9] including sugar-based esters.[10] Our research efforts[11−13] are deeply devoted toward developing building blocks from renewable resources to generate soft nanomaterials such as new surfactants, liquid crystals, organic gelling materials and hydrogels.

Self-assembly has proved to be a powerful strategy to develop molecularly defined and functional materials.[14,15] Hydrogels are one among them, possessing a range of applications in areas such as tissue engineering,[16] controlled-release drug delivery systems,[17−19] and medical implants.[16−20] Design and synthesis of low-molecular-weight hydrogelators has received considerable attention in soft materials research in terms of its potential use in cosmetics, toiletries, and pharmaceutical formulations. A study of the literature reveals that there are only limited

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reports on easily achievable and efficient low-molecular-weight gelators that are able to gel water or even water mixtures with other solvents[21−29] and are often achieved by multistep chemical synthesis. Surprisingly, to the best of our knowledge, to date there are no examples in the literature where low-molecular-weight hydrogelators were synthesized from renewable resources by using regioselective enzyme catalysis.[30] Herein we report biocatalysis as a tool to make the gelators from biomass and their assembly to form hierarchical superstructures in water (formation of hydrogel), encapsulation of hydrophobic drug, as well as enzyme-mediated hydrogel degradation. We believe this approach will give new insights into low-molecular-weight hydrogelator-based drug delivery models.

Controlled-release drug delivery occurs when a polymer, whether natural or synthetic, is judiciously combined with a drug or other active agent in such a way that the active agent is released from the material in a predesigned manner.[31,32] While these advantages can be significant, the potential disadvantages cannot be ignored: the possible toxicity or nonbiocompatibility of the materials used, undesirable byproducts from gel degradation, and the higher cost of controlled-release systems compared with traditional pharmaceutical formulations. Considering the above parameters, we designed sugar amphiphiles generated by regioselective synthesis of amygdalin esters as new hydrogelators.

[*O*-*β*-D-Glucopyranosyl-(1-6)-*β*-D-glucopyranosyloxy]benzeneacetonitrile which is known as D-amygdalin is a naturally occurring glycoside found in many food plants, for example, the kernels of apples, almonds, peaches, cherries, and apricots.[33] Amygdalin (a byproduct of apricot, almonds and peach industry, see Figure S1 (Supporting Information)) has been used as a main ingredient in commercial preparations of laetrile, a purported therapeutic agent.[34,35] In particular, our aim is to synthesize amygdalin derivatives which can form nanoaggregates through self-assembly and encapsulate a hydrophobic drug, followed by release of the encapsulated drug upon enzyme-mediated degradation (an enzyme-triggered drug delivery model). In amygdalin−fatty acid conjugates, the sugar moiety can facilitate the stacking of molecules through hydrogen bonding, the phenyl ring can facilitate intermolecular interactions through $\pi-\pi$ stacking, and the hydrophobic hydrocarbon chain not only decreases the solubility in water but also helps the molecular association through the van der Waals interactions. By keeping these thoughts we developed gelator molecules **1**−**3** with various chain lengths (Figure 1).

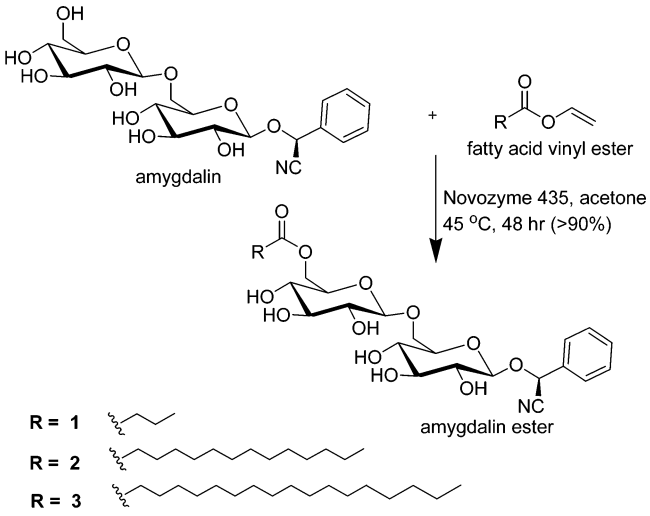

Figure 1. Synthetic scheme of amygdalin-based amphiphiles.

Results and Discussion

Synthesis of Hydrogelators by Enzyme Catalysis. By taking advantage of the supreme control of enzyme catalysis on regioselectivity, we made a series of amygdalin derivatives where we selectively introduced an acyl moiety onto the primary hydroxyl group in excellent yields. Amygdalin is a disaccharide containing one primary hydroxyl group, which forms an ester bond with fatty acids (vinyl esters were used as acyl donors). The detailed synthesis procedures are shown in the Experimental Section (in Supporting Information), and the synthetic route to the amphiphilic amygdalin derivatives is shown in Figure 1. In general, multistep synthesis, arduous separation procedures, and lower yields often keep low-molecular-weight gelators away from commercial use due to high production cost.[36] Strikingly, the hydrogelators we developed were synthesized from renewable resources in a single-step process in high yields (>90%), and unpurified crude products showed unprecedented gelation abilities, similar to the purified products. In particular, this property may provide the opportunity to develop these gelators on industrial scale for various applications.[36]

Gelation Abilities of 1−3. Amygdalin derivatives **1**−**3** encompass all required functional groups such as hydrogen bond-forming "sugar" headgroups, phenyl rings for $\pi-\pi$ stacking, and hydrocarbon chains for van der Waals interactions; these groups together can synergistically act to form strong intermolecular interactions, which lead to the gelation. We compared the gelation abilities of **1**−**3** in water and in organic (polar and nonpolar) solvents (Table S1, Supporting Information). Typically, gelator (0.01−2 mg) in required solvent (0.1−1 mL) was heated until the solid was completely dissolved. The resulting solution was slowly allowed to cool to room temperature, and gelation was visually observed. A gel sample was obtained that exhibited no gravitational flow in inverted tube. All gels obtained are thermally reversible. Above their gelation temperature, the gels dissolved in water, but could be returned

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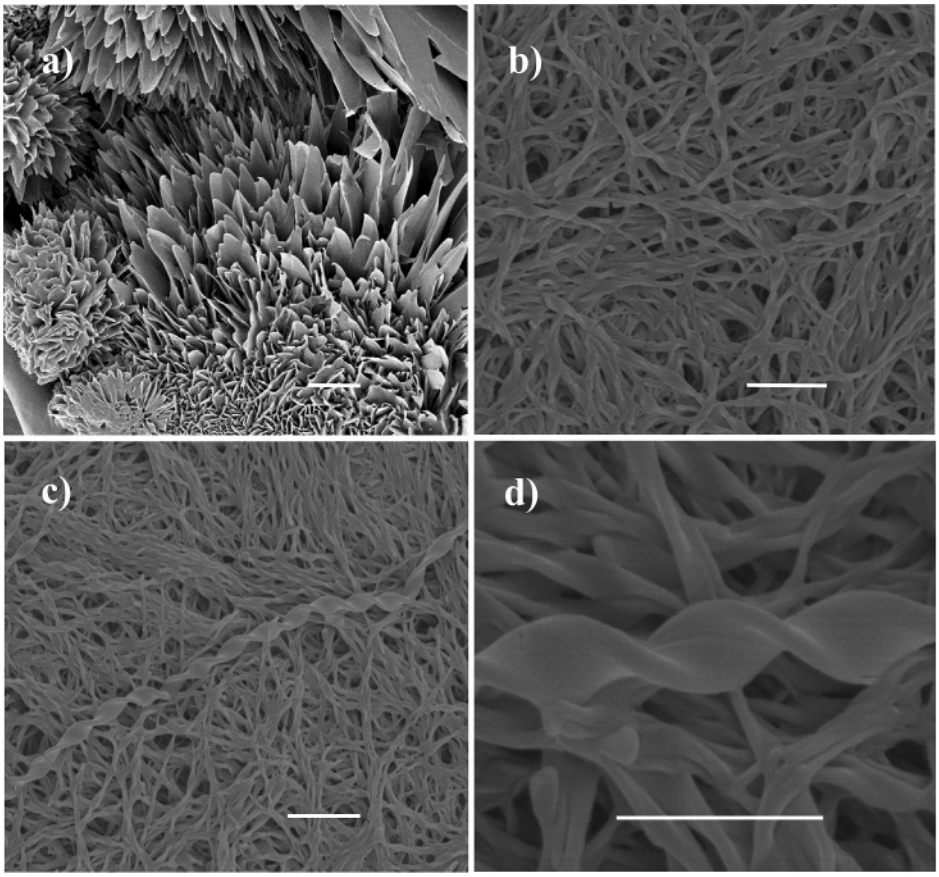

Figure 2. SEM micrographs of the organogel of **1** prepared from acetonitrile (a), aqueous gels from **2** (b) and **3** (c), higher magnification of hydrogel **2** (d). Scale bar is equivalent to 1 μM.

to their original gel state upon cooling. Amygdalin amphiphiles (**1**−**3**) showed unprecedented gelation abilities in a broad range of solvents at extremely low concentrations [0.05−0.2 wt % (MGC)] while displaying excellent thermal and temporal stabilities.

Scanning Electron Microscopic Studies. Molecular self-aggregation features can be observed by an electron microscope, since the initial stage of physical gelation is the self-assembly of gelator monomers. In Figure 2 we present the scanning electron microscope (SEM) images of the organogels formed by **1** (Figure 2a) and aqueous gels formed by **2** and **3** (Figure 2, b and c, respectively). The images of their xerogels reveal two different types of morphologies. The organogel formed in acetonitrile by **1** showed grasslike morphology. Hydrogels of **2** and **3** showed helical ribbon morphology at microscopic level. Analysis of these aggregates clearly showed that the individual fibers are approximately 50 nm wide, pitch is about 100−125 nm, and length is up to several micrometers. These helical nanofibers are entangled and formed a dense fibrous network that results in the immobilization of the solvent. We also made gels in the presence of a drug (curcumin), and SEM images suggest that inclusion of curcumin does not change the basic twisted fibrous morphology of the hydrogel (Figure S2, Supporting Information).

XRD Measurements and Crystal Structure Analysis. From the X-ray diffraction patterns of the xerogels of **1**−**3** prepared from xylene and water, we calculated the long spacings (*d*) and discussed these values to postulate the possible mode of aggregation in the gel state. Possibly lamellar structures were formed by these amphiphiles in gels. Xerogels of **1**−**3** were prepared by a freezing-and-pumping method (see Experimental Section in Supporting Information), and they were spongelike materials. Amygdalin butyrate (**1**) gave a single crystal in water; the obtained single crystal was successfully analyzed by X-ray crystallography. The crystal structure of **1** is shown in Figure 3, and the information obtained from the single-crystal analysis was combined with XRD data to postulate possible molecular packing of amygdalin amphiphiles within the hydro- and organogels.

Drug Encapsulation and Enzyme-Triggered Controlled Release. Solubilization of hydrophobic drugs and developing suitable drug delivery systems is a challenging task in drug discovery research.[20] Herein we demonstrated a conceptually novel approach (single-step enzyme-triggered drug delivery at physiological conditions), where we encapsulated (solubilized without chemical modification) a hydrophobic drug molecule in hydrogel, subsequently released the drug by breaking the gel by using hydrolase enzyme (Lipolase 100L, Type EX). The preformed hydrogel was degraded completely by the lipolase while releasing the encapsulated chemopreventive hydrophobic drug curcumin (for image see Figure S1b,c (Supporting Information). Drug release was monitored by the absorbance spectra of drug. Control of the drug release rate was achieved by manipulating the enzyme concentration and/or temperature. The byproducts formed after the gel degradation was characterized, and thus the cleavage site of the gelator by the enzyme was determined. Gel degradation occurred due to the cleavage of the ester bond in the gelator by the hydrolase enzyme.

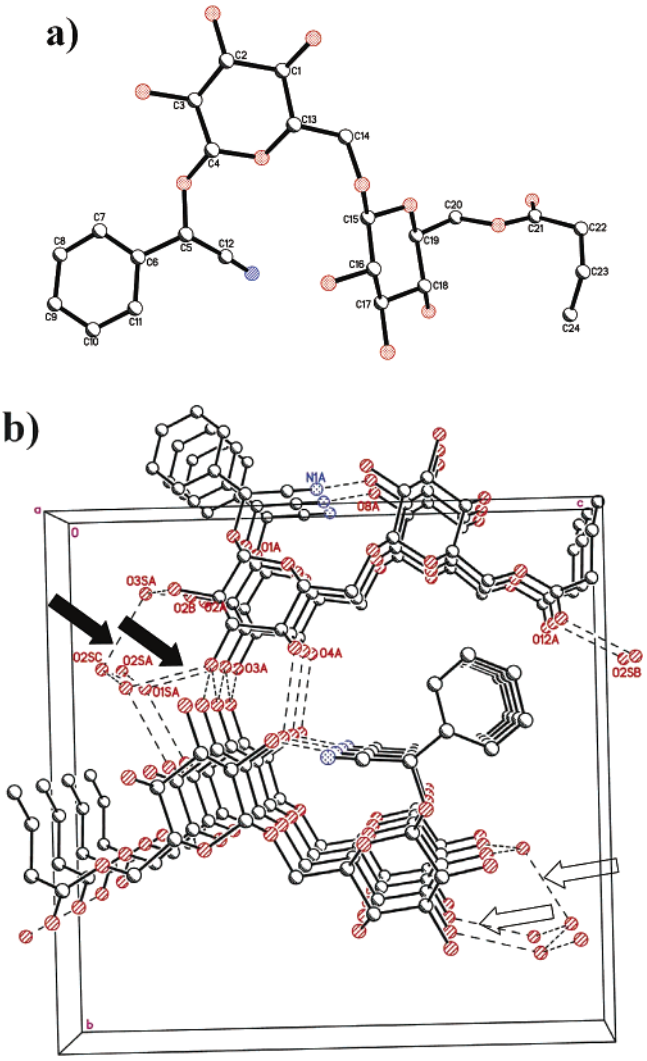

Figure 3. (a) Crystal structure of **1** in water. (b) Top view shows the $\pi-\pi$ stacking of phenyl rings and hydrogen bonding between two amygdalin molecules. Hydrogen bonding acts as bridge between stacked amygdalin molecules along the *b* axis (indicated with blue arrows) and between such two stacks along the *a* axis (indicated with black arrows). (Red) Oxygen. (Blue) Nitrogen. (○) Carbon. (---) Hydrogen-bonds.

Herein we discuss the results in detail. Gelation is the delicate balance between solubility and precipitation. To obtain that, one needs to fine-tune the structural features in the gelator molecules. Amphiphiles **1**−**3** were generated by attaching a fatty acid chain to amygdalin via a regiospecific transesterification reaction on the primary sugar hydroxyl. Inspection of Table S1 (Supporting Information) reveals that amygdalin derivatives are versatile gelators for water and polar/nonpolar organic solvents: **1** formed gels in two solvents out of 10 tested, whereas **3** gelled in all 10 solvents. This would explain us the importance of the chain length on gelation ability. Noteworthy to mention that **2** and **3** do not require any cosolvent to form hydrogel despite its gelation ability in less polar solvents such as benzene, toluene, and xylene. These gelators showed excellent gelation in a broad range of solvents. The robustness of the gelator can be determined by considering three parameters; (i) gelation ability in a broad range of solvents, (ii) low minimum gelation concentration (MGC), and (iii) thermal stability of the gels. For example, these gelators (**2** and **3**) formed gels in highly polar solvents, such as water and methanol, and nonpolar solvents,

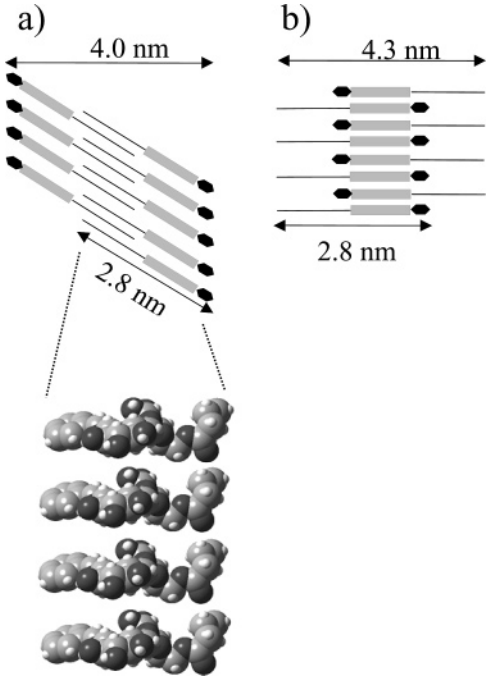

Figure 4. Schematic representation of possible molecular packing models for the hydrogels (a) and organogels (b) of **2**.

such as nonane, benzene, and toluene. MGC of these gels is very low, typically 0.05 and 0.2 wt % for **3** in water and benzene, respectively. This is one of the lowest gelation concentration reported in the literature for any class of gelators.[19,26] Similarly, other derivatives also exhibit the lower MGC values for various solvents; typically between 0.07 and 0.5 wt %. In addition, they showed good thermal and temporal stabilities. Gel-to-solution transition temperature (T_{gel}) was determined by typical "inversion tube method"[21] and from the differential scanning calorimeter (DSC). The T_{gel} values of these gels are in the range between 40 and 85 °C for 0.5 wt % gels, depending on the solvent used. All gels were stable for months. Hence, together all three parameters are satisfied; the reported amygdalin-based gelators could be considered as excellent gelators.

In X-ray diffraction experiments, *p*-xylene gel of **2** showed long distance spacing 4.3 nm, which is larger than the molecular length (2.8 nm from the optimized geometry calculations) and much lower than double the extended molecular length of **2**. Thus, there could be two possible ways to explain how these molecules could self-assemble, which is shown in Figure 4a,b: (1) a highly interdigitated bilayer structure with the alkyl chain tilting with respect to the normal to the layer plane (Figure 4a); (2) hydrophilic parts are facing inside the assembly, and hydrophobic chains are exposed to the outer side of the assembly (Figure 4b). On the other hand, long distance spacing for hydrogel of **2** is 4.0 nm and strongly supports that the interdigitated molecular packing would be possible at nanoscopic level. It is unlikely that hydroxyls containing sugar headgroups will face inside and that lipophilic hydrocarbon chains will face the bulk polar solvent; hence, the model in Figure 4b would be ruled out. Thus, molecular packing in hydrogels of **2** would be similar to that shown in Figure 4a. In this model hydrophilic groups are exposed to the outer solvent while hydrophobic chains are highly interdigitated, which is consistent with previous reports.[11,28] On the basis of long distance spacing of

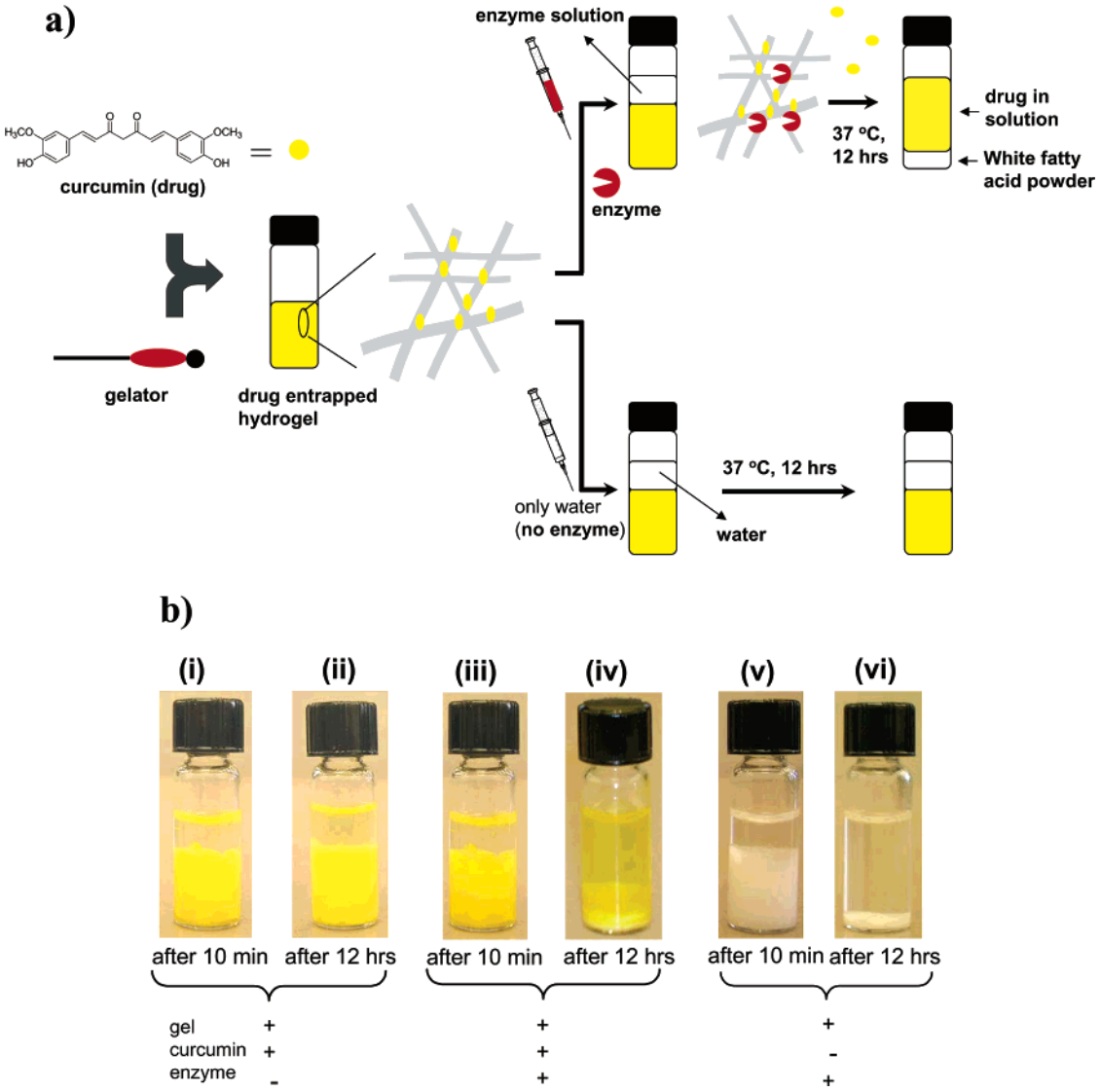

Figure 5. (a) Schematic representation of drug encapsulation in supramolecular hydrogel and subsequent release of drug by enzyme-mediated degradation of hydrogel at physiological temperature. (b) Real images of hydrogels of **3** with (i−iv) and without (v,vi) curcumin [After complete gel degradation, the remaining white, fluffy powder which settled at the bottom was characterized, i.e., water-insoluble fatty acids which formed after gel degradation by enzyme].

the hydro- and organogels of **3**, we propose that most likely the molecular packing of the amphiphiles is similar to that by the gels of **2**; it might be possible that in case of the gels of **3** the alkyl chain tilt would be more than that of **2**. Therefore, we propose layered structures for self-assembly of these gels, which also are supported by solid-state crystal structure.

Amygdalin butyrate (**1**) gave single crystals in water; the isolated single crystal was successfully analyzed by X-ray crystallography. Interestingly, these molecules were well packed in the crystal lattice due to the extensive hydrogen bonding; herein we observed strong well-arranged intra- and intermolecular hydrogen bonding. Intramolecular hydrogen bonding between N (nitrogen) of nitrile group and sugar hydroxyl (O—H) hydrogen helps to form a locked conformation which apparently participates in formation of stacked structures. The stacked layered structures are stabilized by $\pi-\pi$ stacking and van der Waals interactions between the alkyl chains. Such two stacks were arranged in "head-to-tail" fashion to give the extended porous structure (Figure 3b). Water molecules were involved in two types of hydrogen bonding. In one type, water molecules formed hydrogen bonding with sugar hydroxyls while acting as bridged molecules between stacked amygdalin amphiphiles and stabilized the stacked layers (shown in open arrows in Figure 3b). In the second mode, water molecules were involved in hydrogen bonding with sugar hydroxyls while acting as bridged molecules between two different stacks of amygdalin amphiphiles to stabilize the two adjacent layers (shown by filled arrows in Figure 3b). In addition to that, we observed the intermolecular hydrogen bonding between sugar hydroxyls of two amygdalin molecules from opposite stacks, which also indicates the greater ability to form self-assembled structures by amygdalin derivatives. By collecting the information from the crystal structure of **1**, we assume that most likely in the gel state similar self-assembly would be possible. Previously in the literature two reports explained the aggregation modes of the gelators based on single-crystal analysis.[37,38] As we evidenced in crystal structure there are several interactions such as extensive hydrogen bonding, $\pi-\pi$ stacking, and van der Waals

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interactions that exist, and such cooperative interactions play an important role in stabilizing the fiber structures in the gel state.

In further investigations to explore the possible applications of these robust gels, we intended to utilize the hydrophobic pockets within the gel to encapsulate hydrophobic drugs; hence, we tested these hydrogels as a drug delivery vehicle model. In the process of developing drug delivery systems, chemical modification of the drug and cleavage induced by external stimuli such as increasing temperature followed by enzyme-mediated cleavage has been shown recently.[19] Such an approach has limitations while applying to different drugs. Covalently connecting the drugs to the hydrogelators may not be achievable trivially in all types of drugs, and in the process of chemical modification, there is a potential chance of loosing the original drug activity. An ideal system would be to have encapsulated drug models; however, can drug release be triggered by enzymes without the need of altering pH or temperature. Herein we demonstrate an enzyme-triggered drug delivery at physiological conditions, where we encapsulated (solubilized without chemical modification) a hydrophobic drug molecule in hydrogel, subsequently releasing the drug by breaking the gel with hydrolase enzyme (Lipolase 100L, Type EX). The success of this approach for a drug delivery model system for possible in vivo applications relies on a few factors such as (a) selected hydrogels should able to provide the hydrophobic pockets to solubilize the hydrophobic drugs, (b) gel degradation (to release drug) should take place at mild conditions such as physiological pH and temperature, (c) the products formed after degradation should be biocompatible. In the current study as a model drug we selected one of the best-characterized chemopreventive agents, curcumin (or diferuloylmethane)[39] extracted from the root of *Curcuma longa*, which presents strong antioxidative, antiinflammatory, and antiseptic properties.[40] In addition, curcumin also inhibited purified human immunodeficiency virus type 1 (HIV-1) integrace,[40,41] HIV-1 and HIV-2 proteases,[42] and HIV-1 long terminal repeat-directed gene expression of acutely or chronically infected HIV-1 cells.[43] Despite such astounding drug activity, unfortunately curcumin has an extremely low aqueous solubility and poor bioavailability, limiting its pharmaceutical use.[44] One possible way to increase its aqueous solubility is to form inclusion complexes, i.e. to encapsulate curcumin as a guest within the internal cavities of a water-soluble host or encapsulate within the nanoaggregates (formed by self-assembly), which are having hydrophobic pockets within.

A schematic representation of curcumin encapsulation and enzyme-mediated release is depicted in Figure 5a. The release of curcumin into the solution in the presence of enzyme was monitored by measuring the curcumin UV-absorption spectrum. We compared absorption spectrum recorded in aqueous gel solution with curcumin spectra recorded in methanol, and

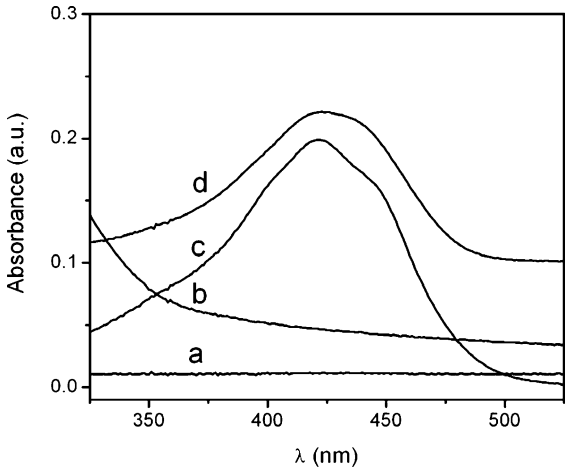

Figure 6. UV absorption spectra of curcumin in various types of solution mixtures. (a) Curcumin entrapped hydrogel in the presence of enzyme. (b) Enzyme added to the hydrogel which does not contain curcumin. (c) Curcumin entrapped hydrogel in absence of enzyme. (d) Methanolic solution of curcumin.

previously it has been reported that the effect of solvent polarity on the absorbance spectrum of curcumin is minimal.[44] High concentration of curcumin (1×10^{-3} M) was solubilized in 0.5 wt % hydrogel of **3** (the reported[45] solubility of curcumin in water is 3×10^{-8} M, i.e. ~33000 times greater than we could solubilize in the hydrogel); the resulting gel was yellow in color (Figure 5b), and due to the hydrophobic nature, curcumin might be located at hydrophobic pockets of the gel. To test this hypothesis we added water to the preformed gel and left it for 12 h and recorded the UV-absorption of the supernatant. The absence of any absorbance peak indicated the unavailability of curcumin on the gel surface by adsorption. First, 0.5 mL of lipase (Lipolase 100L, Type EX, lipase units 100 KLU/g) was added to the preformed gel which was kept at 37 °C (far lower than gel melting temperature); initially the added solution was colorless (Figure 5b, iii), and after 12 h visual changes occurred (Figure 5b, iv), i.e. 100% of the gel was degraded, and the top solution became yellow in color which indicates that upon enzyme-mediated gel degradation encapsulated curcumin has been released into the solution. This was confirmed by spectroscopic experiments. Aliquots were collected after addition of enzyme to the hydrogel (after 10 min and 12 h), and absorbance spectra were recorded; interestingly initial aliquots (after 10 min) did not show any absorbance peak, but aliquots collected after 12 h showed absorption maxima at 425 nm, which corresponds to the absorption peak of curcumin (Figure 6). To find out the role of the enzyme on hydrogel degradation, similar experiments we carried out by adding only water without an enzyme. As we expected, curcumin-encapsulated gel was still intact after incubating few days at 37 °C, there was no visual change in the gel volume and added solution (Figure 5b), and it did not show the absorbance peak corresponding to the curcumin (Figure 6a). In addition to that we performed control experiments with the same hydrogel of **3** without curcumin, which is opaque and white in color (Figure 5b). To that 0.5 mL of lipolase was added and was incubated at 37 °C; after 12 h the gel was degraded completely, and then we recorded the

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absorption spectrum of the solution (Figure 6b). The absence of the absorption peak at 425 nm suggested that, the previously observed peak (Figure 6c) corresponded to the released curcumin into the solution.

To obtain control on the rate of release, we investigated the role of enzyme concentration and temperature on gel degradation or controlled drug release. In the first set of experiments we changed the temperature while keeping enzyme concentration constant. After addition of enzyme to the preformed gel, the vial was kept at room temperature for 2 days, and as we explained previously, curcumin release was monitored by absorption spectra. Interestingly, even after 2 days at room temperature in the presence enzyme there was no gel degradation observed, and thus there was no release of encapsulated curcumin. When the vial was placed at 37 °C in an incubator, after 120 min, slow release of curcumin was observed; within 720 min, encapsulated curcumin was released completely. Similarly when we incubated at 45 °C release was initiated within 30 min, and complete release was observed in 270 min. In the second set of experiments, we changed the enzyme concentration while keeping the temperature constant. A concentration 10 times lower of lipolase (units 10 KLU/g) was added to the preformed curcumin encapsulated gel; at room temperature even after several days no release had occurred. Then when the vial was placed at 37 °C incubation, it took 300 min to start the drug release, which eventually took 4320 min to be released completely. In addition to that, a similar low-enzyme concentration vial was directly incubated at 45 °C, and in this case drug release was started after180 min, and in 2880 min complete release was observed. Hence, at constant temperature we could control the drug release by lowering the enzyme concentration. We summarized the above-mentioned results in Table S2 and Figure S3 (Supporting Information). These results clearly demonstrate the achieved control over the release of the encapsulated drug from hydrogel.

It is important to characterize the products/compounds formed after gel degradation. To find out other components formed after gel degradation we performed thin-layer chromatography (TLC) of the solution, and it has been found that this solution contains amygdalin, curcumin, and enzyme (confirmed by comparing R_f values). This indicates that enzyme is degrading the gel by cleaving the ester bond of **3**. Upon gel degradation, a white, fluffy solid was produced which is not soluble in water, which thus settled down in the vial (Figure 5b, iv). The solid was isolated and characterized by ^{1}H NMR, and it matched with the NMR of pure stearic acid (Figure S4 online). Hence, it undoubtedly suggests that gel degradation is occurring through the cleavage of the ester bond of amygdalin derivatives by lipolase enzyme. These results unambiguously explain the drug encapsulation abilities of hydrogels formed by amygdalin derivatives and enzyme-triggered drug release. Noteworthy, these gelators were generated via enzyme catalysis, and gels were degraded (converting from gelators to starting materials) by yet again using enzyme catalysis in environmentally benign conditions.

Conclusion

In conclusion, we successfully developed hydrogelators from renewable resources, low-molecular-weight hydrogelators were synthesized by regioselective enzyme catalysis for the first time, yields were quantitative, and crude reaction mixtures exhibited equally unprecedented gelation properties such as their counter pure products, and this capability may well allow us to develop hydrogelators on industrial scale for future applications.[36] We clearly demonstrated the hierarchical structural characteristic of supramolecular gels, and explained the self-assembly based on XRD and single-crystal analysis. The gel fibers are self-assembled and stabilized by various interactions such as intra- and intermolecular hydrogen bonding, $\pi-\pi$ stacking, and van der Waals interactions. We also showed the encapsulation of chemopreventive curcumin in the hydrogel, and enzyme-triggered gel degradation was performed to release the encapsulated drug into the solution at physiological temperature. Controlled drug release rate was achieved by manipulating the concentration of enzyme or temperature. The byproducts formed after the gel degradation were characterized and clearly demonstrated the site specificity of degradation of the gelator by enzyme catalysis. Supramolecular chemistry is now a powerful strategy for developing new molecularly defined materials in material and medicinal science. This would be a possible model system for drug encapsulation and enzyme-mediated delivery for in vivo formulations and may have potential applications in pharmaceutical research and molecular design of value-added products from biobased materials, otherwise underutilized.

Experimental Section

For detailed experimental methods, synthesis procedure, and characterization see Supporting Information.

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Supporting Information Available: Tables and graphs of gelation, drug kinetics, crystallographic data and SEM, ^{1}H NMR, detailed experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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