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Self-assembling peptides as building blocks of functional materials for biomedical applications

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Abstract

Self-assembling peptides have been explored as building blocks to construct functional materials that can be used in a broad range of biomedical applications. This account gives an overview of the materials built from biomolecules and summarizes the cell culture and drug delivery applications of nanofibrous and hydrogel materials formed via self-assembly of peptides. The design flexibility of materials composed of calcium ion-responsive peptides, which offer a wide range of applications from cell culture scaffolds to drug releasing devices, is highlighted.

Keywords: Self-assembling peptide / Biomedical application / cell culture scaffold / drug releasing device

1. Introduction

Biomolecules as Building Blocks for Functional Materials

Biomolecules have played a pivotal role as building blocks in engineering functional materials for biomedical applications including tissue engineering and drug delivery systems [1-3]. These molecules include peptides [2], proteins [4], nucleic acids [5], saccharides [6], and lipids [7] that have assembling properties that can form supramolecular nano- and micro-structures. They also have bioactivities that originate from their native roles, and these features have been utilized for the construction of various nanoscopic and macroscopic materials with designed functions.

One of the major applications of these biomaterials is cell and tissue engineering, where the materials are used as scaffolds for cell cultures to support and regulate cellular behaviours toward its ultimate goal, which is the creation of artificial tissues/organs [8-

11]. Artificially prepared cell-scaffold composites could deliver clinical benefits as transplants in regenerative medicine and could be alternatives to animal testing in disease studies and drug development. Here, the scaffolds provide structural supports where the cells attach, grow, and organize into tissues/organs and biological supports to promote and control cellular activities such as cell adhesion, proliferation, and differentiation (Figure 1) [12-15]. These supportive functions are required to replace the role of the natural scaffold for cells, the extracellular matrix (ECM). Artificial ECMs, therefore, should be designed to imitate the common features of natural ECMs, such as networked nano- and micro-fibrous structures and the high water contents. Some specific features, including biological signals and the mechanical strength of the native scaffolds, can also be mimicked depending on the cell and tissue types [16-18]. ECMs extracted from animals have been used as cell culture substrates with a single protein, such as collagen [19, 20] and fibrin [21, 22], or as substrates that contain multiple proteins such as decellularized scaffolds that are obtained by removing cells from native tissues/organs. Although decellularized scaffolds maintain the intricate ECM compositions and structures of the original tissues/organs [23-25], they have potential risks for infection, unknown contaminants and immunogenicity. High production costs and ethical issues concerning the use of animals are also drawbacks. These disadvantages have caused researchers to study artificial ECMs that comprise chemically defined components, such as biodegradable synthetic polymers [26-29], ceramics [30, 31], and peptides/proteins [32-36], that are produced by chemical synthesis or expression systems. Among these molecules, peptides and proteins are known to form materials that highly resemble natural ECMs and have a low toxicity and low immunogenicity because they are biomolecules that are naturally used as the building blocks of ECMs [37, 38]. Smaller peptides have an advantage over proteins in terms of their large-scale chemical synthesis with no biological origin. The cell-scaffold composites resulting from culturing cells on these artificial ECMs can be transplanted into the body to replace and/or induce regrowth of lost tissues [39, 40]. In addition, these composites can also be used as models for disease studies and drug development because the cells cultured in the 3D environments of ECMs are reported to better mimic the cell morphology and functions in the body compared with cells that are conventionally cultured on 2D plastic plates [41-43]. By combining 3D culturing approaches and stem cell technologies to generate specific cells from the pluripotent stem cells of patients, these cell-scaffold composite models are expected to replace animal models for pathological studies and preclinical drug testing as more human-relevant models that do not have ethical issues [44-46].

Another application of biomolecular materials is for drug delivery systems, where materials carry drug molecules to control the spatiotemporal distribution of therapeutics to increase treatment efficacy and efficiency and decrease undesirable side effects [47-50]. Here, biomolecules form drug delivery cargo, such as nanoparticles and vesicles, which retain drug molecules inside [51-53] and/or deliver therapeutics to specific cells/tissues by exploiting their interactions with other biomolecules in the body [54-57]. Although these carriers for drug delivery applications have also been developed from metals [58,59], ceramics [60, 61], and synthetic polymers [62-65], they have a limited biocompatibility/biodegradability compared with biomolecules and are basically delivered only via "passive targeting," which occurs mainly through blood circulation and leaky vasculatures, known as the enhanced permeability and retention (EPR) effect [66-68]. However, biomolecules have a relatively high biocompatibility/biodegradability that averts undesired reactions, such as strong inflammation and tissue damage, and can also be delivered via "active targeting" that utilizes interactions between cell-/tissue-specific ligands and receptors [69-71]. Both biological and nonbiological molecules can be fabricated into macroscopic materials, such as hydrogels, which are used as drug releasing devices for implanting in the body to locally and continuously exert therapeutic effects [72-74]. Biomolecules are again preferable in terms of their biocompatibility/biodegradability for implantable devices. These drug delivery systems could be applied to various types of treatments including cancer chemotherapy [75-77], immunotherapy [78-80], and regenerative medicine [81-83].

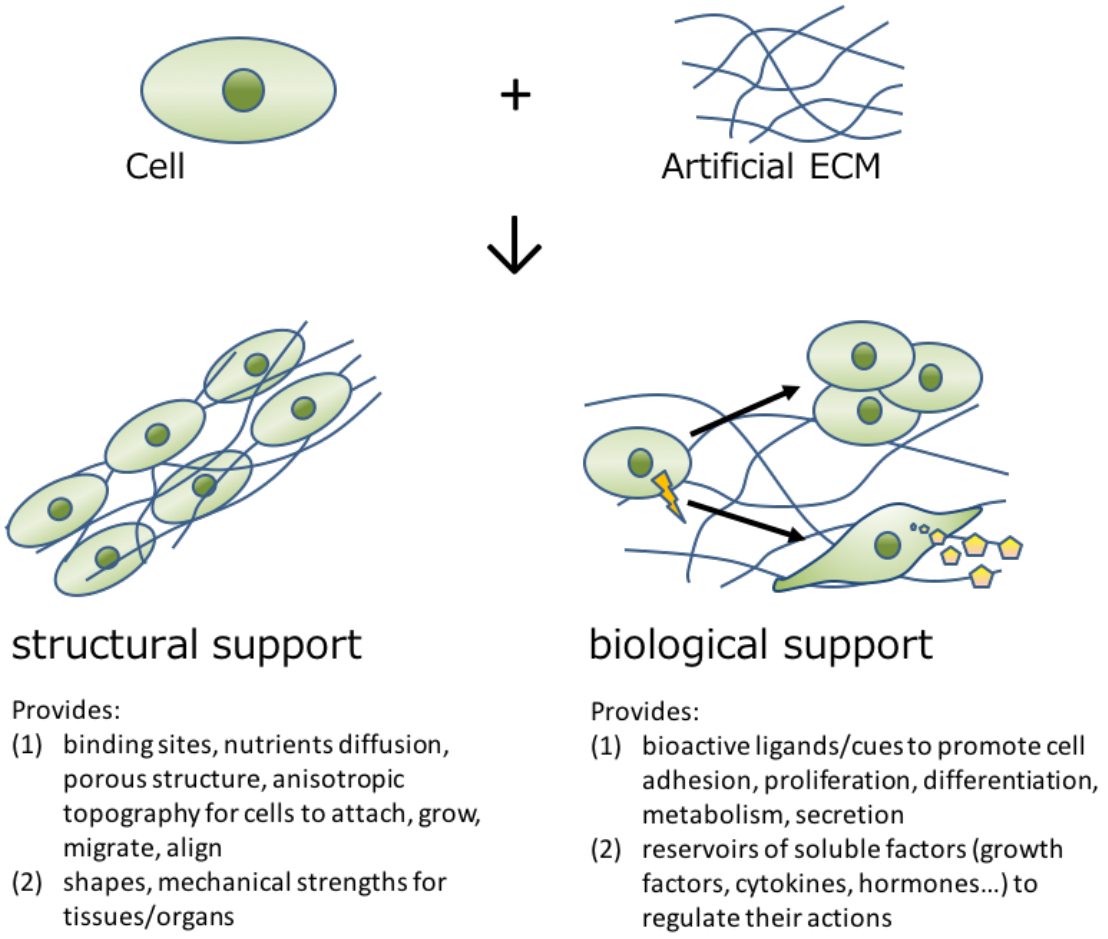

Figure 1. Biomolecular materials for tissue engineering applications: artificial ECMs. Artificial ECMs work as scaffolds that provide both structural and biological supportive functions for cells to regulate cellular behaviors and generate artificial tissues/organs. The yellow thunder and pentagon symbols express signals from ECMs to the cells and soluble factors released from the cells, respectively.

2. Self-Assembling Peptides

Self-assembling peptides are promising biomolecules as building blocks for constructing materials for the biomedical applications described above because of their good biocompatibility, ability to form a variety of nanostructures, and design flexibility to produce molecular diversity (Figure 2) [84-96]. These peptides form various nanostructures, such as nanofibers [97-99], nanotubes [100-104], nanospheres [105-108], and nanoparticles [109, 113], via supramolecular assembly. The assembling properties depend on their amino acid sequences, and a wide variety of self-assembling peptides

have been designed to fabricate nanoscopic and macroscopic materials for molecular arrays [114-116], molecular electronics [117, 118], antibacterial/antitumor agents [119-121], cell and tissue engineering [122-125], and drug delivery applications [126-128]. In particular, many of them that assemble into networked nanofibers in water mimic the fibrous structures of natural ECMs and form hydrogels as artificial ECMs for cell culture and tissue engineering applications. Peptide hydrogels have a high biocompatibility and biodegradability similar to protein hydrogels, and the smaller molecular size of peptides enables easy chemical synthesis that ensures simpler production, lower costs and reduced risks of undesirable contaminants for peptide-based materials compared with protein-based materials. Moreover, the supramolecular features have been reported to provide shear-thinning and self-healing properties in which the hydrogels are injectable through a needle for use in vivo applications [129-131]. Thus, the materials comprising self-assembling peptides are useful for biomedical applications such as tissue engineering and drug delivery systems.

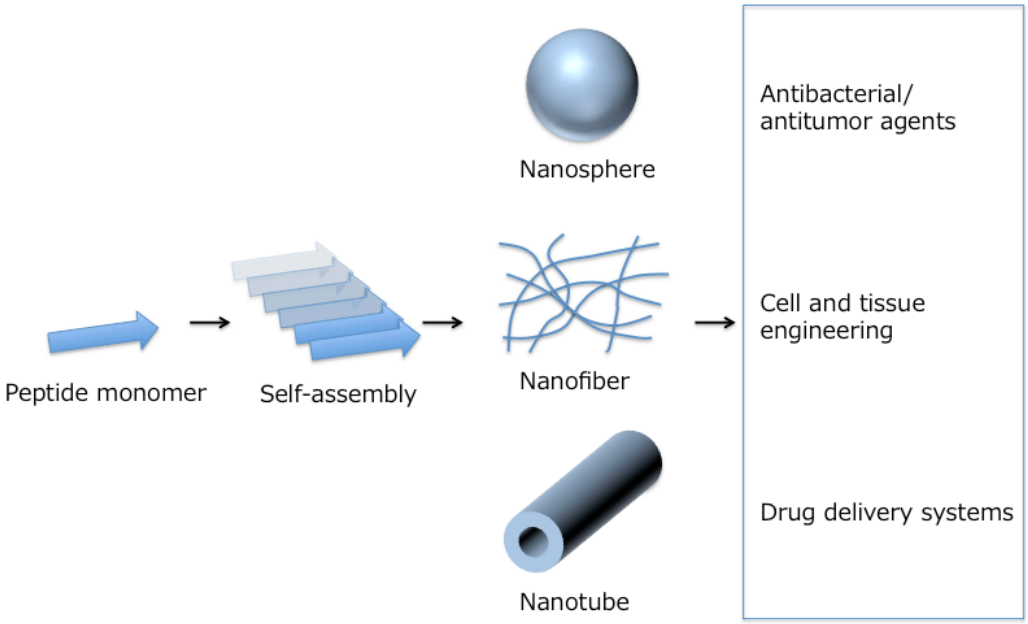

Figure 2. Self-assembling peptides for biomedical applications.

Self-assembling peptides form nanostructures such as nanosphere, nanofiber, and nanotube that work as functional materials for various applications, including antibacterial/antitumor agents, cell and tissue engineering, and drug delivery applications.

E1Y9 Peptide

We have recently developed a de novo designed peptide, E1Y9 (Ac-EYEYKYEYKY-NH_2), which forms networked nanofibers in water by supramolecular assembly and produces hydrogels in the presence of calcium ions [132] (Figure 3). This peptide originated from a peptide named Y9 (Ac-YEYKYEYKY-NH_2), which was found by screening a library of designed peptides that have alternate hydrophobic and hydrophilic residues and self-assembles into nanofibers with a β-sheet conformation [133]. The additional glutamate residue at the N-terminus of E1Y9 appears to play a crucial role in the calcium-ion responsiveness because E1Y9, but not Y9, exhibits bundling of nanofibers and hydrogelation in response to calcium-ion treatment. This suggests cross-linking of the E1Y9 nanofibers via coordination bonds between the carboxyl groups of the N-terminal glutamate side chains and calcium ions. The calcium-ion responsiveness allows fabrication of string- and sphere-shaped hydrogels by injecting E1Y9 solutions via pipette tips into media containing calcium ions. The shaped hydrogels provide cell-compatible environments where cells attach and grow as with other peptide hydrogels. Therefore, E1Y9 hydrogels can work as artificial ECMs that have controlled shapes that would be beneficial for the construction of cell-scaffold composites to imitate the shapes of natural tissues/organs. The E1Y9 hydrogels are stable and keep their shapes during cell culture, suggesting the relatively strong packing of the peptides. More complicated shapes could be fabricated through manufacturing technologies, such as 3D printers, because alginate, the other calcium-ion responsive material, has been employed as a "bioink" to print 3D structures [134-137]. While alginate materials themselves do not supply binding sites for cell adhesion, E1Y9 hydrogels are cell-compatible alternatives that could be combined with alginates to produce cell-adhesive bioinks for 3D printers. Thus, E1Y9 is a promising peptide that can be used to create cell-compatible hydrogel materials that could be useful as artificial ECMs with controlled shapes for cell culture and tissue engineering applications.

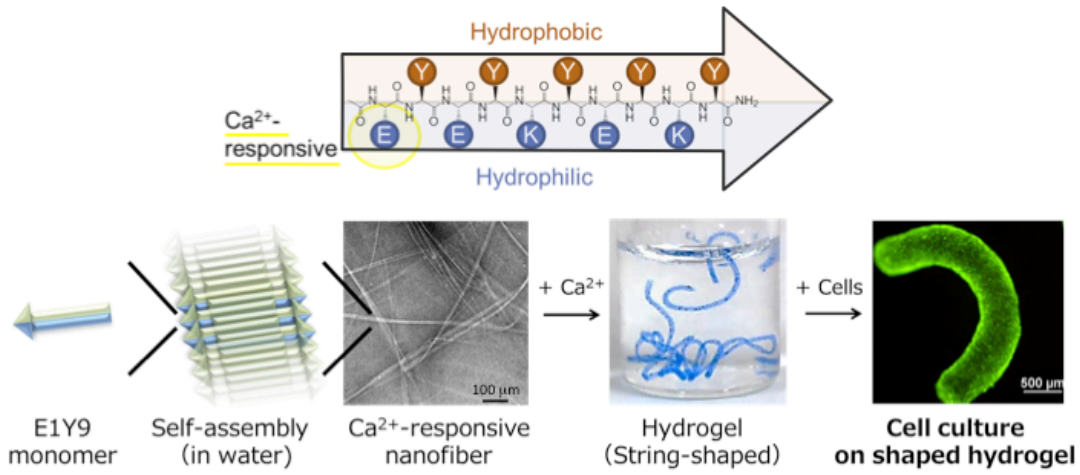

Figure 3. Self-assembly and hydrogelation of E1Y9 peptide.
E1Y9 peptide self-assembles into supramolecular nanofibers in water and forms hydrogels in response to calcium-ion treatment. Shaped hydrogels can be fabricated by injection of E1Y9 solution into medium containing calcium ions, and cells attach and grow on the shaped hydrogels. Thus, E1Y9 hydrogels could be useful as artificial ECMs with controlled shapes for the construction of 3D structures of tissues/organs. Modified from reference [132] with permission from the publisher.

3. E1Y9 Peptide with Functional Moieties for Cell Culture

As described above, the E1Y9 peptide hydrogels with desired shapes may be utilized as artificial ECMs due to their cell compatibility and calcium-ion responsiveness, offering good structural supports for cells. However, to create complex cell-scaffold composites that resemble natural tissues/organs, biological supports that enhance cellular activities, such as cell adhesion and differentiation, are vital for ECM materials. To further functionalize E1Y9 hydrogel materials and to generate artificial ECMs with bioactivities, E1Y9 derivatives that maintain self-assembling properties and have enhanced bioactivities that facilitate cell adhesion and differentiation were designed, synthesized, and employed as scaffolds for cell culture applications [138]. The E1Y9 derivatives were designed to bear bioactive peptides that are derived from natural ECM proteins at the C-terminal of E1Y9. These bioactive motifs have been extensively used to provide bioactivities with a vast range of artificial ECMs that are composed of polymers [139], polysaccharides [140], metals [141], ceramics [142], proteins [143], and peptides [144]. The RGDS sequence in fibronectin is known to bind to integrins [145] and facilitate cell

adhesion when present on the surfaces of materials [146]. The IKVAV sequence derived from laminin reportedly enhances cell adhesion [147], neuronal differentiation [148], and angiogenesis [149]. Thus, an E1Y9 derivative conjugated with the RGDS sequence at the C-terminus via a GGG linker, namely E1Y9-RGDS (Ac-EYEYKYEYKY-GGG-RGDS-NH_2), and an E1Y9 derivative containing the IKVAV sequence, namely E1Y9-IKVAV (Ac-EYEYKYEYKY-GGG-IKVAV-NH_2) were designed to promote cell adhesion and differentiation, respectively. These modified peptides self-assembled into networked nanofibers in a similar manner to E1Y9 with β-sheet secondary structures, indicating the robust self-assembly of the E1Y9 sequence. Polystyrene surfaces coated with E1Y9-RGDS nanofibers exhibited the superior cell adhesion of 3T3-L1 fibroblasts to surfaces coated with E1Y9 nanofibers (Figure 4A). E1Y9-IKVAV-coated substrates exhibited facilitated neuronal differentiation of PC12 cells compared with E1Y9-coated substrates (Figure 4B). Therefore, functionalized E1Y9 derivatives with bioactive moieties can form stable nanofibers and promote cellular activities. Disk- and string-shaped hydrogels comprised of E1Y9 and E1Y9-IKVAV were then fabricated as artificial ECMs with enhanced bioactivity to facilitate neuronal cell differentiation [150]. E1Y9 and E1Y9-IKVAV co-self-assembled into networked nanofibers in water and produced hydrogels in response to calcium ion stimulus, indicating that the mixed E1Y9 derivative does not disrupt the hydrogelation. PC12 cells were cultured on the disk-shaped hydrogels, and hydrogels with appropriate E1Y9-IKVAV contents efficaciously promoted their neuronal differentiation (Figure 4C). Hydrogels comprised of E1Y9 and a scrambled control of E1Y9-IKVAV, namely E1Y9-VVIAK (Ac-EYEYKYEYKY-GGG-VVIAK-NH_2), did not significantly enhance the neuronal differentiation, indicating that the specific interaction between the IKVAV ligand and cell surface receptors is critical for facilitating cell differentiation on E1Y9/E1Y9-IKVAV mixed hydrogels. String-shaped hydrogels containing E1Y9-IKVAV also promoted cell differentiation in an IKVAV density-dependent manner, and the hydrogel with 25% E1Y9-IKVAV contents exhibited the highest activity. Moreover, on the string-shaped hydrogels, PC12 cells extended their neurites along the long axis of the string shape, suggesting that cellular alignment may be controlled by the hydrogel shape (Figure 4D). Thus, these peptide hydrogels with bioactive motifs and desired shapes could be useful as highly functionalized artificial ECMs for regulating multiple cellular behaviors, such as cell differentiation and cellular orientation. Furthermore, E1Y9 peptide hydrogels mixed with E1Y9 and other bioactive sequences have also been reported to facilitate bone cell maturation such as osteoblastic growth and differentiation [151].

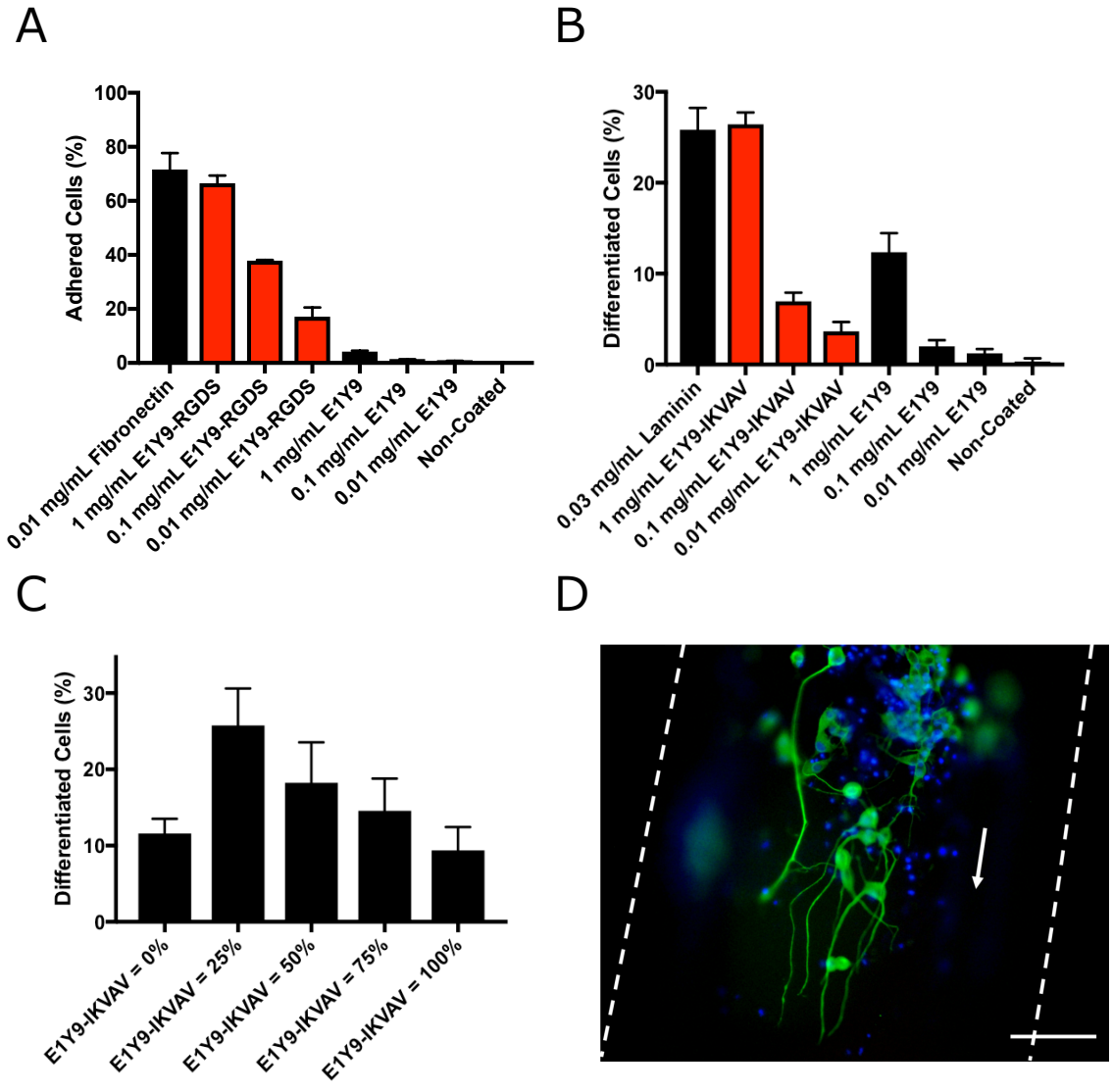

Figure 4. Cell culture on E1Y9 materials.

E1Y9 materials modified with ECM-derived peptides exhibit enhanced bioactivities. (A) Percentages of 3T3-L1 cells well adhered to substrates coated with different concentrations of E1Y9, E1Y9-RGDS, or fibronectin (n = 3, mean ± SD). (B) Percentages of neuronally differentiated PC12 cells that have neurites longer than cell body diameter on substrates coated with different concentrations of E1Y9, E1Y9-IKVAV, or laminin (n = 5, mean ± SD). (C) Percentages of neuronally differentiated PC12 cells that have neurites longer than cell body diameter on disk-shaped E1Y9/E1Y9-IKVAV mixed hydrogels with various contents of the E1Y9 derivative (n = 3, mean ± SD). (D) Neuronally differentiated PC12 cells extend neurites along the long axis of the string-

shape when they are cultured on string-shaped E1Y9/E1Y9-IKVAV mixed hydrogels (Bar = 100 μm). Cells were immunostained with anti-β-tubulin (green) and conterstained with DAPI (blue). White dashed lines indicate the edges of the strings. Modified from reference [139] and [140] with permission from the publisher.

4. **E1Y9 Peptide Hydrogels as Drug Releasing Devices**

In addition to the shaped hydrogels being fabricated from E1Y9 and E1Y9 derivative peptides to investigate their performance as artificial ECMs, the hydrogel materials have been further developed as injectable drug releasing devices for the local delivery of therapeutics, which is a new application of the E1Y9 peptide (Figure 5). Considering the calcium-ion rich environments of extracellular fluids [152], it is hypothesized that an E1Y9 solution mixed with drug molecules can be injected into the body to form a drug releasing hydrogel at the injection site to exploit the calcium-ion responsiveness of the peptide. This strategy improves the efficacy and efficiency of the released therapeutic because this type of localized delivery is known to maximize the treatment effects via a continuous slow release and is known to reduce adverse side effects by minimizing systemic exposure [153–155]. In addition, drug delivery devices that continuously release therapeutic molecules over a long period will allow a decrease in the injection times compared with conventional periodic injections, reducing patient stress and risk of infection related to drug injection [156]. Various implantable devices to deliver drugs have been created from synthetic polymers [157, 158], ceramics [159, 160], proteins [161, 162], and peptides [163, 164]. E1Y9 hydrogels were expected to be effective drug releasing devices that are injectable through syringe needles, and therefore do not require surgical implantation to be placed inside the body. Moreover, their drug releasing properties were anticipated to be easily tuned for each drug molecule and medical condition, by designing and mixing E1Y9 derivatives, to enable the most effective treatment. Hence, an E1Y9 derivative conjugated with a hydrophilic moiety, namely E1Y9-KGES (Ac-EYEYKYEYKY-GGG-KGES-NH_2), was designed to modify the releasing profile of doxorubicin, a hydrophobic chemotherapeutic agent, from E1Y9/E1Y9-KGES mixed hydrogels. E1Y9-KGES maintains self-assembling ability of E1Y9 and formed hydrogels in response to calcium ion treatment, although it displayed different appearance of the self-assembled nanostructure, flake-like structures comprising shorter nanofibrils. E1Y9/E1Y9-KGES mixed solutions containing doxorubicin can be injected into medium with calcium ions to form doxorubicin-releasing hydrogels (Figure 6A), and the maximum released amount of doxorubicin molecules from the hydrogels was increased as the E1Y9-KGES contents increased (Figure 6B). E1Y9/E1Y9-KGES

mixed hydrogels with doxorubicin have shear-thinning property and can therefore be loaded onto and injected through syringes. This property could be employed for injections into the sites that have poor or no calcium ions. E1Y9 and E1Y9-KGES hydrogels containing doxorubicin were then peritumorally injected on a syngeneic tumor mouse model, a BALB/c mouse bearing CT 26 tumor. Mice treated with the single injection of the E1Y9 hydrogel formulation exhibited significantly delayed tumor growth compared to mice treated with free doxorubicin and the E1Y9-KGES formulation (Figure 6C). 24 h after injection, hydrogels and skins at the injection sites were collected to quantify in vivo retention of doxorubicin molecules, and the E1Y9 formulation displayed significantly enhanced retention of doxorubicin (22%) compared to the E1Y9-KGES formulation (12%) and free doxorubicin (13%) (Figure 6D). Therefore, the local chemotherapy by the E1Y9 hydrogel formulation of doxorubicin improved the efficacy of the doxorubicin treatment, indicating that the improved retention of doxorubicin molecules and their gradual release at the injection site may raise the concentration of doxorubicin in the tumor milieu for prolonged time period to effectively kill the tumor cells. Thus, E1Y9 hydrogel materials would be promising injectable drug releasing devices to locally deliver therapeutics, and the drug releasing properties could be controlled to maximize the treatment effects, by designing and mixing E1Y9 derivatives that alter physical properties of hydrogels and/or have binding affinity for the drug molecules.

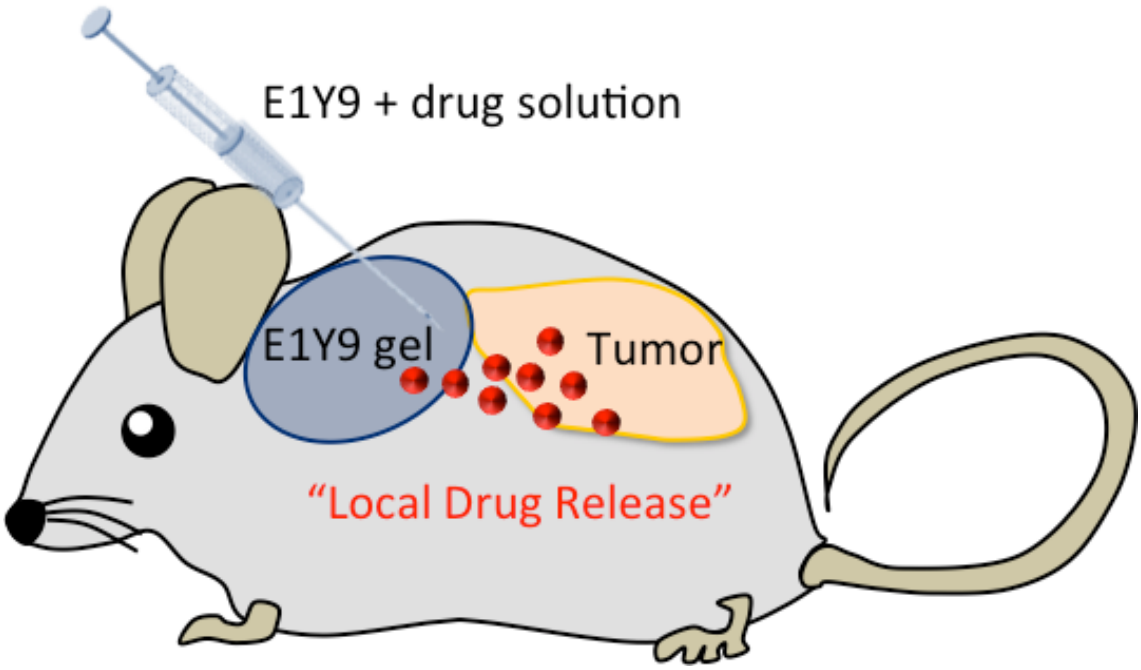

Figure 5. E1Y9 hydrogels as injectable drug delivery devices.
E1Y9/drug mixed solutions are injected into the body, for example, peri-tumorally, and they form hydrogels in response to calcium-ion rich environments. The hydrogels then gradually release the therapeutics (represented by red circles) at the injection site. The localized release would increase the concentration of the drug molecules at the injection site over a certain period of time to have effective treatment, and would decrease systemic exposure of the drug to avoid treatment-related side effects.

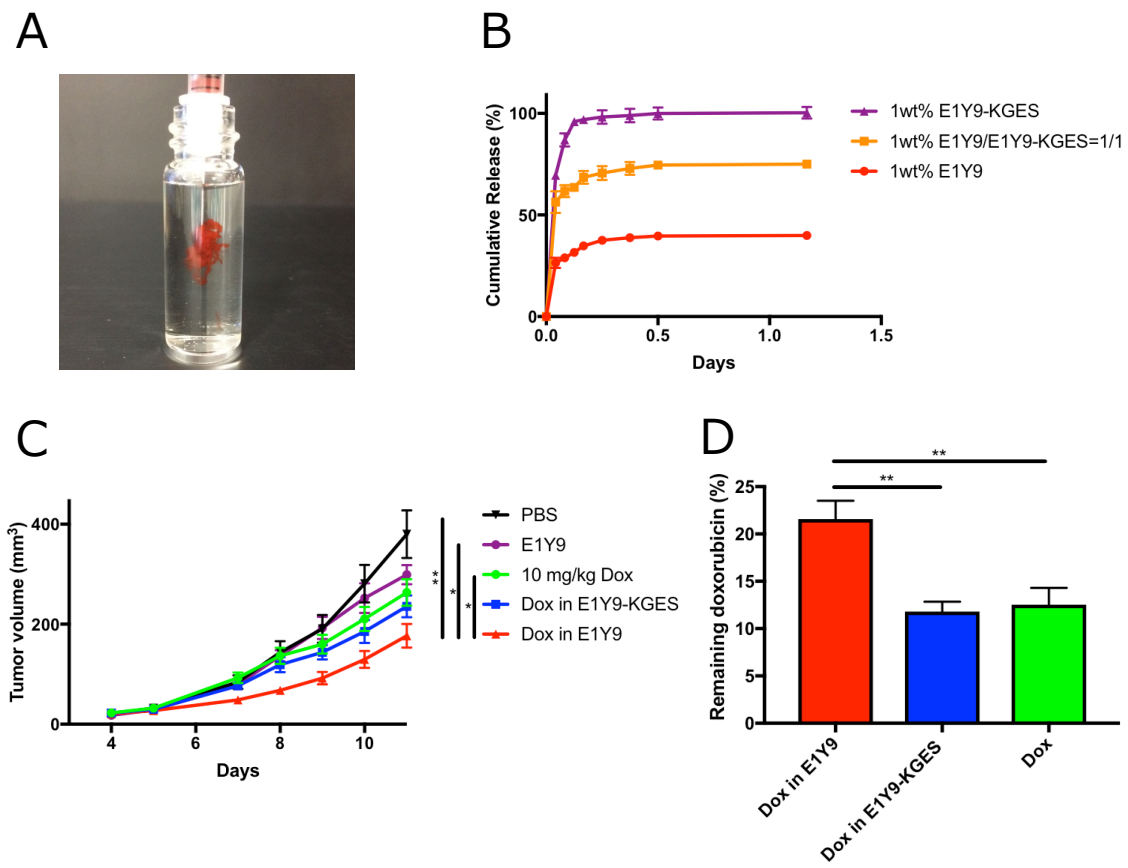

Figure 6. Local chemotherapy with injectable E1Y9/Drug mixed hydrogels.
E1Y9/doxorubicin mixed hydrogels serve as an injectable drug delivery device for local chemotherapy. (A) E1Y9/doxorubicin mixed solution forms a hydrogel when injected into medium containing 2 mM calcium ions via a syringe needle. (B) E1Y9/E1Y9-KGES mixed hydrogels (1 wt% peptide content) with doxorubicin (E1Y9-KGES = 0, 50, and 100%) gradually release doxorubicin in the medium that contains calcium ions. (n = 3, mean ± SD) . (C) Single peritumoral injection (on day 5) of 10 mg/kg doxorubicin with 1 wt% E1Y9 delayed tumor growth on mice inoculated with CT26 tumor (5×10^5 cells on day 1), compared with injections of doxorubicin with PBS or with 1 wt% E1Y9-KGES (n = 10 (Dox in E1Y9, E1Y9-KGES), n = 9 (PBS, Dox in PBS), or n = 7 (E1Y9 alone), mean ± SEM). (D) Single peritumoral injection (on day 5) of doxorubicin with E1Y9 exhibits enhanced retention around the injected site (on day 6), compared with injections of doxorubicin with PBS or with E1Y9-KGES (n = 4, mean ± SEM). Statistical analyses were done using one-way ANOVA with Tukey's post-hoc test. *$P < 0.05$, **$P < 0.01$. The data presented in this figure are original and unpublished.

Conclusion

Self-assembly of designed peptides has been employed to create various nanoscopic and macroscopic materials for biomedical applications. Nanofibrous and hydrogel materials composed of self-assembling peptides closely resemble the environments of natural ECMs and are therefore useful as artificial ECMs for cell culture and tissue engineering applications. Peptide hydrogels are also exploitable as drug delivery devices that retain and release drug molecules to maximize therapeutic effects and to minimize adverse side effects. E1Y9, a de novo designed self-assembling peptide that forms β-sheet nanofibers in water, produces hydrogel materials in the presence of calcium ions. E1Y9 hydrogels have a cell-compatible environment where cells can attach and grow, and they can easily be shaped into desirable structures via a calcium-ion treatment, providing artificial ECMs with controlled shapes. E1Y9 derivatives conjugated with bioactive moieties facilitate cellular activities, such as cell adhesion and differentiation, when they are mixed with E1Y9 to form functionalized artificial ECMs. Moreover, E1Y9 peptides and drug molecules can be mixed to form drug-releasing hydrogels that are injectable to locally deliver therapeutics and to enhance treatment effects. Thus, self-assembling peptide materials are beneficial for a broad range of biomedical applications.

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