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Development of a biocompatible and biodegradable hybrid hydrogel platform for sustained release of ionic drugs

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In this study, we reported the development of a new drug encapsulation strategy and a robust hybrid hydrogel platform for controlled and sustained release of small and large molecule ionic drugs. A biodegradable, biocompatible and temperature stimuli responsive hybrid hydrogel platform was fabricated from arginine based unsaturated poly (ester amides) (Arg-UPEA), Pluronic diacrylate (Pluronic-DA) and alginate by UV photo-crosslinking method, combining the favorable properties of hydrogel and polyelectrolyte complex. The hydrogels were systematically characterized, including the swelling mechanics, mechanical property, biodegradation and interior morphology. *In vitro* biocompatibility study showed that the hydrogels could support the cell attachment and proliferation well. Some model drugs, such as hydrochloride salts of hydralazine, insulin and interleukin-12, were encapsulated into the hydrogels and the drug release behavior was investigated using HPLC, LC-MS, BCA assay and ELISA assay. The obtained release profiles indicated that the Pluronic/Arg-UPEA/alginate hybrid hydrogels could release ionic drugs over weeks *in vitro* via a sustained manner. Hydrogels' structure-function study indicated that the polymer structure, hydrogel composition and environmental temperature had strong effects on the hydrogel property and their drug release profiles.

Introduction

Different from microspheres and nanoparticles[1-4], hydrogels have attracted wide interests for controlled drug release because of their biocompatibility, high water content, organic solvent free environment and permeability of molecules[5-12]. Other advantages of hydrogel for drug delivery include easy and effective drug encapsulation and enhanced stability of many types of proteins or nucleic acids[6]. Furthermore, for the environmental stimuli responsive hydrogels, their drug release behavior could be regulated by environmental stimuli, such as temperature or pH changes[8, 13, 14]. However, for most of the hydrogels, due to the large interior pore size and hydrophilicity, their releasing rates of ionic drugs (small and large molecules, including proteins and nucleic acids) are normally rapid due to the 3D microporous structure of hydrogel and fast diffusion of drug molecules in aqueous environment. Some strategies or systems have been developed for this shortcoming, such as cyclodextrin[15] based hydrogels. But this kind of system normally has relative strict requirement for the drug types. Therefore, there is still a high demand for development of new functional hydrogels that could release ionic drugs without many drug size/type requirements (small or large molecules) through a sustained and controllable manner while maximally maintaining the original hydrogel properties.

A potential solution is to introduce a secondary functional component or system into the hydrogels. One example is the introduction of microspheres or nanoparticles into hydrogels for drug delivery applications[16-19]. However, the reported systems still had some limitations, such as the non-satisfactory loading efficacy and release performance[16-19]. Based on the charged nature of ionic drugs, polyelectrolyte complex (PEC) has been considered as one of the suitable candidates due to the reported ability to suppress the initial burst release and have extended release behavior for proteins and other charged drugs[20-23]. Recently, PECs have been incorporated into hydrogels and the resulting hybrid hydrogels could deliver protein in a controlled and sustained manner[24, 25]. However, for many reported PEC hydrogel systems, some challenges still exist, including

biocompatibility, biodegradability, manufacturing difficulty and drug loading efficiency.

In this study, for sustainable and controllable release of ionic drugs, especially for the small molecule ionic drugs, a multi-functional hybrid hydrogel system was developed, containing cationic arginine based unsaturated poly (ester amides) (Arg-UPEA), anionic alginate and thermo-responsive Pluronic. The hydrogel was fabricated from the mixture of Pluronic-DA, alginate and Arg-UPEA aqueous solutions by UV photo crosslinking. Photo crosslinking has been widely used to prepare hydrogels [6-11] and it could realize better spatial and temporal control over the reaction and gelation reactions can be performed under very mild conditions, including aqueous solution, room temperature, body pH, and even *in situ* in a minimally invasive manner. Pluronics are biocompatible polymers with lower critical solution temperature (LCST) behavior [26-29] and Pluronic based hydrogels have shown to retain their temperature responsive property[26-29]. Anionic alginate has excellent biocompatibility and has been widely fabricated into hydrogels or utilized as one component of many PEC systems[30]. Arg-UPEAs are newly developed cationic, water soluble and biodegradable polymers and Arg-UPEA based hydrogels have shown excellent biocompatibility[31-34]. However, its drug delivery applications have never been reported. In this study, Arg-UPEA would not only crosslink with Pluronic-DA to form the hydrogel scaffold, but also bring positive charge to the resulting hybrid hydrogels and interact with anionic alginate inside the hydrogel to form a 3-D macro PEC hydrogel. A small molecule ionic drug, hydrochloride salts of hydralazine (Figure S1), was used as a model drug since it has been used for cardiovascular therapy and wound healing[35]. Insulin and IL-12 (an anti-tumor cytokine) were also selected in this study as the model protein drug. IL-12, insulin and hydralazine were pre-loaded into the hydrogels before photo-crosslinking. Then various hydrogel formulations have been characterized in terms of *in vitro* release kinetics. In addition, the physicochemical properties and biocompatibility of the hydrogels were also systematically evaluated.

Experimental

Materials

Pluronic (F127, MW 12,600 and 70 % PEG content), acryloyl chloride, triethylamine, sodium alginate, hydrochloride salt of hydralazine and insulin were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Mouse IL-12 and mouse IL-12 ELISA kit were purchased from R&D systems. Water soluble UV initiator: 2-Hydroxy-l- [4-(hydroxyethoxy) phenyl]-2-methyl-l-propanone (Irgacure 2959), L-Arginine (L-Arg), p-toluenesulfonic acid monohydrate, fumaryl chloride, ethylene glycol, 1, 4-butanediol and *p*-nitrophenol were all purchased from Alfa Aesar (Ward Hill, MA) and used without further purification. Other chemicals and reagents if not otherwise specified were purchased from Sigma-Aldrich (St. Louis, MO).

Synthesis of Hydrogel Precursors

Pluronic-DA (F127-DA) precursors were synthesized according to a previously reported method[28]. The Arg-UPEAs were synthesized by the same procedures reported before[31, 32]. Briefly, the synthesis of Arg-UPEAs could be divided into the following three steps: the preparation of unsaturated di-*p*-nitrophenyl ester of dicarboxylic acid (**I**); the preparation of tetra-*p*-toluenesulfonic acid salts of bis (L-arginine), α, ω-alkylene diesters (**II**); and the synthesis of Arg-UPEAs (**III**) via solution polycondensation of (**I**) and (**II**). For the specific Arg-UPEA, it's named as x-UArg-y-z, while x and y is the CH_2 number of diacid and diol part of the polymer repeating unit, respectively. And z means the salt type. The more details about the Arg-UPEA could be found in previous report[31]. The Arg-UPEA and Pluronic-DA precursors were purified first by dissolving the precursors in distilled water and were dialyzed against deionized water (MW cut off 4,000) under dark environment for 2 days. After that, the solutions were lyophilized for 2 days using a Virtis Freeze Drier (Gardiner, NY) under vacuum at -48 °C.

Preparation of Hydrogels

Arg-UPEA/Pluronic-DA/alginate hybrid hydrogels were prepared by the photo-polymerization of two precursors (Arg-UPEA and Pluronic-DA) at different weight ratios in aqueous solution with alginate and initiator. An example of the fabrication of a hybrid hydrogel using 2-UArg-2-S (S means the salt type is *p*-toluenesulfonic acid type) as Arg-UPEA was given below: 0.10 g of 2-UArg-2-S, 0.40 g F127-DA and 5.0 mg of photoinitiator Irgacure 2959 (1.0 wt % of total amount of precursors) were added into a glass vial and dissolved in 2.0 mL of deionized water to form a clear homogeneous solution. Then 1.0 ml 4.0 wt% alginate deionized water solution was added into the precursors' solution and mixed well. The solution mixture was then transferred to a custom-made 20 well Teflon mold (diameter 12 mm and thickness ≈ 4 mm for each well). Then the precursor solution in the mold was irradiated by a long-wavelength UV lamp (365 nm, 100 W) for specified time (normally 2-5 min) at room temperature. The irradiation distance is 5-10 cm. After fabrication, the resultant hydrogels were moved from the mold and dried in vacuum at room temperature for 24 hours before further characterization. For drug loaded hydrogels, pre-determined amount of drugs were mixed with alginate solution first and then the mixture was added to the precursor solution before photo-crosslinking. All other fabrication conditions were same.

Equilibrium Swelling Ratio & Swelling Kinetics of Hydrogels

The equilibrium swelling ratio (Q_{eq}) of the hydrogel is calculated by the following equation: $Q_{eq} = [(W_e - W_d)/ W_d] \times 100$ %, where W_e is the weight of a swollen hydrogel at equilibrium and W_d is the weight of the corresponding dry hydrogel at t = 0. All swelling ratio results were obtained from triplicate samples and data were expressed as the means ± standard deviation. The swelling kinetics of the Arg-

UPEA/F127-DA/alginate hydrogels was measured over a period of 4 days at room temperature. Each dry Arg-UPEA/F127-DA/alginate hydrogel sample was weighed and immersed in 20 mL of solutions with different parameters for predetermined periods. Before weighing, the samples were taken from the solutions and blotted with filter papers to remove excess surface water. The swelling ratio (Q) of the hydrogel at time t is calculated by the following equation: $Q = [(W_t - W_d)/ W_d] \times 100$ %. W_t is the weight of the corresponding dry hydrogel at time t.

Compressive Modulus of Hydrogels

The mechanical property of the Arg-UPEA/F127-DA/alginate hydrogels was measured by a 2980 Dynamic Mechanical Analyzer (DMA) (TA Instruments Inc., New Castle, DE) in a ''controlled force'' (CF-mode) mode. The swollen hydrogel samples in circular disc shape were submerged in distilled water and mounted between the movable compression clamp (diameter 30 mm) and the fluid cup with a 0.1 N preloading force. A force ramp from 0.1 N at a rate of 0.3 or 0.5 N/min was applied. All measurements were carried out at room temperature. The compression elastic modulus (E) of the swollen hydrogel was extracted by plotting the compressive tress versus strain. All compression elastic modulus data in this study were obtained from triplicate samples and data were expressed as the means ± standard deviation.

***In vitro* Enzymatic Biodegradation of Hydrogels**

The *in vitro* biodegradation of the Arg-PEA/F127/alginate hybrid hydrogels was evaluated. Briefly, freshly prepared and purified hydrogel was placed into a glass vial containing 10 mL PBS buffer (pH 7.4, 0.067 M) or 0.1 mg/mL trypsin in 10.0 mL PBS buffer. The hydrogel was then incubated at 37 °C with a constant reciprocal shaking (ca. 100 rpm). The trypsin solution was changed every other day and at each measuring time point. At the end of predetermined period, the hydrogels were removed (or collected by filtration if broken into small parts), then washed with 10 mL distilled water for 5 minutes for 3 times, and then dried in freeze drying machine for 24 h to completely remove the residue water.

Interior Morphology of Hydrogels

Interior morphology of Arg-UPEA/Pluronic-DA/alginate hydrogels was investigated by scanning electron microscope (SEM). The swollen hydrogel samples, after reaching their maximum swelling ratio in distilled water at room temperature, were quickly frozen in liquid nitrogen and then freeze-dried under vacuum at -48 °C for 3 days until all water inside the hydrogel was sublimed. The freeze-dried hydrogel samples were then cut and fixed on aluminum stubs and then coated with gold for 30 seconds for interior morphology observation with a SEM instrument (Leica S440, Germany).

Cell attachment and proliferation on hydrogel surface

The cell attachment on the Arg-UPEA/F127-DA/alginate hybrid hydrogel surface was evaluated and pure F127-DA hydrogel was selected as the hydrogel control. The cells used for this study were BAECs and Detroit 539 human fibroblast. The purified swollen hydrogels were cut into round shape with the diameter that just filled the well of 24-well cell culture plates at 37 °C in cell culture media. Before being put into the 24-well cell culture plates, the hydrogels were sterilized under UV light (in the cell culture hood) for 2 h. After that, the hydrogels were washed twice by PBS buffer and cell culture media. Then, the hydrogels were placed into the wells of the cell culture plate and fixed by sterilized rubber ring which has the same diameter as the well of cell culture plate. BAECs or fibroblast cells were seeded at an appropriate cell density (10,000 cells/well) and incubated overnight. After 48 h incubation, the cell attachment and proliferation on the hydrogel surface was record by an optical microscope.

Controlled Release of Ionic Drugs via Hydrogels

For hydralazine, its release from the Arg-UPEA/Pluronic-DA/alginate hydrogels was carried out in a PBS buffer at 37 °C or room temperature. Hydralazine was preloaded into the hydrogel samples according to the following protocol: pre-determined amount of hydralazine was directly mixed alginate first, the mix with Arg-UPEA, Pluronic-DA precursors and photo-initiators in distilled water, and the solution mixture was UV irradiated for 3-5 mins to form drug loaded hydrogels. The drug loaded hydrogels were then placed inside small vials containing 10.0 mL PBS solution (one piece of hydrogel per vial). The vial was incubated at 37 °C or room temperature with a constant reciprocal shaking (ca. 100 rmp). The hydralazine contents were then analyzed by a high performance liquid chromatography (HPLC). The following are the details of measuring hydralazine concentration via HPLC. To determine the release amounts of the drug at predetermined time points, 50 μL of the immersion solution was removed from the vial and added into a HPLC vial followed by adding 950 μL PBS buffer to dilute the solution. The solution was thoroughly vortexed before tested by a HPLC (HP 1100 model, Palo, Alto, CA, USA) equipped with a diode array detector. A C18 reversed phase column (5 um, 4.6X250 nm, Alltech Adsorbosphere XL) was used as the stationary phase, while the mobile phase consisted of acetonitrile and water (pH 3.0) in the volume ratio 63:37. The injection volume was 50 or 100 μL based on the released hydralazine concentration and the mobile phase was pumped at a flow rate of 2.0 mL/min. Detection was at 365 nm with a UV detector. ChemStation (Palo, Alto, CA, USA) software was used for data analysis. All drug release tests at each time point were carried out in triplicate and variation was expressed as a standard error of the mean. The confirmation of hydralazine chemical structure was carried out by GC-MS method and the major diagnostic MS fragments were analyzed. For insulin and IL-12 release study, the released contents were measured by BCA assay and ELISA assay, respectively. All other steps were same as the protocol for hydralazine release study.

Results and Discussion

To achieve sustained release of ionic therapeutics, especially for small molecule ionic drugs, we developed a novel biocompatible and biodegradable hybrid hydrogel platform utilizing a new simple fabrication strategy, which would maximally keep the original hydrogels properties and be suitable for a variety of ionic drugs with different MW range. This hybrid hydrogel system would not only combine the favorable properties of hydrogel and PECs, but also utilize the special characteristics of arginine (high pKa) and Pluronic (temperature responsive). The wide variety of hydrogel components (various precursors) and material parameters (molecular weight, composition ratio, type and degree of cross linking) may allow us to engineer the hydrogels with a wide range of ionic drug release kinetics. And the understanding of these structure-function relationships would be very helpful to develop new generations of hydrogels having finely controllable and sustained drug release performance.

Synthesis of Precursors

In this report, Pluronics were chosen as a precursor component due to their excellent biocompatibility, water solubility, low toxicity, low immunogenicity and temperature responsive property[26-29]. Among a variety of Pluronics, F127 was chosen because of its suitable chain length for chemical modification and hydrogel fabrication. The synthesis scheme of the F127-DA could be found in the supporting information (Figure S2).

Unsaturated di-*p*-nitrophenyl ester of dicarboxylic acid (Monomer **I**, di-*p*-Nitrophenyl Fumarate (NF), x=2) was prepared by reacting fumaryl chloride with *p*-nitrophenol as previously reported[31]. Two types of *p*-toluenesulfonic acid salt of L-arginine diesters (Monomer **II**) were prepared in this study: tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) ethane diesters Arg-**2**-S (y=2); tetra-*p*-toluenesulfonic acid salt of bis (L-arginine) butane diesters, Arg-**4**-S (y=4). Arg-UPEAs were prepared by the solution polycondensation of (**I**) and (**II**) monomers (NF and Arg-2-S, Arg-4-S) at different combinations[31]. The Arg-UPEAs synthesized with different combinations of diacids and diols building blocks were: 2-UArg-2-S, 2-UArg-4-S. The two Arg-UPEAs synthesized here have high water solubility (> 2 wt%) and low hydrophobicity and they would not form visible precipitates with alginate in aqueous solutions. Other Arg-UPEAs with longer CH_2 segment in the repeating unit were not used in this study due to their low water solubility and high hydrophobicity. The synthesis scheme of the monomers and polymers could be found in the supporting information (Figure S3).

Fabrication of Hydrogels

In this report, Pluronic-DA was selected as a co-precursor to facilitate photo-crosslinking of Arg-UPEA with Irgacure 2959 as the photo initiator in an aqueous system. Besides the previously mentioned functions of Pluronic-DA, one more important role of Pluronic-DA in the hydrogel fabrication was to work as a polymer surfactant buffer to reduce the interactions between positively charged Arg-UPEA and negatively charged alginate. The specially designed Arg-UPEAs have good water solubility (> 2.0 wt%) and the molecular weight is below 15.0 KDa. After testing, it's been confirmed that mixing of Arg-UPEAs and alginate would not form visible insoluble or precipitated complexes. And the large amount of the Pluronic-DA would further reduce the interaction and avoid the formation of insoluble or precipitated complexes of Arg-UPEA and alginate. During the mixing step, the Arg-UPEAs would mix with Pluronic-DA first, then the alginate with drug would be added for further mixing. Since the charge density of Arg-UPEA and alginate is around 2.5 mol/kg and 5.7 mol/kg, respectively, the weight ratio of Arg-UPEA to alginate was fixed at 2.5 in this report so that the PEC could be formed well while the net charge of the hydrogel is a little bit positive, which would be good for the cell attachment and proliferation. The naming of the hybrid hydrogel in this study is: Arg-UPEA/F127-DA/alginate (m/n, w/w). The m/n is the weight ratio of Arg-UPEA to F127-DA (the weight ratio of Arg-UPEA/alginate has been fixed at 2.5).

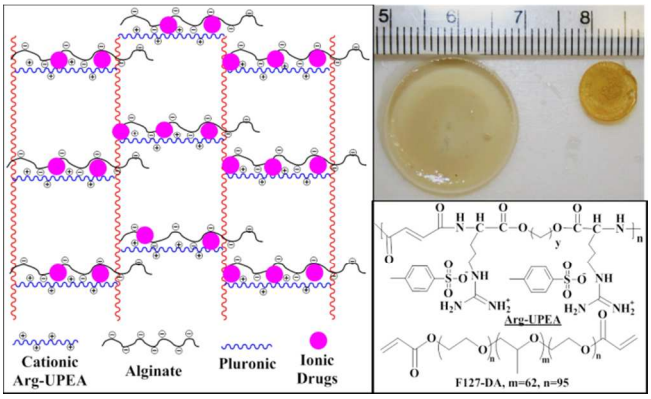

Figure 1, Illustration of the 3-D structure of drug loaded hydrogel (left) and precursors, chemical structure of Arg-UPEA and F127-DA, and the photo image of 2-UArg-2-S/F127-DA/alginate (1:4, w: w) hydrogel (swollen (left) and dry (right))

Photo-crosslinking time was suggested to be as short as possible so that the bioactivity of the therapeutics would not be affected. For this goal, a powerful 100W UV light is used so that the photo-crosslinking could be completed within mins. After optimization, the crosslinking time was set at 2-5 mins, depending on the composition of the hydrogel. More Arg-UPEA component in the hydrogel would need a little bit longer time due to the relative weak reactivity of the double bonds of Arg-UPEA. Figure 1 showed the chemical structure of Arg-UPEA and F127-DA, illustration of 3-D structure of ionic drug loaded hybrid hydrogel (left) and the photo image of a 2-UArg-2-S/F127-DA/alginate hydrogel (swollen (left) and dry (right)). All the fabricated Arg-UPEA/F127-DA/alginate hydrogels in this report were transparent or light white after reaching their swelling equilibrium. The successful fabrication of Arg-

UPEA/F127-DA/alginate hybrid hydrogels was confirmed by elemental analysis (Table 1). By measuring the nitrogen element percentage of dried hydrogels, it was confirmed that most of the Arg-UPEAs were successfully introduced into the hydrogels. The measured N contents in Table 1 were slightly lower than the theoretical value (in the parentheses). The theoretical value was estimated according to the molecular structure of polymer repeating unit and hydrogel composition (before crosslinking).

Equilibrated Swelling Ratio & Swelling Kinetics of Hydrogels

The swelling behavior of the hybrid hydrogels was systematically examined in terms of equilibrated swelling ratio and swelling kinetics and as a function of hydrogel compositions (the weight feed ratio of Arg-UPEA to F127-DA) and y parameter of Arg-UPEA.

Table 1, Arg-UPEA/F127-DA Hybrid Hydrogels and Their Physicochemical Properties

Sample	Compressive Modulus* (kPa)	N (%)
2-UArg-2-S/ F127-DA/alginate (1:4, w/w)	6.23±0.45	2.49 (2.60)
2-UArg-2-S/ F127-DA/alginate (2:3, w/w)	4.02±0.36	4.62 (4.84)
2-UArg-4-S/ F127-DA/alginate (1:4, w/w)	5.68±0.34	2.36 (2.51)
2-UArg-4-S/ F127-DA/alginate (2:3, w/w)	3.54±0.47	4.53 (4.68)

* Compressive modulus was measured in DI water at room temperature.

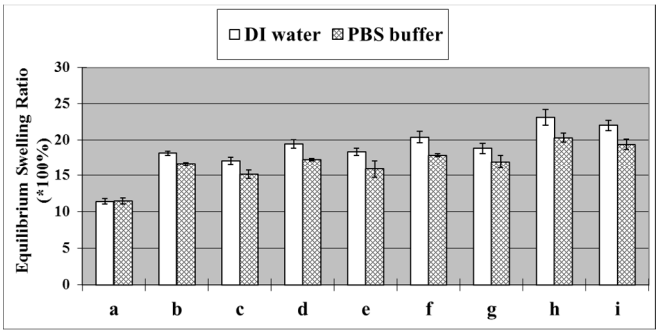

Figure 2, Equilibrated swelling ratio of Arg-UPEA/Pluronic-DA/alginate hydrogels in PBS (pH=7.4) solution and DI water at room temperature. a) Pure F127-DA hydrogel (control); b) 2-U-Arg-2-S/F127-DA (1/4, w/w); c) 2-UArg-2-S/F127-DA/alginate (1/4, w/w); d) 2-U-Arg-4-S/F127-DA (1/4, w/w); e) 2-UArg-4-S/F127-DA/alginate (1/4, w/w); f) 2-U-Arg-2-S/F127-DA (2/3, w/w); g) 2-U-Arg-2-S/F127-DA/alginate (2/3, w/w); h) 2-U-Arg-4-S/F127-DA (2/3, w/w); i) 2-U-Arg-4-S/F127-DA/alginate (2/3, w/w)

For the swelling ratios at equilibrium, they were tested in both deionized water (DI water) and 1X PBS solution. Figure 2 showed the equilibrated swelling ratios at room temperature for the hybrid hydrogels with varied compositions. F127-DA and Arg-UPEA/F127-DA hydrogels were used for comparison. First, the equilibrium swelling ratios of Arg-UPEA/Pluronic-DA/alginate hybrid hydrogels in DI water were always slightly higher than that of the corresponding hydrogels in PBS solution, but no significant difference in equilibrium swelling ratio between PBS and DI media was observed for the pure F127-DA hydrogel. This result suggested that the electrolytes in PBS might interfere with the interaction between water molecules and charged groups in the hybrid hydrogels. Second, the equilibrated swelling ratio of all Arg-UPEA/Pluronic-DA/alginate and Arg-UPEA/Pluronic-DA hybrid hydrogels were higher than that of pure Pluronic-DA hydrogel (1,144 ± 17% and 1,150 ± 40 % in DI water and PBS, respectively), while the Arg-UPEA/Pluronic-DA/alginate hybrid hydrogel showed a little bit smaller equilibrated swelling ratio than that of the corresponding Arg-UPEA/Pluronic-DA hydrogel. These results implied that the incorporation of Arg-UPEA segment did enhance equilibrated swelling ratio of the hydrogels and the introduction of alginate may slightly reduce the equilibrated swelling ratio of the hybrid hydrogels. One of the possible reasons could be due to the electrostatic interaction between the Arg-UPEA and alginate. Third, the equilibrated swelling ratios of the hybrid hydrogels generally increase with an increase in the weight feed ratio of Arg-UPEA to F127-DA either in DI water or PBS solution. For example, in DI water and at room temperature, the hydrogel of 2-UArg-2-S/F127-DA/alginate (1/4, w/w) had an equilibrated swelling ratio of 1711±90%, while the hydrogel of 2-UArg-2-S/F127-DA/alginate (2/3, w/w) had an increased equilibrated swelling ratio of 1889±70%. Fourth, the equilibrated swelling ratios of the hybrid hydrogels increase with an increase of the y value of the Arg-UPEA either in DI water or PBS solution at a constant weight ratio of Arg-UPEA to F127-DA. For example, at the feed weight ratio of 1 to 4, in DI water and at room temperature, from 2-UArg-2-S/F127-DA/alginate (1/4, w/w) to 2-UArg-4-S/F127-DA/alginate (1/4, w/w), the equilibrated swelling ratio increased from 1711±90% to 1843±50%.

Furthermore, the effects of pH and temperature on the equilibrium swelling ratios of the Arg-UPEA/Pluronic-DA/alginate hybrid hydrogels were examined as shown in Figure 3. The preliminary results indicated that the swelling ratios of the hybrid hydrogels were sensitive to the change of pH (Figure 3a). For example, for the hybrid hydrogels at a feed ratio of 1:4, the equilibrium swelling ratio of 2-UArg-2-S/F127-DA at an acidic pH (pH = 4) was much higher than that at a basic condition (pH = 10). However, the introduction of alginate slightly reduced this pH effect on the equilibrium swelling ratios. Figure 3b showed that the swelling ratios of the hybrid hydrogels were also sensitive to the change of environmental temperature. Due to the temperature responsive property of F127, all the hydrogels showed temperature sensitivity: high temperature would cause the shrinking of hydrogel and low temperature would cause the swelling of hydrogel. The introduction Arg-UPEA and alginate slightly

reduced the temperature sensitivity of the hydrogel since the percentage of F127-DA decreased slightly after the introduction, and the whole decreasing trends did not show obvious changes.

The swelling kinetics of the Arg-UPEA/Pluronic-DA/alginate hydrogel was studied over a period of 4 days in DI water at room temperature. For example, Figure 4 showed the hydrogels of varied compositions (different types of Arg-UPEAs or feed weight ratio of Arg-UPEA to F127-DA) had a high swelling rate during the initial 3 hours. After the initial 3 hours, the swelling rate levelled off, and finally reached their swelling equilibrium within about 12-18 hours. Compared with the pure F127-DA hydrogel and Arg-UPEA/Pluronic-DA hydrogel[31], the corresponding Arg-UPEA/Pluronic-DA/alginate hydrogel did not show obvious swelling rate difference.

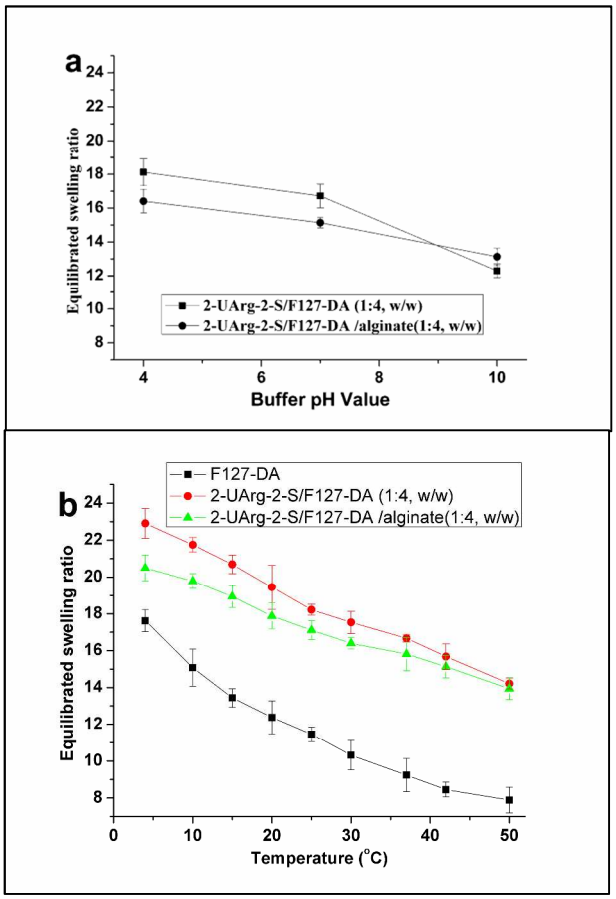

Figure 3, Equilibrium swelling ratios of the hybrid hydrogels in aqueous solution as a function of the pH value (a, at room temperature) and temperature value (b, in DI water)

Biodegradation of Hydrogels

Figure 5 showed some biodegradation results of 2-UArg-2-S/F127-DA/alginate hydrogels. In PBS buffer, without enzyme, the hydrogels showed no obvious degradation within 1 month. Therefore, in PBS buffer, the biodegradation was slow and would not affect the drug release mechanism greatly. However, the 0.1 mg/mL trypsin enzyme would help to degrade the hydrogel completely for 2-3 weeks. 2-UArg-2-S/F127-DA/alginate (2:3, w/w) hydrogel showed faster degradation than the 2-UArg-2-S/F127-DA/alginate (1:4, w/w), indicating that more percentage of 2-UArg-2-S/alginate would cause faster degradation. The degradation curves for trypsin treated hydrogels ended before 100% because the degraded hydrogel debris was very hard to collect at that time. With the uncrosslinked but electronic absorbed alginate, the possible physical structure of the hybrid hydrogels could be close to a semi-IPN or double network. Some reports have discussed the enzymatic biodegradation of semi-IPN hydrogels and the degradation behaviour/rate depends on the hydrogel type, composition, enzyme type and concentration[36-38]. For the Arg-UPEA/Pluronic-DA/alginate hybrid hydrogel platform, the biodegradation mechanism is complicated due to the complicated structure and composition of this hybrid hydrogel platform. The role of Pluronic-DA in biodegradation is also important since it's the main component and forms the major part of the hydrogel network. Therefore, further systematic investigation is needed for the details of the enzymatic degradation of this platform.

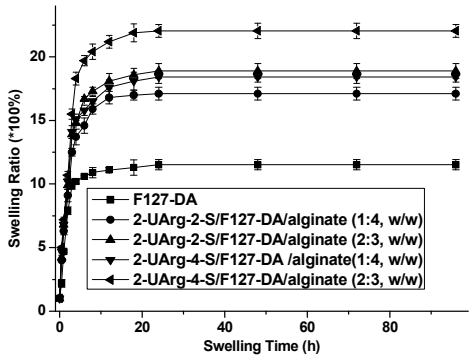

Figure 4, Swelling kinetics of the Arg-UPEA/Pluronic-DA/alginate hydrogel

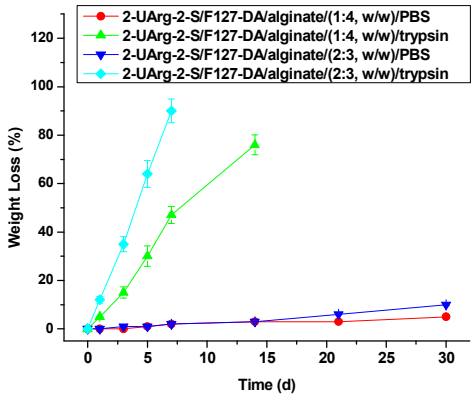

Figure 5, Biodegradation of the Arg-UPEA/Pluronic-DA/alginate hydrogel at 37 °C

Interior Morphology (SEM) of Arg-UPEA/F127-DA Hydrogels

The cross-sectional interior morphology of the freeze dried Arg-UPEA/F127-DA/alginate hydrogel was examined by SEM to further understand the 3-D structure of hybrid hydrogel. Even though the interior structure/morphology of hydrogel after freeze drying would be different from the real state of the swelling hydrogel, it is still a very helpful index for obtaining first hand detail of the hydrogel 3-D interior micro structure. As shown in Figure 6a, compared with Arg-UPEA/F127-DA hydrogels[31], the Arg-UPEA/F127-DA/alginate hydrogel has similar average pore size and cell wall thickness, but a little bit more nanofiber webs entangled with the hydrogel cells. Figure 6b showed an example of the interior morphology change of Arg-UPEA/F127-DA/alginate hybrid hydrogel after 30 days' incubation at 37 °C in PBS buffer.

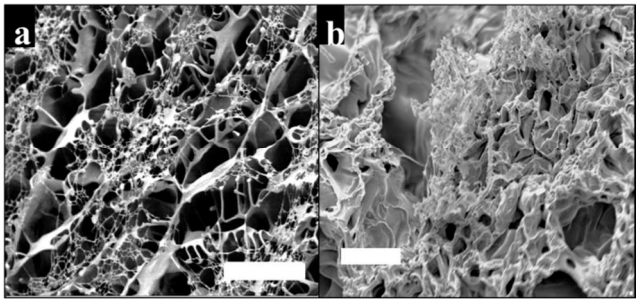

Figure 6, SEM images of freeze dried 2-UArg-2-S/F127-DA/alginate (1:4, w/w) hybrid hydrogel (a: 0 day incubation in PBS; b: 30 days incubation in PBS). White scale bar is 30 μm.

Cell Attachment and Proliferation on Hydrogel Surface

Excellent biocompatibility of hydrogel is a prerequisite for its potential biomedical/clinical applications. Here the cellular interaction with Arg-UPEA/F127-DA/alginate hybrid hydrogels was studied to preliminarily evaluate the biocompatibility of hydrogels. The fibroblast and BAEC cells were cultured on the surface of Arg-UPEA/F127-DA hybrid hydrogels to investigate the cell attachment and proliferation performance. Figures 7a and 7b showed one example of the Detroit 539 human fibroblast cells cultured for 48h on the surface of pure F127-DA hydrogel and 2-UArg-2-S/F127-DA/alginate (1/4, w/w) hybrid hydrogel, respectively. Compared with the pure F127-DA hydrogel control, the hybrid hydrogel had better cell morphology and much higher amounts of the attached/proliferated fibroblast cells after 48 h, and the density and morphology of the attached/ proliferated fibroblast cells were in the same level as the 2-UArg-2-S/F127-DA hydrogel[31]. This result indicated that the introduction of alginate did not change the biocompatibility of the Arg-UPEA/F127-DA hydrogel. Figures 7c and 7d showed another example of the BAEC cells cultured for 48 h on the surface of pure F127-DA hydrogel and 2-UArg-2-S/F127-DA/alginate (1/4, w/w) hybrid hydrogel, respectively. Normally, endothelial cells are more sensitive to the cell culture environment than the fibroblast cells. For the BAEC cell morphology, there is significant morphology change between the F127-DA hydrogel control (Figure 7c) and hybrid hydrogel surface (Figure 7d) after 48 h's culture. For the cells cultured on the hybrid hydrogel surface, there were no visible signs of cell rounding or membrane blebbing which would indicate cell death. The BAEC cells attached onto the pure F127-DA hydrogel surface, however, did show some morphology change and the BAEC cells did not completely attach and spread on the F127-DA hydrogel surface. The light dots are air bubbles. Therefore, the cell culture results indicated the 2-UArg-2-S/F127-DA/alginate could support the cell attachment and proliferation well, which would bring this platform a great potential as a new type of scaffolds for drug delivery applications.

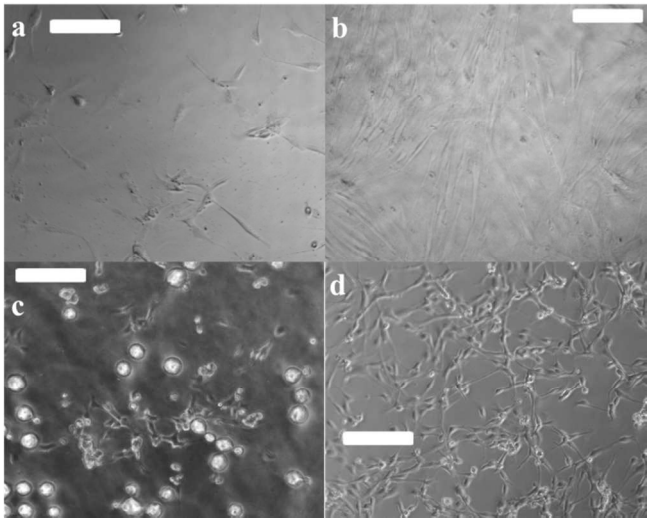

Figure 7, Representative micrographs of Detroit 539 human fibroblast and BAEC cells after 48 h culture on the hydrogel surface, 10x, white scale bar is 200 μm. a) Detroit 539 human fibroblast cells cultured on the surface of pure F127-DA hydrogel; b) Detroit 539 human fibroblast cells cultured on the surface of 2-UArg-2-S/F127-DA/alginate (1/4, w/w) hydrogel; c) BAEC cells cultured on the surface of pure F127-DA hydrogel; d) BAEC cells cultured on the surface of 2-UArg-2-S/F127-DA/alginate (1/4, w/w) hydrogel.

Controlled release of hydralazine, insulin and IL-12 via Arg-UPEA/F127-DA/alginate Hybrid Hydrogels

As we discussed before, the sustained release of ionic drugs, especially small molecule ionic drugs, is still a big challenge for most of the hydrogel system. Combine the unique properties of hydrogel and PEC, this platform is expected to be able to release a variety of ionic drugs in a sustained manner.

Hydralazine (Apresoline, Figure S1), as a model small molecule ionic drug, was preloaded into the hydrogel samples (small pellet). The loading amount of hydralazine was set at 5 or 10 wt% of Arg-UPEA plus alginate. The drug content was analyzed by high performance liquid chromatography (HPLC). The elution time of the hydralazine chlorine salt peak is around 2.35-2.37 mins, which was consistent with the previous reports [39, 40]. The confirmation of the chemical structure of the released hydralazine was carried out by LC-MS method and the major diagnostic MS fragments were analyzed[39, 40]. The result (Table S1) showed that the released hydralazine chlorine salt did not

have any change. A standard curve was made for the calibration (supporting information, Figure S4). The Figure 8a showed some release data of hydralazine via Arg-UPEA/F127-DA/alginate hydrogels in PBS buffer. The release data from pure F127-DA hydrogel and Arg-UPEA/F127-DA showed that all the drugs were released within 4-6 hours. The reasons could be due to the large hydrogel pore size, small MW/size of hydralazine salt and same charge property of hydralazine and Arg-UPEA (electrical repulsion). For Arg-UPEA/F127-DA/alginate, around 20-25 wt% hydralazine would be released within 4-6 hours, and then the release time would be within weeks depending on the hydrogel formulation or temperature. For examples, higher percentage of Arg-UPEA /alginate (2-UArg-2-S/F127-DA/alginate (2/3, w/w) hydrogel) would cause slower release of hydralazine than the 2-UArg-2-S/F127-DA/alginate (1/4, w/w) hydrogel due to the stronger electrostatic density/interaction, while room temperature would cause faster release of hydralazine than 37 °C due to the larger pore size (larger equilibrated swelling ratio). Therefore, this hybrid hydrogel platform could achieve the sustained release and easy regulating the release profiles of small molecule ionic drugs.

Due to the significantly different properties of insulin and IL-12, the release profiles of insulin and IL-12 from Arg-UPEA/F127-DA/alginate hydrogels were different from the hydralazine. Insulin has a MW around 5,800 Da and isoelectric point about 5.3, while the mouse IL-12 has a MW around 75 KDa and isoelectric point about 4.3. The loading amount of insulin was set at 20 wt% of Arg-UPEA plus alginate and the loading amount of IL-12 was set at 5 μg per hydrogel pellet. Figure 8b showed the insulin release profiles from hydrogels in PBS buffer at 37 °C. The insulin content was analyzed by BCA assay. Since the hydrogel show no biodegradation in PBS buffer (without enzyme) for at least 1 month. The Arg-UPEA component will not affect the BCA assay results much. The release data from pure F127-DA hydrogel showed that the insulin release was completed around 1 day. For the 2-UArg-2-S/F127-DA (1/4, w/w) hydrogel, the insulin release was completed around 7 days due to the strong electrostatic interaction between Arg-UPEA and insulin and relative big size of insulin. For Arg-UPEA/F127-DA/alginate hydrogel, around 10-15% insulin would be released within 4-6 hours, then the sustained release was obtained for around 2-3 weeks for 2-UArg-2-S/F127-DA/alginate (1/4, w/w) hydrogel. Figure 8c showed some release data of IL-12 from hydrogels in PBS buffer at 37 °C. The IL-12 was analyzed by ELISA assay. Compared with insulin release profile, the IL-12 release profile showed slower release rate due to the much larger MW/size and sustained release were observed for both 2-UArg-2-S/F127-DA (1/4, w/w) and 2-UArg-2-S/F127-DA/alginate (1/4, w/w) hydrogels. The 2-UArg-2-S/F127-DA/alginate (1/4, w/w) hydrogel showed slower release rate than the 2-UArg-2-S/F127-DA (1/4, w/w) hydrogel.

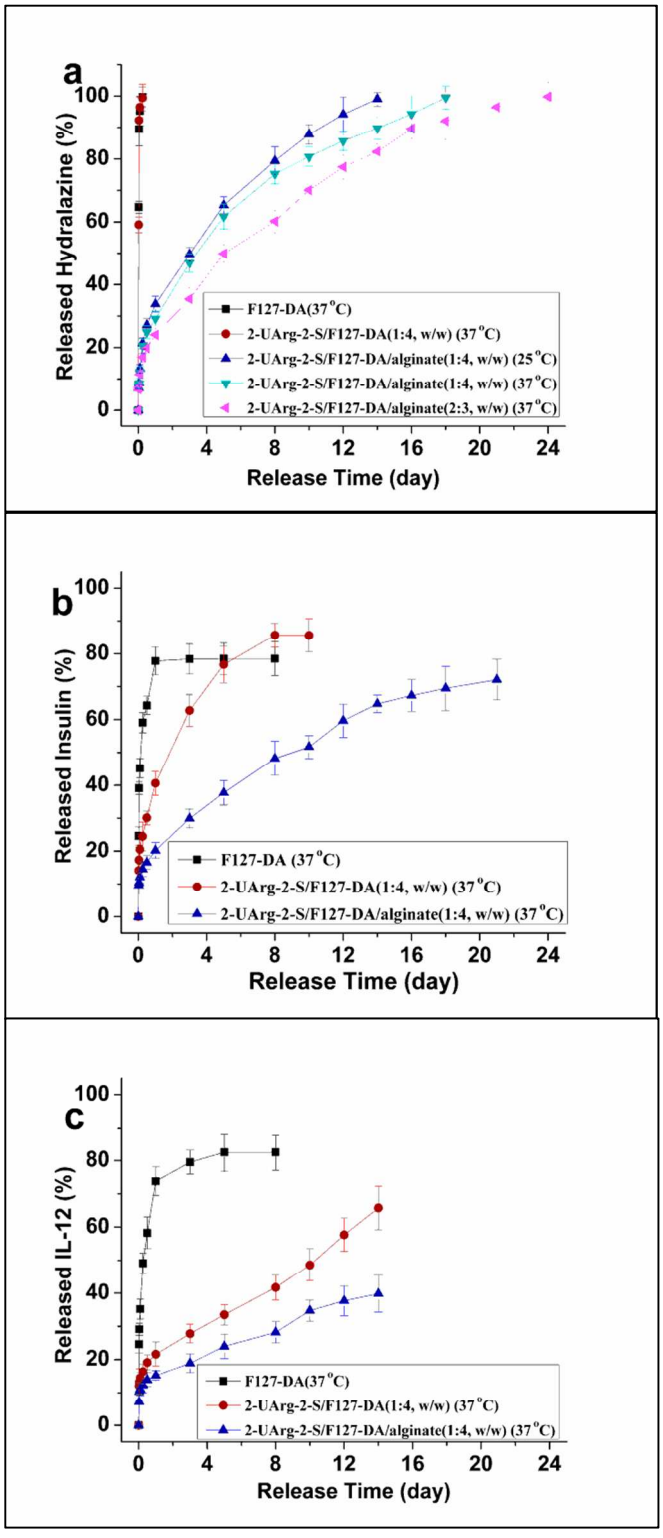

Figure 8, Controlled ionic drug release from hydrogels. a) Hydralazine chlorine salt; b) insulin; c) IL-12

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Notes and references

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Development of a biocompatible and biodegradable hybrid hydrogel platform for sustained release of ionic drugs

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We have developed and characterized a new hydrogel platform for ionic drug delivery, incorporating cationic, anionic, and temperature responsive precursors. *In vitro* biological assays show that these polymers have excellent biocompatibility, controllable biodegradability, and sustained ionic drug release capability.