Cationic Hybrid Hydrogels from Amino-Acid-Based Poly(ester amide): Fabrication, Characterization, and Biological Properties

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A family of biodegradable, biocompatible, water soluble cationic polymer precursor, arginine-based unsaturated poly (ester amide) (Arg-UPEA), is reported. Its incorporation into conventional Pluronic diacrylate (Pluronic-DA) to form hybrid hydrogels for a significant improvement of the biological performance of current synthetic hydrogels is shown. The gel fraction (G_f), equilibrium swelling ratio (Q_{eq}), compressive modulus, and interior morphology of the hybrid hydrogels as well as their interactions with human fibroblasts and bovine endothelial cells are fully investigated. It is found that the incorporation of Arg-UPEA into Pluronic-DA hydrogels significantly changes their Q_{eq}, mechanical strength, and interior morphology. The structure–property relationship of the newly fabricated hybrid hydrogels is studied in terms of the chemical structure of the Arg-UPEA precursor, i.e., the number of methylene groups in the Arg-UPEA repeating unit. The results indicate that increasing methylene groups in the Arg-UPEA repeating unit increases Q_{eq} and decreases the compressive modulus of hydrogels. When compared with a pure Pluronic hydrogel, the cationic Arg-UPEAs/Pluronic hybrid hydrogels greatly improve the attachment and proliferation of human fibroblasts on hydrogel surfaces. A bovine aortic endothelial cells (BAEC) viability test in the interior of the hydrogels shows that the positively charged hybrid hydrogels can significantly improve the viability of the encapsulated endothelial cell over a 2 week study period when compared with a pure Pluronic hydrogel.

1. Introduction

In recent years, hydrogels have attracted many interests because of their good biocompatibility, high water content, 3D microporous structure, permeability for oxygen and nutrients, and tissue-like elastic properties.[1–6] Among the reported hydrogel systems, synthetic hydrogels have received a lot of attention because they are more reproducible, and can offer better control over the structure, physical integrity and mechanical properties.[1–6]

For many pure (single component) synthetic hydrogels, the lack of tunable property/functionality and unsatisfied cellular interaction greatly limits their biomedical applications. To solve these problems, hybrid hydrogels, which contain two or more components (precursors), have been developed. The introduction of new functional precursors can help to tune or bring new property/functionality, such as charge property, hydrophobic/hydrophilic property, pH and temperature responsive properties, and functional groups.[7–19] For example, Chao et al. used maleic chitosan to bring negative charge property, carboxylic acid functional groups and polysaccharide component into PEG hydrogel.[16] Guo et al. and Pang et al. incorporated hydrophobic phenylalanine based poly (ester amide)s (Phe-PEA) into PEG hydrogel to make the hydrogel more hydrophobic and cell friendly.[17,18] Wu et al. reported the integration of a cationic hydrophilic precursor, 2-(acryloxy)ethyl]trimethylammonium chloride precursor with a hydrophobic *N*-vinylcaprolactam precursor to achieve a hydrophilic-hydrophobic balanced hybrid hydrogels for the delivery of ovalbumin.[19]

In this study, we reported the development of a family of cationic arginine based precursors (Arg-UPEA, **Scheme 1**) to test the hypothesis that the introduction of cationic Arg-UPEA into single-precursor-based hydrogels can improve their cellular interactions because of the cationic property and biocompatibility of Arg-UPEA. Pluronic hydrogel was selected as a model synthetic hydrogel for testing our hypothesis because it has been used in many reported studies.[20–24] Human fibroblast and bovine endothelial cells were used to compare the cellular response difference between the Pluronic hydrogel and Arg-UPEA/Pluronic hybrid hydrogel in terms of cell attachment on hydrogel surface and cell encapsulation inside the hydrogel.

Arg-UPEAs is a relatively new member of the amino acid based poly (ester amide)s (AA-PEAs) family. Recently, AA-PEAs, a newly developed biodegradable biomaterial family, have shown very low cytotoxicity and muted inflammatory response,

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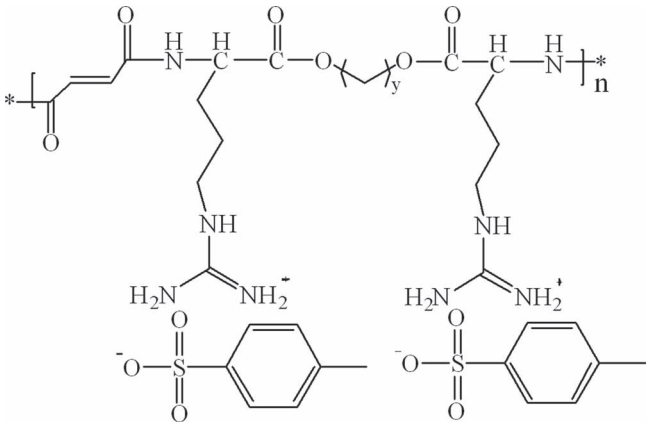


Scheme 1. Chemical structure of unsaturated Arg-PEA (Arg-UPEA).

and support natural wound healing.[25–37] For this study, a type of water soluble unsaturated AA-PEAs based on L-arginine (Arg) (Arg-UPEA) have been prepared[38] to overcome the aqueous solubility problem so that the hybrid hydrogels could be fabricated in an aqueous medium which would be advantageous for cell encapsulation or impregnation of biologically originated species such as proteins.

2. Results and Discussion

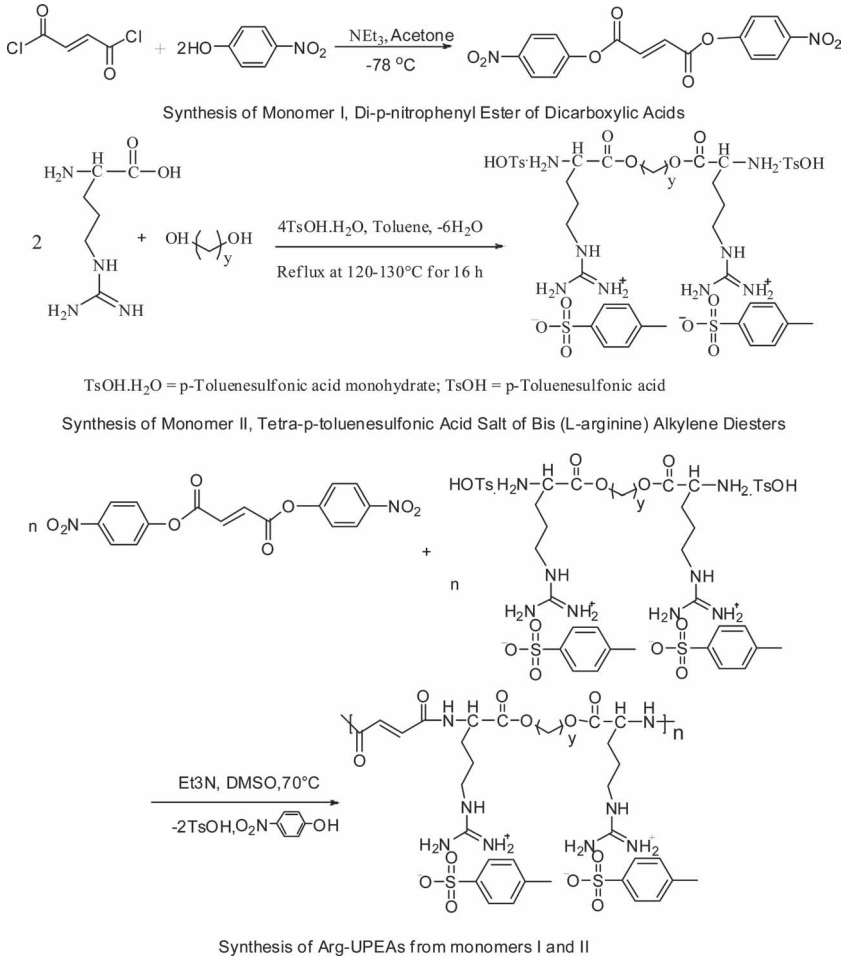
2.1. Preparation and Characterization of Polymer Precursors

The Pluronic (F127), with an average molecular weight (MW) of 12 600, was used here to prepare one of the two precursors for the photo-crosslinking. The synthesis of F127-DA followed the previously reported procedures.[23] The chemical structure of F127-DA was confirmed by ^{1}H-NMR and Fourier transform infrared (FTIR) spectroscopy. The final product was white powder and can dissolve in distilled water to form a clear and transparent solution at room temperature or 4 °C. The final product should be re-purified by dialysis before any biological tests.

The unsaturated di-*p*-nitrophenyl esters of dicarboxylic acids (NF) and tetra-*p*-toluenesulfonic acid salts of bis (L-arginine) alkylene diester monomers were synthesized and characterized as previous reports.[28,30,38] The only difference among the four types of monomers **II** (Arg-2-S, Arg-3-S, Arg-4-S, and Arg-6-S) prepared is the methylene chain length (y) in the diol part between the two adjacent ester groups: number of CH_2 varies from 2 (Arg-2-S) to 6 (Arg-6-S). The chemical structures of these 4 monomers **II** were confirmed by FTIR and ^{1}HNMR, and the details were described and discussed elsewhere.[38]

Four types of Arg-UPEA precursors differing in y number were prepared according to the chemical scheme in **Scheme 2**. The yield of the final product was high, i.e., >85%. The chemical structure of Arg-UPEAs was confirmed by FTIR and H-NMR, and their yields, water solubility, charge density, glass transition temperature (T_g), molecular weight of repeating unit, molecular weight are given in **Table 1**. The Arg-UPEAs were moisture sensitive and should be stored in sealed bottles at 4 °C or lower temperature in dark place.

For the solubility of Arg-UPEA in common organic solvents, 1.0 mg/mL was used as solubility criteria to judge whether a polymer is soluble or insoluble at room temperature. Due to their strong polar nature, Arg-UPEAs tended to dissolve in polar solvents. All the synthesized Arg-UPEAs were soluble in polar organic solvents such as DMSO, DMF and methanol, but insoluble in non-polar or weak polar organic solvents such as ethyl acetate, THF or chloroform. The effect of y material parameters on Arg-UPEA water solubility (Table 1) revealed that y had a major impact on the water solubility of Arg-UPEAs; and an increase in the methylene chain length in the diols (y) part reduced the water solubility significantly due to the increasing



Scheme 2. Synthesis of monomers and Arg-UPEA polymers.

Table .1 Chemical and physical properties of Arg-UPEAs.

	2-UArg-2-S	2-UArg-3-S	2-UArg-4-S	2-UArg-6-S
Yield [%]	87	91	93	89
Charge Density [mol kg^{-1}]	2.50	2.46	2.42	2.34
Molecular Weight of Repeating Unit [g mol^{-1}]	798.9	812.9	826.9	855.0
Molecular Weight, M_n [kg mol^{-1}]	12.93	14.56	15.71	14.82
Molecular Weight, M_w [kg mol^{-1}]	14.01	16.14	17.49	16.33
Polydispersity, M_w/M_n	1.08	1.11	1.11	1.10
T_g [°C]	112	103	94	88
Water Solubility [mg/mL]	20 ± 2	30 ± 2	10 ± 1	2 ± 0.5

hydrophobicity. The Arg-UPEAs showed reduced water solubility when compared to the reported saturated Arg-PEAs.[30,38] The MW data in Table 1 indicated that all the Arg-UPEAs had M_n between 12.5 kg mol^{-1} and 16.0 kg mol^{-1} with a narrow polydispersity of 1.07–1.10. The y values did not have any significant impact on MW and polydispersity of Arg-UPEAs. When compared with saturated Arg-PEAs, the MW of the Arg-UPEAs did not show any big difference and were in the same range.

For the thermal property of Arg-UPEAs, the melting point (T_m) could not be detected, which was consistent with the reported saturated Arg-PEAs.[38] The T_g's of Arg-UPEAs (Table 1) were in the range of 88–112 °C, which had the same range as the T_g of phenylalanine-based unsaturated PEAs (Phe-UPEAs).[28] Due to the presence of double bonds in the repeating unit of the Arg-UPEA backbone, much higher T_g values were observed when compared with the saturated Arg-PEAs.[38] For example, 2-UArg-2-S had a T_g around 112 °C, while 2-Arg-2-S had a T_g 52 °C, more than 100% increase in T_g by simply introducing unsaturated C=C bond in the polymer backbone. Pang et al. reported that the location of C=C bonds may also have a significant effect on T_g.[32] For example, if the C=C bonds were located in the side chain, the T_g value may decrease significantly.[32] Similar magnitude of T_g change was also reported for the Phe-based PEAs.[25,28] An examination of the effect of the number of methylene groups in the y part of the Arg-UPEAs revealed that an increase in y led to a lower T_g. The T_g decreased from 112 °C to 88 °C when the y value was increased from 2 to 6. This reduction in T_g with increasing y (or x) was attributed to the increasing chain flexibility and hence free volume.

2.2. Fabrication and Characterization of Arg-UPEA/Pluronic-DA Hybrid Hydrogels

In this study, Irgacure 2959 was used as the photo-initiator in an aqueous system via the UV photomeans. The initiator was previously reported to cause minimal toxicity (cell death) over a broad range of mammalian cell types and species ranging from human fetal osteoblasts to bovine chondrocytes.[39]

All the fabricated Arg-UPEA/F127-DA hydrogels were transparent after reaching their swelling equilibrium as shown in Figure S1 (Supporting Information), which is an example of the 2-UArg-2-S/F127-DA hydrogel at the feed weight ratio 1/4. The right image was a corresponding dried hydrogel. The swelling behavior, compression modules and interior morphology of these hybrid hydrogels were systematically examined as a function of the weight feed ratio and the y parameter in the Arg-UPEA precursor. **Table 2** summarizes the gel fraction (G_f), compression modulus, equilibrium swelling ratio (Q_{eq}) and elemental analysis (N). The successful fabrication of Arg-UPEA/F127-DA hybrid hydrogels was confirmed by the elemental analysis. By measuring the nitrogen element percentage of dried hydrogels, it was confirmed that most of the Arg-UPEA was successfully crosslinked with F127-DA as F127-DA doesn't have any N element. The measured N contents in Table 2 were slightly higher than the theoretical values (in the parentheses). The gel fraction data (G_f) data showed that a pure F127-DA hydrogel had a higher G_f value (more than 90%) than the Arg-UPEA/F127-DA hybrid hydrogels (around 80%). The reason for this G_f difference is that F127-DA has a higher activity than Arg-UPEA. For the effects of y and feed ratio on G_f, the results in Table 2 showed that y value has almost no effect on the G_f; while the feed ratio of F127-DA to Arg-UPEA has some effect on the G_f, and a higher feed ratio of F127-DA to Arg-UPEA resulted in a higher G_f. The reason could be due to the weak

Table 2. Arg-UPEA/F127-DA hybrid hydrogels and their physicochemical properties.

Sample	Weight Ratio	G_f [%]	Q_{eq} [a)] [%]	Compressive Modulus[a)] [KPa]	N [%]
F127-DA	100:0	92	1144 ± 17	12.27 ± 0.34	0.00 (0.00)
F127-DA/2-UArg-2-S	4:1	83	1785 ± 74	4.85 ± 0.41	2.80 (2.61)
F127-DA/2-UArg-3-S	4:1	80	1843 ± 77	4.43 ± 0.23	2.76 (2.54)
F127-DA/2-UArg-4-S	4:1	81	1917 ± 59	3.77 ± 0.24	2.71 (2.55)
F127-DA/2-UArg-6-S	4:1	79	2158 ± 98	2.58 ± 0.25	2.62 (2.50)
F127-DA/2-UArg-2-S	3:2	77	2014 ± 63	1.97 ± 0.21	5.61 (5.40)
F127-DA/2-UArg-3-S	3:2	79	2191 ± 94	1.91 ± 0.11	5.52 (5.44)
F127-DA/2-U-Arg-4-S	3:2	76	2287 ± 63	1.63 ± 0.19	5.43 (5.27)
F127-DA/2-UArg-6-S	3:2	75	2407 ± 117	0.77 ± 0.17	5.24 (5.10)

[a)] Q_{eq} and compressive modulus were measured in DI water at room temperature.

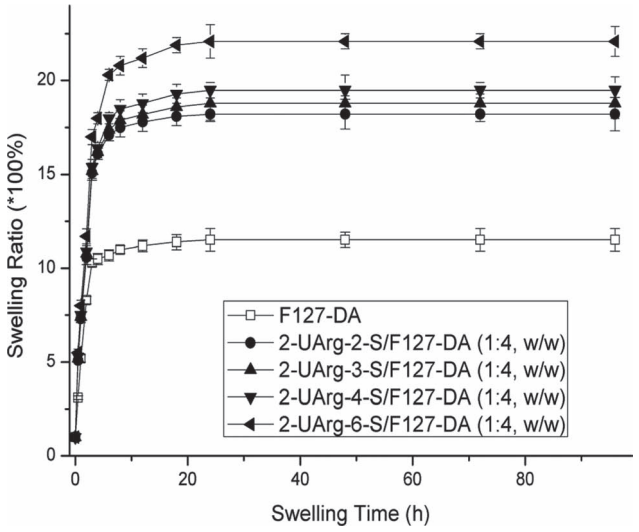


Figure 1. Swelling kinetics of Arg-UPEA/Pluronic-DA hydrogels in distilled water at room temperature.

crosslinking ability of Arg-UPEA precursor as it can't form hydrogels by itself. The upper limit of the weight feed ratio of F127-DA to Arg-UPEA in the hybrid hydrogels for a successful fabrication is 2:3 (60 wt% of Arg-UPEA) and the hydrogels under this composition is very soft. Our unpublished data showed the thermo-responsive property of the hybrid hydrogels since Pluronic is thermoresposive (LCST behavior). This property did affect the hydrogel properties, such as swelling ratios, but did not affect the cellular interaction significantly. Since it's not the focus of this report, it will be discussed in another report.

The equilibrium swelling ratio tests were performed in deionized water (DI water) and the Q_{eq} were also summarized in Table 2. During the tests of equilibrium swelling ratio, the swelling kinetics of hydrogels (how fast the hydrogel swells) was also measured. **Figure 1** showed the swelling kinetics for a series of hybrid hydrogels as a function of the y parameter of the Arg-UPEA precursor at room temperature in DI water. Within first several hours, all the hydrogels showed very high swelling rate, and these early stage swelling rates appear to be independent of the composition of the hybrid hydrogels. After that, the hydrogels' swelling rates were much slower and will eventually reach equilibrium state after around 20 h. These swelling kinetics observed in the Arg-PEA/F127 hybrid hydrogels are consistent with other types of AA-PEA-based hybrid hydrogel systems.[17,18] For example, both Pang et al. and Guo et al. reported the swelling kinetics study of Phe-UPEA hybrid hydrogels,[17,18] and within the first several hours, the Phe-UPEA hybrid hydrogels showed very high swelling rate, and then tapped off after 24 h.[17]

The equilibrated swelling ratios of the hybrid hydrogels generally increase with an increase in either the weight feed ratio of Arg-UPEA to F127-DA precursors or the y value in the Arg-UPEA precursor. For example, 2-UArg-2-S/F127-DA (1/4 w/w) had an equilibrated swelling ratio of 1785 ± 74%, while 2-UArg-2-S/F127-DA (2/3 w/w) had a higher equilibrated swelling ratio of 2014 ± 63%, a 13% increase in Q_{eq}. The reason could be due to the relatively very high hydrophilicity of the Arg-UPEA precursor which could lead to a more loose 3D network structure upon swelling, i.e., higher equilibrium swelling ratio with those hybrid hydrogels having a higher percentage of the Arg-UPEA components.

We further investigated the effect of molecular structure (y value) of the Arg-UPEA on the swelling property of the hybrid hydrogels at a constant weight feed ratio of Arg-UPEA to F127-DA. As shown in Table 2, the swelling ratio increased as the number of methylene groups in the repeating unit of Arg-UPEA increased. For example, at the weight feed ratio of 1 to 4, the equilibrated swelling ratio increased from 1785 ± 74% from 2-UArg-2-S/F127-DA (1/4 w/w) to 2158 ± 98% from 2-UArg-6-S/F127-DA (2/3 w/w). Pang et al. also reported the similar effect of the methylene chain length on hydrogel swelling in their study of the Phe-based PEA and polyethylene glycol diacrylate hybrid hydrogels with.[17] They reported that a shorter CH_2 segment from C8 to C2 decreased the Q_{eq} value slightly from 860% to 750%.[17] They suggested that this relationship between the shorter methylene chain length and lower hybrid hydrogel equilibrium swelling could be due to the formation of a tighter network structure from denser double bond contents due to shorter methylene chain spacer between two adjacent double bonds.[17]

2.3. Mechanical Properties (Compressive Modulus) of Arg-UPEA/F127-DA Hybrid Hydrogels

The mechanical property (compressive modulus) of the Arg-UPEA/F127-DA hybrid hydrogels is summarized in Table 2. The pure F127-DA has a compress modulus of 12.27 KPa and the Arg-UPEA/F127-DA hybrid hydrogels showed much lower compress moduli than a pure F127-DA hydrogel, in the range between 0.77 KPa and 4.85 KPa. For the effect of molecular structure (y value) of Arg-UPEA on the compressive modulus of the hybrid hydrogels, it was found that the compressive modulus decreased with an increase in y value (Table 2). For example, at a fixed feed weight ratio of Arg-UPEA to F127-DA of 1:4, an increase of y from 2 (2-UArg-2-S/F127-DA), 4 (2-UArg-4-S/F127-DA) to 6 (2-UArg-6-S/F127-DA) caused their compressive moduli decreased from 4.85 ± 0.41, 3.77 ± 0.24 to 2.58 ± 0.25 KPa, respectively. The compressive modulus of Arg-UPEA/F127-DA hydrogel is much less than our previous reported Phe-UPEA/PEG8000-DA system.[17,18] For example, for the hybrid hydrogel system of Arg-UPEA/F127-DA with a weight feed ratio of 1:4, its compressive moduli is in the range of 2-5 KPa; while Phe-UPEA/PEG8000-DA at the same feed ratio had the compressive moduli 560 KPa, about 100 times of the Arg-UPEA/F127-DA system. Such a big difference is mainly caused by the significant difference between Arg-UPEA and Phe-UPEA precursors as the Arg-UPEA precursor is far more hydrophilic and ionic than the hydrophobic and non-ionic Phe-UPEA precursor used in the Guo et al. study.[28] Pang et al. also discussed the compression modulus of some other types of Phe-PEA hybrid hydrogels whose photo-reactive double bonds were in the pendant group of the allyl glycine unit (AG)[17] rather in the Phe-PEA backbone as in the Guo et al. study.[18]

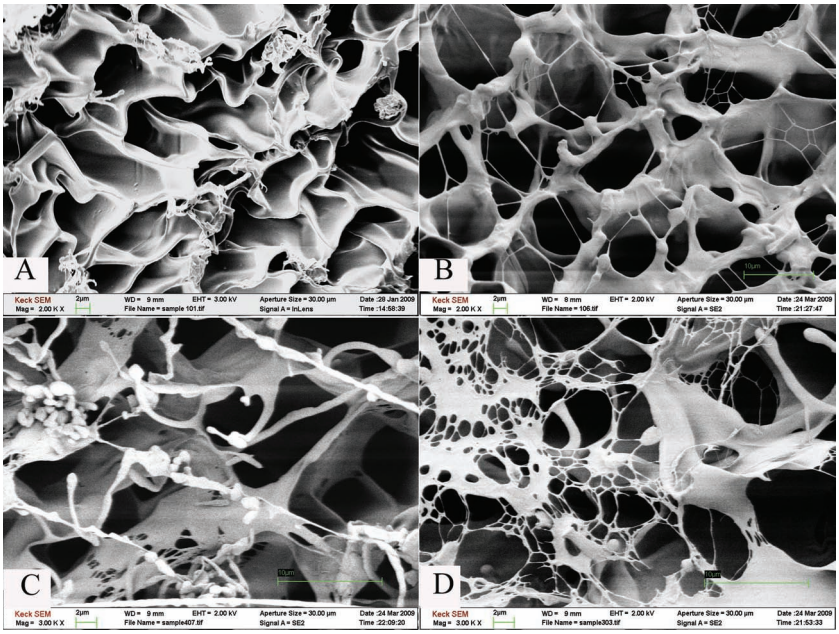


Figure 2. SEM images of Arg-UPEA/F127-DA hybrid hydrogels at weight feed ratio of Arg-UPEA/F127-DA of 1/4. A) Pure F127-DA hydrogel, B) 2-UArg-2-S/F127-DA hybrid hydrogel, C) 2-UArg-4-S/F127-DA hybrid hydrogel, and D) 2-UArg-6-S/F127-DA hybrid hydrogel. The scale bar is 5 μm in (A–D).

Pang et al. reported that the compressive modulus of their Phe-PEA hybrid hydrogels increased with the increasing of the AG (allyl glycine) contents in the Phe-PEA-AG precursor.[17] As we know, the mechanical property of hydrogels was very important for their interaction with cells.[2] This Arg-UPEA/Pluronic-DA hybrid hydrogel system offered a variety of mechanical property choices for tissue engineering applications by adjusting the material parameter of the precursor as well as its feed ratio to the co-precursors.

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

To further understand the 3D structure of Arg-UPEA/F127-DA hybrid hydrogels, the cross-sectional interior morphology of the hybrid hydrogels was examined and shown in **Figure 2**. When compared with a pure F127-DA hydrogel (Figure 2A), these Arg-UPEA/F127-DA hybrid hydrogels (Figure 2B–D) have larger average pore size and thinner cell wall. For example, a pure F127-DA hydrogel had an average pore size 8.0-10.0 μm, while all Arg-UPEA/F127-DA hybrid hydrogels had the average pore size 10.0-15.0 μm. One of the distinctive morphology of these Arg-UPEA/F127-DA hybrid hydrogels from a pure F127-DA hydrogel was that the Arg-UPEA/F127-DA hybrid hydrogels showed some nano-size fiber webs entangled with the cells. At a fixed feed ratio of the 2 precursors, no significant difference of the pore size was observed among the Arg-UPEA/F127-DA hybrid hydrogels regardless of the difference of y material parameter in the Arg-UPEA precursors. Guo et al. and Pang et al. also reported the SEM images of the interior morphology of other AA-PEA hybrid hydrogels.[17,18] Compared with Guo and Pang's AA-PEA hybrid hydrogel systems, the Arg-UPEA/F127-DA hybrid hydrogels studied here showed some unique nano-size fiber webs entangled with the cells, while Guo and Pang's did not. All the prepared Arg-UPEA/F127-DA hybrid hydrogels are biodegradable, Figure S2 (Supporting Information) showed an example of the interior morphology change of Arg-UPEA/F127-DA hybrid hydrogel after 60 days of degradation.

2.5. Human Fibroblast Cell Attachment and Proliferation on Hydrogel Surfaces

To study the cellular interaction with Arg-UPEA/F127-DA hybrid hydrogels, the Detroit 539 human fibroblast cells were cultured on the surface of Arg-UPEA/F127-DA hybrid hydrogels to investigate the cell attachment and proliferation performance. Detroit 539 human fibroblast cells cultured onto the 24 well cell culture plate without any other treatment were used as the blank control, and fibroblast cells cultured on the pure F127-DA hydrogel was used as a hydrogel control. **Figure 3** showed a representative example of the Detroit 539 human fibroblast cells cultured on the surface of 2-UArg-2-S/F127-DA (1/4 w/w) hybrid hydrogel. When compared with the

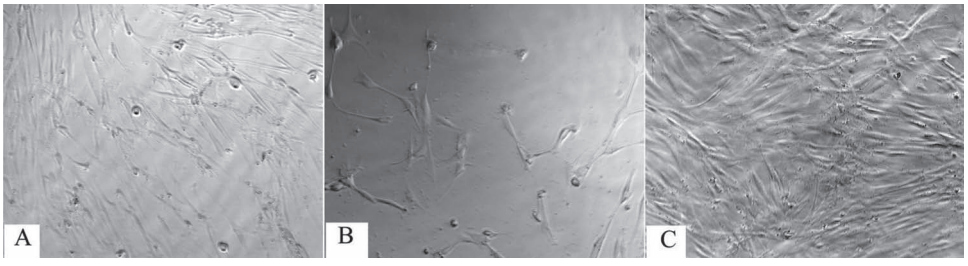


Figure 3. Representative microscopy images of Detroit 539 human fibroblast cells after 48 h of culture, 10×: A) cells cultured in 24 well cell culture plate without any treatment (control), B) cells cultured on the surface of pure F127-DA hydrogel, and C) cells cultured on the surface of 2-UArg-2-S/F127-DA(1/4, w/w) hydrogel.

pure F127-DA hydrogel (Figure 3B) control, the hybrid hydrogel (Figure 3C) had much higher amounts of the attached/proliferated fibroblast cells, and the amounts of the attached/proliferated fibroblast cells were slightly better than the blank control (Figure 3A). From the aspect of the fibroblast cell morphology, no significant morphology change was observed between the hybrid hydrogel surface (Figure 3C) and the blank control (Figure 3A) after 48 h of culture. There were no visible signs of cell dying or unhealthy cells. The Detroit 539 human fibroblast cells attached onto the pure F127-DA hydrogel surface, however, did show some morphology change (Figure 3B). The data in Figure 3B show that the Detroit 539 human fibroblast cells did not completely attach and spread on the F127-DA hydrogel surface. Therefore, the introduction of Arg-UPEA to Pluronic-DA hydrogel can significantly enhance the hydrogel's cell attachment and proliferation.

These qualitative cell morphological data were also confirmed by the quantitative MTT assay for the attached/proliferated fibroblast cells (Figure S3, Supporting Information). Both Figure 3 and Figure S3 (Supporting Information) showed that the blank and the hybrid hydrogel have similar amounts of the attached Detroit 539 human fibroblast cells and both had significantly higher amounts of attached cells than the pure F127-DA hydrogel.

The possible reasons for the observed encouraging Detroit 539 human fibroblast cell attachment and proliferation data onto the 2-UArg-2-S/F127-DA hybrid hydrogel system can be attributed to the excellent biocompatibility and cationic nature of the Arg-UPEA component rather than the F127. Many reported studies have used F127 or F127 derivatives to fabricate hydrogels for tissue engineering applications.[7–15] However, those reported studies indicated that F127 hydrogel itself can not support the cell attachment/proliferation well, and many chemical modification methods have been applied to F127 hydrogel system to improve the cellular interaction of F127 hydrogels.[7–15] For example, Park et al. reported using chitosan-F127 hydrogels for cartilage regeneration.[7] Jung et al. reported using TGF-β1-conjugated biodegradable Pluronic F127 hydrogel for adipose-derived stem cells culture.[13] Lippens et al. reported using alanine modified F127 hydrogel for mesenchymal stem cells culture.[15] Lin et al. reported using poly(dimethyl siloxane-urethane)/Pluronic F127 for L929 fibroblast cell culture,[10] and their cell morphology data indicated that the attachment performance of L929 fibroblast on F127/poly(dimethyl siloxane-urethane) hybrid hydrogels was considered non-cytotoxic, but still exhibited lesser L929 fibroblast confluence from the cell culture plate negative control.

The significantly better fibroblast attachment and proliferation on a cationic charged hydrogel system as observed in the Arg-UPEA/F127 hydrogel system is consistent with other reported positively charged hydrogel systems, such as chemically modified PEGDA and HEMA hydrogels.[40–42]Schneider et al. reported that the positively charged HEMA or PEG hydrogel system[40] showed the highest support of 3T3 fibroblast attachment and spreading than either RGD modified, anionic, neutral or unmodified HEMA and PEG hydrogels in the presence or absence of serum, Unfortunately, no 3T3 fibroblast attachment and proliferation data on tissue culture plates (TCP) were given in the Schneider et al. study to draw a conclusion whether their cationic HEMA or PEG hydrogel systems would exhibit the similar fibroblasts attachment and proliferation as the TCP control as we demonstrated in our Arg-UPEA/Pluronic-DA hydrogel system in Figure 3. Hynes et al. discussed how to utilize the PEG/polylysine hydrogel scaffold to improve the survival and promote the differentiation of neural stem cells.[41,42] These excellent cell attachment and proliferation performances of Arg-UPEA/F127-DA hybrid hydrogels suggest they may have a great potential as a new type of scaffolds for various biomedical applications. Of course, these cationic Arg-UPEA/Pluronic-DA hybrid hydrogel systems can be further improved with cell-adhesive ligands for additional enhanced cell adhesion.

2.6. BAEC Cell Viability Inside Arg-UPEA/F127-DA Hybrid Hydrogels

In order to understand the effects of Arg-UPEA on the long-term encapsulated cell behavior, we tested the long term BAEC cell viability encapsulated within the Arg-UPEA/F127-DA hybrid hydrogel for 2 weeks at 37.0 °C and 5.0% CO_2. The live-dead assay was used to evaluate the BAEC cell viability and was performed according to the manufacturer protocol (LIVE/DEAD Cell Viability Assay Kit from Invitrogen). As shown in **Figure 4**, after 2 weeks almost all of the remaining encapsulated BAEC cells within 2-UArg-2-S/F127-DA hybrid hydrogel

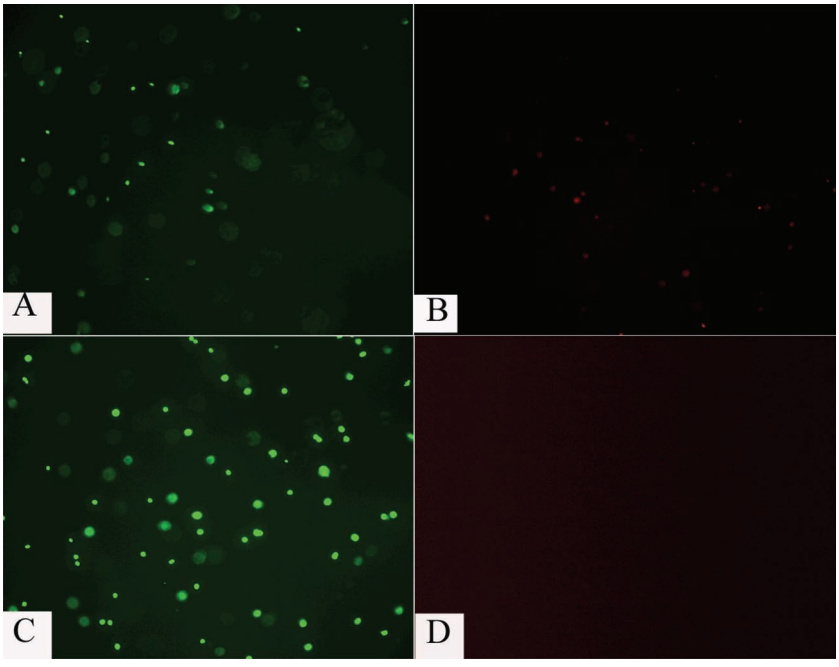


Figure 4. Live-dead assay for BAEC cells encapsulated in the pure F127-DA hydrogel (A,B) and 2-UArg-2-S/F127-DA hybrid hydrogel (C,D). Green dots in the (A,C) are for the living cells and red dots in (B,D) are for the dead cells

(1:4 weight feed ratio) and the pure F127-DA hydrogel were viable and healthy (stained in green, Figure 4A,C), but the pure F127-DA hydrogel shows a lot of less green dot density but a lot of unhealthy or dead cells (stained in red, Figure 4B), while the 2-UArg-2-S/F127-DA hybrid hydrogel shows far higher green dot density and hardly any red dots (Figure 4C,D), The green dots of different brightness and sizes mean that the living BAEC cells were at the different depth of the 3D hydrogel. This finding indicates that the incorporation of the cationic Arg-UPEA moiety into F127 system could significantly facilitate the proliferation and long-term viability of the encapsulated cells within the 3D hydrogel interior.

3. Conclusions

A new family of cationic charged biocompatible Arg-UPEA/Pluronic-DA hybrid hydrogel was successfully fabricated in an aqueous medium via UV photocrosslinking with a photo-initiator. The physicochemical, swelling, mechanical, and morphological properties of the resulting hybrid hydrogels were investigated. The newly designed and fabricated Arg-UPEA/Pluronic-DA hybrid hydrogel system can offer many advantages in terms of water soluble precursors for easier pre-loading of cells or therapeutic proteins, cationic charge for better cell attachment and proliferation, mechanical property, a range of hydrophobicity vs. hydrophilicity balance, and pore size. We demonstrated that by varying the feed ratio of Arg-UPEA to Pluronic-DA and the type of Arg-UPEA, we can finely tune the swelling, mechanical, and morphological properties of such a cationic hybrid hydrogel system. These results contribute to the understanding of the structure–property relationship of Arg-UPEA. Cell culture studies indicated that the incorporation of Arg-UPEA to Pluronic-DA hydrogel significantly increase the cell attachment, proliferation, and viability of both Detroit 539 human fibroblasts and bovine aortic endothelial cells.

4. Experimental Section

Materials: Pluronic (F127, MW 12 600 and 70% PEG content), acryloyl chloride, triethylamine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. 2-Hydroxy-l-[4-(hydroxyethoxy)phenyl]-2-methyl-l-propanone (Irgacure 2959) was donated by Ciba Specialty Chemicals Corporation. L-Arginine (L-Arg), *p*-toluenesulfonic acid monohydrate, fumaryl chloride, ethylene glycol, 1,3-propanediol, 1, 4-butanediol, 1,6-hexaniol 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) was synthesized according to a previously reported method[23] (Scheme S1, Supporting Information). The Arg-UPEAs were synthesized by the same procedures reported before,[25,28,30] except the unsaturated diacids were used to provide unsaturated moiety in the AA-PEA backbone. Briefly, the synthesis of Arg-UPEAs can 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**).

Unsaturated di-*p*-nitrophenyl ester of dicarboxylic acid (monomer **I**, di-*p*-nitrophenyl fumarate (NF)) was prepared by reacting fumaryl chloride with *p*-nitrophenol as previously reported.[28] Four 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) propane diesters, Arg-**3**-S (y = 3); tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) butane diesters, Arg-**4**-S (y = 4); tetra-*p*-toluenesulfonic acid salt of bis(L-arginine) hexane diesters, Arg-**6**-S (y = 6). y indicates the number of methylene group in the diol segment of the Arg-UPEA. Arg-UPEAs were subsequently prepared by the solution polycondensation of (**I**) and (**II**) monomers (NF and Arg-2-S, Arg-3-S, Arg-4-S, or Arg-6-S) at different combinations (Scheme 2 and Table S1, Supporting Information). The resulting Arg-UPEAs are labeled as x-UArg-y-S, where x and y are the number of CH_2 groups in diacid and diol segments, respectively, and U means the Arg-PEA is unsaturated. x was fixed at 2 because only one type of unsaturated monomer **I** (NF) was used. The Arg-UPEAs synthesized with different combinations of diacids and diols building blocks are: 2-UArg-2-S, 2-UArg-3-S, 2-UArg-4-S and 2-UArg-6-S (Table S1, Supporting Information).

Fabrication of Arg-UPEA/Pluronic-DA Hybrid Hydrogels: Arg-UPEA/Pluronic-DA hybrid hydrogels were prepared by the photo-polymerization of two precursors (Arg-UPEA and Pluronic-DA) at different weight feed ratios in an aqueous solution with a water soluble photo-initiator. The Arg-UPEA and Pluronic-DA precursors were purified first by dissolving the precursors in distilled water and were dialyzed against deionized water (molecular weight cut off 4000) for 2 days. After that, the solutions were lyophilized for 3 days using a Virtis Freeze Drier (Gardiner, NY) under vacuum at −48 °C. An example of the fabrication of a hybrid hydrogel was given below: 2-UArg-2-S (80.0 mg) and F127-DA (320.0 mg) (1/4 weight ratio of 2-UArg-2-S/F127-DA) were added into a glass vial and dissolved in deionized water (2.0 mL) to form a clear homogeneous solution with light yellow color. Photoinitiator Irgacure 2959 (4.0 mg which was 1.0 wt% of the total amounts of precursor) was added into the precursors' solution and dissolved completely. The precursor solution was then transferred to a custom-made 20 well Teflon mold (diameter 12.0 mm and depth ≈ 4.0 mm of each well) using a micropipette. The precursor solution in the molds was irradiated by a long-wavelength UV lamp (365 nm, 100 W) for specified time (5.0 min) at room temperature. The irradiation distance was 5.0–10.0 cm. The resultant hydrogels were moved from the mold and immersed in distilled water at room temperature for 48 h to remove any residual chemicals. The distilled water was replaced periodically. After this purification process, the hydrogel was soaked in distilled water to reach swelling equilibrium, and dried in vacuum at room temperature for 48 h before further characterization and application. The gel fraction (G_f) of the resulting hydrogels was calculated by the following equation: $G_f = (W_d/W_p) \times 100\%$, where W_d is the weight of dry hydrogel and W_p is the total weight of the two precursors and the photo-initiator.

Hydrogel Equilibrium Swelling Ratio: The equilibrium swelling ratio (Q_{eq}) of hydrogels was calculated by the following equation: $Q_{eq} = [(W_e - W_d)/W_d] \times 100\%$, where W_s 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.

Compressive Modulus Measurement by Dynamic Mechanical Analyzer (DMA): The mechanical property of the Arg-UPEA/F127-DA hydrogels was measured by a DMA 2980 Dynamic Mechanical Analyzer (TA Instruments Inc., New Castle, DE) in a controlled force mode (CF-mode). The swollen hydrogel samples in circular disc shape were submerged in distilled water and mounted between the movable compression clamp (diameter 30.0 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 $N.min^{-1}$ 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 stress versus strain. All compression elastic modulus data in this study were obtained from triplicate samples and data were expressed as the mean ± standard deviation.

Cell Attachment and Proliferation on Arg-UPEA/F127-DA Hybrid Hydrogels Surface: The cell attachment and proliferation on the Arg-UPEA/F127-DA hybrid hydrogel surfaces was evaluated by cell morphology. Pure F127-DA hydrogel was chosen as the hydrogel control, and the cell culture plate without any treatment was used as the blank control. The cells used for this study were Detroit 539 human fibroblasts. The purified hydrogels were cut into round shape with the diameter that just filled the well of 24-well cell culture plates. The hydrogels were sterilized under UV light (from the cell culture hood) for 1.0 h. After that, the hydrogels were washed twice by PBS buffer and cell culture media. Then, the hydrogels were placed into the wells of cell culture plate and fixed by sterilized rubber ring which has the same diameter as the well of cell culture plate. Detroit 539 human fibroblasts were seeded at an appropriate cell density (10,000 cells/well) and incubated overnight. After incubation (48 h), the cell attachment and proliferation on the hydrogel surface was record by an optical microscope. For MTT assay, the attached cells were first detached from the hydrogel surface or cell culture plate surface by trypsin (0.1 mg mL^{-1}) treatment, then the detached cells were transferred into a new 96 cell culture place, after incubation (12 h), the MTT assay were processed.

Cell Viability inside Arg-UPEA/F127-DA Hybrid Hydrogels: In order to test the cell viability inside the Arg-UPEA/F127-DA hybrid hydrogels, the BAECs were encapsulated into hydrogels by the following steps: Purified hydrogel precursors and initiators were dissolved in a PBS buffer, and then the cells (10^7 mL^{-1} in media), FBS, antibiotics and other nutrients of complete cell culture media were added. The final mixture solution has 20.0 wt% precursors, 60 000 cells mL^{-1}, and 10.0 wt% FBS inside. All other components of complete cell culture media in the mixture have the exactly same concentration as the normal BAEC cell culture media recommended by ATCC. The mixture were injected into 24-well cell culture plate (0.5 mL per well) by a pipette and crosslinked under irradiation (100 W UV) for 5 min and the irradiation distance is 5 cm. After crosslinking, complete cell culture media (0.5 mL) was added into each well. The BAEC-loaded hydrogels were incubated for 2 weeks at 37 °C, 5% CO_2. Cell culture media was changed every other day. The live-dead assay was then performed according to the manufacturer protocol (LIVE/DEAD Cell Viability Assay Kit from Invitrogen).

Measurements: The physicochemical properties of the monomers, polymers and hydrogels were characterized by various standard methods. ^{1}H NMR spectra were recorded with a Varian Unity Inova 400-MHz spectrometer (Palo Alto, CA). Deuterated water (D_2O-d_2) or deuterated dimethyl sulfoxide (DMSO-d_6) (Cambridge Isotope Laboratories, Andover, MA) with tetramethylsilane as an internal standard was used as the solvent. MestReNova software was used for the data analysis. Elemental analyses of the polymers/hydrogels were performed with a PE 2400 CHN elemental analyzer by Atlantic Microlab (Norcross, GA). The thermal property of the Arg-UPEAs was characterized with a DSC 2920 (TA Instruments, New Castle, DE). The measurements were carried out from -10.0 to 200.0 °C at a heating rate of 10.0 °C min^{-1} and at a nitrogen gas flow rate of 25.0 mL min^{-1}. TA Universal Analysis software was used for thermal data analysis. The solubility of Arg-UPEAs in common organic solvents at room temperature was assessed by using 1.0 mg mL^{-1} as solubility criteria. The quantitative solubility of Arg-UPEAs in distilled water at room temperature was also measured by adding distilled water to the polymer sample step by step until the clear solution was obtained. Slight heating was needed for some types of Arg-UPEAs. For the MW measurement, Arg-UPEAs were prepared at a concentration of 1.0 mg mL^{-1} in a 0.1% (w/v) LiCl in DMAc solution. The sample MWs were determined from a standard curve generated from polystyrene standards with MWs ranging from 841.7 kDa to 2.93 kDa that were chromatographed under the same conditions as the samples. The standard curve was generated from a third order polynomial fit of the polystyrene standard MWs. Interior morphology of Arg-UPEA/Pluronic-DA hydrogels was investigated by 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 s for interior morphology observation with a scanning electron microscope (Leica S440, Germany).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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