

Nucleobase-grafted polycaprolactones as reversible networks in a novel biocompatible material†

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A series of nucleobase-grafted polycaprolactones have been prepared which exhibits a high complementary ability in solution and solid states owing to the formation of uracil–adenine pairs by induced hierarchical self-assembly. In addition, they can self-organize into spherical structure and result in excellent biomaterial properties.

Supramolecular self-assembly continues to be one of the most studied subjects in polymer science due to the interesting physical properties and the wide potential applications.¹ These materials utilize non-covalent interactions similar to those found in complex biological systems such as protein, DNA, and RNA, to direct and modulate their 3-D topology.² DNA is a highly influential structure in nature utilizing supramolecular chemistry to store, transmit, and replicate information to offer the opportunity of fabricating versatile nanostructures. Biomimetic polymers consisting of multiple hydrogen-bonding telechelic polymers show unique physical properties, such as high specificity, controlled affinity, reversibility and spontaneous self-assembly behavior.^{3–13} However, reports on synthesis of biocompatible and biodegradable polymers containing nucleobase molecular recognition sites are still rare.^{14–21} Thus, the combination of non-covalent interactions and their higher structures at the molecular level is a key to appending further function in the design aspect of bio-inspired macromolecules.

Biocompatible and biodegradable polymers containing multiple hydrogen bonding have received significant interest in applications such as surgical fixation devices, controlled drug delivery, and tissue engineering scaffolds.²² Poly(ϵ -caprolactone) is member of the aliphatic polyester family which is biocompatible with biomedical and pharmaceutical applications. Utilization of the biocompatible and biodegradable polymers in supramolecular chemistry offers the potential for advanced biopolymers with biomimetic properties. Nevertheless, lack of pendent functional groups along these polyester main chains is a major limitation to a large range of applications. We have demonstrated earlier the biocomplementary interaction of a DNA-like side-chain poly[1-(4-vinylbenzyl)thymine] (PVBT) homopolymer with the PVBT block complexed with alkylated nucleobases *via* hydrogen-bonded thymine–adenine (T–A) base pairs.²³ The system forms well-ordered lamellar structures and its *d*-spacings are controlled by the degree of crystallization of the alkyl side chains. Recently, we also reported the synthesis and assembly behavior of heteronucleobase-functionalized poly(ϵ -caprolactone).²⁴ Attachment of multiple hydrogen-bonding units to chain ends of PCL results in phase separation and substantial increase in the viscosity. Therefore, we expect that introducing nucleobase units into the side-chain copolymer may also influence their phase behavior considerably. In this study, aliphatic polyesters with pendent chloride were synthesized by controlled ring-opening copolymerization²⁵ and subsequently used for grafting nucleobase units by the Cu(I)-catalyzed addition of azides and alkynes, a typical of “click” chemistry.²⁶ These hydrogen bonding segments not only form the reversible cross-links in this blends, but they can also be used to introduced functionalities *via* specific molecular recognition. In the present paper we show that this variation in the polymer structure does not significantly affect the biomaterial properties. Moreover, these recognition-induced aggregates interact with guests featuring appropriate recognition functional and provide a novel means for the control of self-assembled structures.

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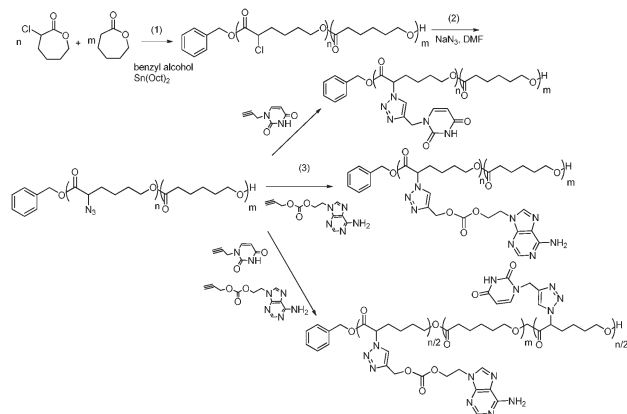
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¹³C NMR in carbonyl region of the random copolymer. See DOI: 10.1039/c3ra41412d



Scheme 1 Synthetic route of the poly(ϵ CL-g-nucleobase) copolymer. Reagents and conditions: (1) benzyl alcohol, Sn(Oct)₂, toluene, 120 °C; (2) NaN₃, DMF, 60 °C; (3) alkynyl nucleobase, CuBr, PMDETA, DMF, 60 °C.

Syntheses and characterizations of nucleobase-functional polycaprolactone

Nucleobase-functional polycaprolactone was synthesized in three steps as shown in Scheme 1 (See supporting information† for further details).

First, the ring-opening copolymerization of α Cl ϵ CL and ϵ CL was carried out using tin octoate as transesterification catalyst. This metal catalyst is one of the most common catalysts for the polymerization of lactones and lactides. The pendent chlorides of poly(α Cl ϵ CL-*co*- ϵ CL) was then converted into azides by reacting with excess sodium azide in DMF at 60 °C overnight. The IR spectrum shows a new absorption at 2106 cm⁻¹ characteristic of the azide in Fig. S5, ESI†. ¹H NMR confirms that the CHCl proton peak shifts from 4.25 ppm to 3.8 ppm (Fig. S6, ESI†). Finally, the click reaction was performed first by introduction of the azide-functional prepolymer as a concentrated solution in DMF to a vigorously stirring solution of poly(α N₃ ϵ CL-*co*- ϵ CL) at 60 °C, followed by the addition of CuBr and PMDETA. Uracil-grafted random copolymer with 1,2,3-triazole linkages were passed through neutral aluminum oxide, followed by precipitation into methanol. Successful incorporation of side-chain uracil moieties was confirmed using ¹H NMR and FT-IR spectroscopy. Upon triazole formation with propargyl uracil, the azide stretch at 2106 cm⁻¹ was completely disappeared, direct evidence of complete functionalization of all the azide sites of the poly(α N₃ ϵ CL-*co*- ϵ CL). Fives new resonances characteristic peaks of the poly(ϵ CL-g-Uracil) were observed in ¹H NMR spectrum at 11.28 ppm (o, -CO-NH-CO-), 8.19 (k, -N-CH-), 7.71(m, -N-CH-), 5.57 (n, -CO-CH-), and 4.95 (l, -N-CH₂-). In addition, several new resonances characteristic peaks of the poly(ϵ CL-g-Adenine) were observed in ¹H NMR spectrum at 8.1–8.2 (k, 2H, HC=C-), 8.0–8.1 (o and p, 2 H, ArH), 7.4–7.5 (q, 2H, s, NH₂), 5.4–5.5 (a, -CO-CH-), 5.1–5.2 (l, -COO-CH₂-), 4.1–4.2 (n, 2H, t, -N-CH₂-), and 3.3–3.4 (m, 2H, t, -C=O-O-CH₂-). Molecular weights and molecular weight distributions of these nucleobase-functional polycaprolactones are listed in Table S1, ESI†. In addition, the number of repeat units for α Cl ϵ CL and ϵ CL segments are also calculated from the ¹H NMR integration of

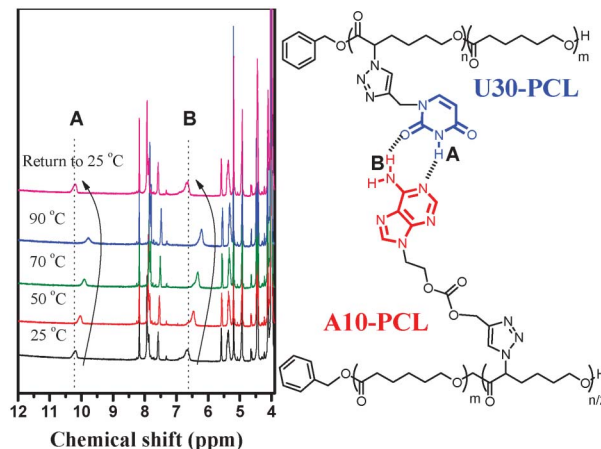


Fig. 1 ¹H NMR NH chemical shift of the U30-PCL/A10-PCL complexes as a function of temperature (concentration : 10 mg L⁻¹).

the signal at 4.2–4.3 ppm [poly(α Cl ϵ CL-*co*- ϵ CL), ¹H per repeating unit, -CO-ClH-] and 4.1–4.2 ppm [poly(α Cl ϵ CL-*co*- ϵ CL), 2H per repeating unit, -COO-CH₂-]. In terms of nomenclature, the descriptor U30-PCL, for example, represents a copolymer containing 30 mol% of uracil. Table S1, ESI† summarizes the monomer feed ratios and the compositions, molecular weights, glass transition temperatures, and thermal decomposition temperatures of the synthesized copolymers.

Reversibility behaviors in supramolecular polymers

The reversibility of supramolecular polymer formation was investigated by using variable-temperature ¹H NMR spectroscopy in 1,4-dioxane-*d*₈ because the ability to form or break the hydrogen bonding by external stimuli is a key reason for the interest in these materials. The temperature dependence of the NH proton chemical shift of the U30-PCL/A10-PCL complex at 10 wt% in 1,4-dioxane-*d*₈ is shown in Fig. 1. The NH resonance shifts to upfield region systematically from 10.3 to 9.7 ppm as the temperature is raised from 25 to 90 °C. The gradual decrease in the intensity of the NH resonance with increasing temperature can be attributed to the partial rupture of complementary hydrogen bonds.²⁷ However, as soon as the sample is cooled from 90 to 25 °C, the NH resonance returns to its original position at 10.3 ppm, suggesting that the nucleobase-grafted supramolecular complexes are thermoreversible in 1,4-dioxane-*d*₈. Similar results were verified in ¹H NMR titration experiments of the U30-PCL/A10-PCL blends (Fig. S7, ESI†). The position of the uracil N-H resonance in U30-PCL shifts with increasing the concentration of A10-PCL. These observations are consistent with the formation of the complexes driven by the adenine–uracil (A–U) recognition.

To further investigate the self-assembly, we performed dynamic light scattering (DLS) to determine the supramolecular structures formed in DMF solution at 20 °C (Fig. S10, ESI†). We carried out the experimental correlation function using the cumulant method and the CONTIN algorithm as described previously.²⁸ The Stokes–

Einstein approximation was used to estimate diffusion coefficients for each sample that exhibited hydrodynamic diameter (D_h). According to Fig. S10, ESI†, we attributed these larger particles of U30-PCL and A30-PCL, having D_h values ranging from around 360 nm to 480 nm, to the large aggregates formed through self-complementary uracil-uracil (U-U) and adenine-adenine (A-A) interactions, respectively. The DLS analysis of the 50/50 U30-PCL/A30-PCL complex shows substantial changes in size and distribution of the aggregates. The appearance of a broad distribution peak with an average particle size of 514 nm comes from the complexation of U30-PCL with A30-PCL and causes disappearance of U30-PCL and A30-PCL peaks. Comparative experiment on the Cl30-PCL solution resulted in no detectable DLS signal, which is a good solvent for Cl30-PCL homopolymer; therefore, the change in size of the micellar aggregates within U30-PCL/A30-PCL blend is shifted to higher D_h values, indicating the formation of A-U pairs by induced hierarchical self-assembly.²⁹

In addition, variable-temperature FTIR spectroscopy was also employed to investigate specific interactions between polymers at various temperatures (from 25 to 180 °C, Fig. S9, ESI†). This observation further indicates that the U30-PCL/A30-PCL blend form highly stable hydrogen-bonded complexes. (variable-temperature FTIR results are described in more detail in the supplementary information†).

Self-assembly of biomimetic copolymer polycaprolactone in bulk state

Because poly(ϵ CL-*g*-Uracil) and poly(ϵ CL-*g*-Adenine) could form a bio-complementary system, we examined the self-assembly of these complexes in the bulk state using DSC, WAXD and AFM measurements. Fig. 2A displays DSC curves recorded at temperatures ranging from -80 to 150 °C for Cl30-PCL and nucleobase-grafted PCL. The glass transition temperature (T_g) of Cl30-PCL is *ca.* -55 °C and the onset of melting zone is at 5 °C. The endothermic peak of Cl30-PCL shifts to lower temperature, suggesting that the pendant chloride retards or even inhibits

PCL crystallization. The glass transition temperatures of these two copolymers both increase upon increasing the contents of nucleobase units.³⁰ It is notable that incorporation only 30% of uracil and 30% of adenine into the PCL copolymer chain results in T_g increase of 32 °C relative to that of pure PCL, implying that this system features physical cross-linkage through strong multiple hydrogen bonds.

A single compositionally dependent glass transition implies full miscibility of the blend at dimension on the order of 20–40 nm. Fig. 2B displays DSC thermograms of A-PCL/U-PCL = 50/50 blends, where the A-PCL and U-PCL components contain various contents of adenine and uracil, respectively. These binary blends of U-PCL and A-PCL exhibit only one glass transition temperature, revealing that they are completely miscible. For instance, the T_g of pure A30-PCL and U30-PCL are *ca.* 7 and 3 °C, respectively. The T_g of the 50/50 A30-PCL/U30-PCL blend at 13 °C is higher than the those to most of their original components, implying that A30-PCL associated strongly with its complement U30-PCL, and that A-U interactions were more favorable than either U-U or A-A interactions. In addition, the T_g behavior of copolymers also show similar trend as the blend system, the T_g of AU30-PCL copolymer (32 °C) is higher than T_g of Cl30-PCL (-55 °C) (Fig. 2A). In other words, the value of T_g shifts higher upon increasing the adenine content in the copolymer through strong multiple hydrogen bonding interactions between the A and U units.

We observed similar results in the WAXD analyses presented in Fig. S8, ESI†, where the Cl30-PCL exhibited two intensive peaks at 21.6 and 23.9°, corresponding to the (110) and (200) planes of the orthorhombic crystal form.³¹ The Cl30-PCL displays the same crystalline structure as linear PCL as shown in Fig. S8, ESI†, indicating that the pendant chloride does not change the crystalline conformation of PCL. Az30-PCL also displays the same crystalline structure as Cl30-PCL according to the results in Fig. S8, ESI†. The diffraction pattern of PCL crystallization disappears for U30-PCL, implying that the nucleobase component retards or even inhibits the PCL crystallization in the complex system. These results demonstrate that the thermal properties of the nucleobase-grafted polymers depends on the introduction of the nucleobase units to some extent reduce crystallinity in the materials.

The crystalline morphologies of linear PCL, Cl30-PCL, Az30-PCL, A30-PCL, U30-PCL and A30-PCL/U30-PCL were investigated by polarized optical micrographs. According to Fig. S11, ESI†, the linear PCL shows a typical large spherulitic morphology and Maltese cross patterns, indicating that the PCL homopolymer has strong crystallizability. As for the Cl30-PCL, the size of spherulites is significantly smaller than that of the linear PCL, and no Maltese cross pattern is found in Fig. 3. This observation suggested that the presence of pendant chloride in the copolymer interfere the natural growth of the spherulites. The crystalline morphology of the Az30-PCL is similar to the Cl30-PCL, many small crystals are observed. The graft structure of the copolymer and the mutual influence between the PCL and pendant groups reduce the movement ability of PCL chains, therefore, the formation of large spherulites is difficult and only small crystals are formed. However, careful examination shows the disappearance of these large spherulites and the appearance of new aggregated spheres.

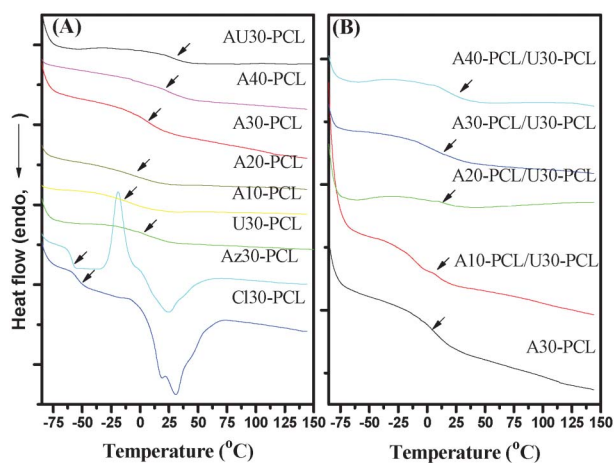


Fig. 2 (A) DSC thermograms of Cl30-PCL, Az30-PCL, A30-PCL, U30-PCL, and AU30-PCL copolymers. (B) DSC thermograms of A-PCL/U-PCL binary blends.

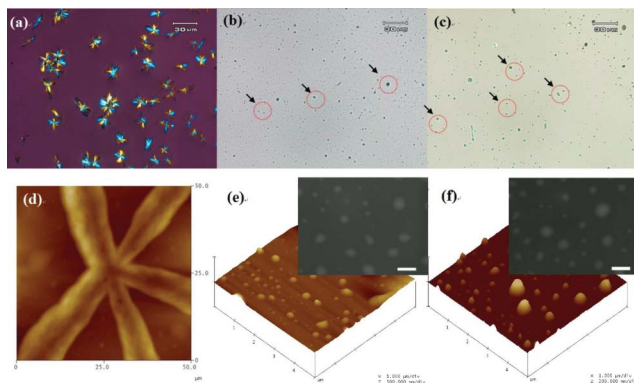
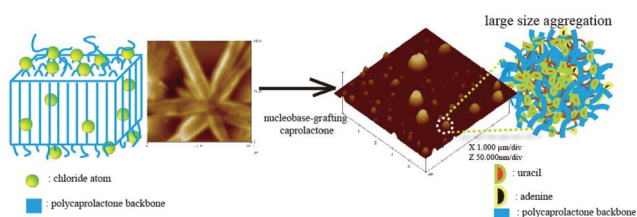


Fig. 3 Polarized optical micrographs of (a) Cl30-PCL (b) U30-PCL (c) A30-PCL/U30-PCL. AFM height images of (d) Cl30-PCL (e) U30-PCL (f) A30-PCL/U30-PCL. Shown in the insets are TEM images. The scale bars in TEM images: 1 μm .

We used atomic force microscopy (AFM) to further characterize the different crystalline morphologies of polymer with different structures. As shown in Fig. S12, ESI†, the spherulites are relatively larger for the linear PCL, indicating that it has higher crystallizability. In contrast, Cl30-PCL forms star-like objects instead of spherical (Fig. 3), suggesting that the PCL crystals grow preferentially in some directions due to the presence of chloride group at the polymer side chain. This observation can be explained in terms of the reduction in the fraction of crystallization of copolymer. For those nucleobase-grafted copolymers and their blends, two distinct aggregations corresponding to complexation-induced morphologies are observed, implying the formation of hydrogen bonding between A-U base pairs within polymer systems. AFM and TEM images in Fig. 3 show the formation of sphere structure of 200–600 nm in diameter. These results revealed that the A30-PCL/U30-PCL systems exhibited a larger and more densely packed aggregation of particles. In other word, the highly cross-linked structure is restrained due to the intermolecular fixing effect from complementary adenine–thymine (A–T) hydrogen-bonding. Thus, we can also depict a lamellar crystallization in which the molecules are arranged into star-like morphology. Furthermore, nucleobase-graft polycaprolactone is able to form heterocomplexes *via* biocomplementary hydrogen bonds (Scheme 2).



Scheme 2 Graphical representation of star-like morphology and hydrogen bonding-mediated aggregate formation.

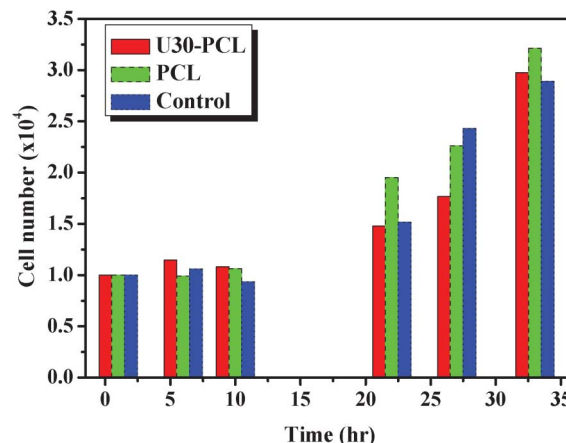


Fig. 4 The number of mouse fibroblast L929 cells during culture after 2 days on (a) U30-PCL, (b) PCL, and (c) Control (Cell Desk LF1).

Biocompatibility of polyester-graft-nucleobase copolymers

An evaluation of novel supramolecular polymers possessing biocompatibility are critically for exploring the potential of new materials in biomaterial applications such as tissue engineering. In this study, L929 cells were chosen as the test cells to evaluate the cytotoxicity of the novel polyester-graft-nucleobase copolymer. This was accomplished by first growing a monolayer of fibroblasts (L929 mouse fibroblasts obtain from Japanese Collection of Research Bioresources) to near confluence in modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum, then introducing the polyester-graft-nucleobase with fresh medium to give a final concentration of 10 mg mL⁻¹. The cell numbers on the PCL and U30-PCL were evaluated after 2 days of incubation in Fig. 4, resulting in both higher numbers than the FC-1 commercial cell as a control. U30-PCL also held fewer cells than the pure PCL. It could be considered that both hydrophilic and physical cross-linking factors were simultaneously reflected on these surfaces. However, the additional requirement for a potential hydrogen-bond group suggestions that the presence of uracil did not affect the cell adhesion significantly. After 24 h incubation, the monolayer was observed microscopically for changes in morphology. Fig. 5 shows the phase-contrast micrographs for graft polyester (positive and negative controls are also shown). The cells adhered and spread better and proliferated faster on dilute solution than on origin solution. No obvious change in monolayer confluence and morphology was observed in graft polyester as compared to the negative control, indicating no significant cytotoxic response of the L929 cells for the nucleobase-grafted polyesters. Therefore, the hydrogen-bonding units leave room for a modular approach to biofunctionalization of the materials, combining the advantages of tuning the thermal properties of the material and the nature of the bioactive compound used. We are continuing to study these systems to elucidate the factors affecting the relationship between the relative magnitudes of non-covalent interactions and biocompatibility activity that feature reversible cross-linking networks mediated by

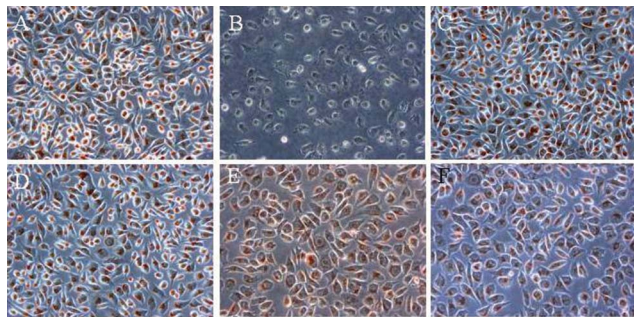


Fig. 5 Phase contrast optical microscopy from minimal essential medium cell culture experiments using L929 fibroblasts in (A) Dulbecco's Phosphate-Buffered Saline (negative control); (B) 1% phenol (positive control); (C) polyester-graft-uracil copolymer (origin solution); (D) polyester-graft-uracil copolymer (two-fold dilution of the origin solution); (E) polyester-graft-adenine copolymer (origin solution); (F) polyester-graft-adenine copolymer (two-fold dilution of the origin solution).

nucleobase hydrogen bonding. Our results will be reported in due course.

In summary, ring-opening polymerization and click chemistry were employed to synthesize biotailored graft copolymers poly(ϵ CL-*g*-Uracil), poly(ϵ CL-*g*-Adenine), and poly(ϵ CL-*g*-Uracil/Adenine). Grafting these multiple hydrogen bonding motifs onto poly(ϵ -caprolactone) dramatically retards crystallization due to the formation of supramolecular polymers. From molecular recognition experiments, the interactions between adenine and uracil through stronger cooperative hydrogen bonding has been identified. Morphological development of the nucleobase-functionalized copolymers was studied using AFM and TEM, which revealed discrete nano-scale spherical aggregates compared to the individual precursors. The biomimetic graft polyesters prepared in this study are shown to be biocompatible by *in vitro* cytotoxicity evaluation, suggesting their suitability for potential biomedical engineering applications.

Acknowledgements

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