

Synthesis and Characterization of Chitosan-graft-Poly(lactic acid) Copolymer^{*}

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Abstract Chitosan-graft-poly(lactic acid) (CS-g-PLA) copolymer was synthesized through emulsion self-assembly in a water-in-oil (W/O) microemulsion. The water phase was composed of CS aqueous solution, while the oil phase was made up of PLA in chloroform. The W/O microemulsion was fabricated in the presence of surfactant span-80 and the self-assembly was performed between PLA and CS under the effect of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl). FTIR and ¹H-NMR analysis indicated PLA was grafted onto the backbone of CS *via* the reaction between the carboxyl groups in PLA and the amino groups in CS. ¹H-NMR characterization further revealed the grafting content of PLA was 16%. The obtained CS-g-PLA could self-assemble to form micelles, their size distributed in the range of 125–375 nm with average diameter of 142 nm. The present design integrates the favorable biological properties of CS and the excellent mechanical properties of PLA, which makes CS-g-PLA copolymer a promising candidate as a carrier for targeted bioactive molecules delivery.

Keywords: Poly(lactic acid); Chitosan; Copolymer; Self-assembling; Grafting.

INTRODUCTION

Composite materials play an increasingly important part in diverse biomaterial-related applications, ranging from drug delivery systems and cell adhesion mediators to control of enzyme function and gene expression^[1–3]. Particularly, amphiphilic composites derived from natural and synthetic biodegradable polymers, capitalizing on the favorable properties of these two kinds of biomacromolecules, are the prerequisite of biomaterial-induced tissue regeneration^[4–6]. However, the development of such amphiphilic composites remains a great challenge due to the immiscibility of the component polymers^[7]. Even so, several attempts were made to prepare the natural-synthetic amphiphiles by using traditional solution polymerization^[8, 9]. Regrettably, to the best of our knowledge, the composites synthesized by the conventional methods exhibited irreproducible material properties as a result of unavoidable heterogeneous reaction.

In this paper, an emulsion self-assembly technique was brought forward, with the purpose of developing

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natural-synthetic amphiphilic composite comprised of chitosan (CS) grafted with poly(lactic acid) (PLA). It should be noted that, CS and PLA could effectively offset the drawbacks with each other if they were well blended together^[10]. CS, a natural extracellular matrix polysaccharide, is extensively investigated for a variety of biomedical applications due to its non-toxicity and favorable cell affinity, antimicrobial activity and other biological properties^[11, 12]. However, it is thermolabile and has poor mechanical and processing properties^[13]. Besides, it is insoluble in common organic solvents and pure water and only dissolvable under weak acidic conditions^[14]. The complementary polymer used in this study, PLA, is a synthetic biodegradable aliphatic polyester, which is also widely studied and used as biomaterials^[15, 16]. Although PLA is an eco-friendly macromolecule with excellent processability and biocompatibility, it has disadvantages as well, predominant by strong hydrophobicity, uncontrollable degradation, and lack of reactive lateral chain groups^[17, 18]. The present design provides an amphiphilic composite where individual deficiencies of each biopolymer can be complemented by the presence of the other one. Particularly, PLA can possibly enhance the mechanical strength of CS and in turn, the latter can provide the former with properties such as hydrophilicity, functional groups (primary amine and hydroxyl groups). Besides, as a weak alkaline polysaccharide, CS can also neutralize the acidity of PLA during degradation and further regulate its degradation rate^[19, 20].

EXPERIMENTAL

Materials

PLA ($M_w = 10$ kDa) was prepared by using melt ring-opening polymerization of lactide initiated by stannous octoate at 140 °C in vacuum, and purified according to the method reported previously^[17]. CS (viscosity 0.2–0.8 Pa·s) with 75%–85% degree of deacetylation and EDC·HCl were purchased from Sigma-Aldrich Co., and the former was refined by dissolving in aqueous acetic acid solution and precipitating from dilute ammonia. Chloroform was distilled from phosphorus pentoxide shortly before use. Span-80, 1-hydroxybenzotriazole (HOBt) and all other chemicals were analytical grade and used as received.

CS and HOBt Aqueous Solution System

CS alone is insoluble in deionized water. Here, in order to make it dissolved in aqueous solution, a constant amount of CS (0.10 g) was mixed with various quantities of HOBt in 20 mL deionized water. The mixture was magnetic stirred overnight at ambient temperature. Afterward, the optimized proportion was achieved according to the solubility of CS and HOBt in deionized water.

Synthesis of Chitosan-graft-poly(lactic acid) (CS-g-PLA) Copolymer

The whole emulsion self-assembly of CS-g-PLA copolymer involved two sequential steps. First, a W/O microemulsion was prepared by homogenizing water and oil phases in the presence of surfactant span-80. In a representative experiment, PLA (6.0 g) was dissolved in 60 mL chloroform and used as oil (outer) phase. The surfactant span-80 (1.20 g) and coupling agent EDC·HCl (0.34 g) were also added in oil phase. CS (0.10 g) and HOBt (0.08 g) were dispersed in 20 mL deionized water. The obtained transparent CS-HOBt aqueous solution was passed through paper filter to remove the possible insoluble substances and served as water (inner) phase. Under vigorous magnetic stirring, the water phase of CS-HOBt system was added dropwise to the oil phase of PLA solution. The mixture was emulsified at 10000 r/min for 3 min in a homogenizer and the W/O microemulsion was formed. Secondly, the self-assembly was carried out between amino groups of CS and end carboxyl of PLA under the effects of EDC. The reaction was allowed to proceed at ambient temperature for 24 h with constant magnetic stirring. Thereafter, the microemulsion was demulsified and the upper water phase solution was dropped into excess amounts of anhydrous alcohol. The obtained precipitate was further washed with anhydrous alcohol for two times, followed by lyophilizing with a freeze dryer. The various experimental parameters, including CS, PLA and span-80 concentration, emulsifying speed and time, were investigated to get the optimal reaction conditions.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR spectra of CS, PLA and CS-g-PLA graft copolymer were collected on an attenuated total reflectance Fourier transform infrared spectrometer (Nicolet 560, USA). Every spectrum was recorded in transmission mode with a resolution of 4 cm^{-1} and a spectral range of $4000\text{--}400\text{ cm}^{-1}$.

Proton Nuclear Magnetic Resonance ($^1\text{H-NMR}$) Characterization

The $^1\text{H-NMR}$ spectra were recorded using a JNM-ECA600 spectrometer (JEOL, Japan). Both CS and CS-g-PLA copolymer were dissolved in $\text{CF}_3\text{COOD}/\text{D}_2\text{O}$ solvent.

Aggregating Behavior

CS-g-PLA copolymer micelles were prepared by using phase separation dialysis method. Briefly, a certain amount of CS-g-PLA were dissolved in DMF to a final concentration of 2.5 g/L and 1 mL copolymer solution was added dropwise into 10 mL deionized water under magnetic stirring. The resultant solution was transferred into a dialysis membrane (molecular weight cut-off 12 kDa) and dialyzed against deionized water for 48 h to remove DMF. Thereafter, size and size distribution of the obtained micelles were measured by a Zetasizer 3000HS (Malvern, UK) at $25\text{ }^\circ\text{C}$ with a wavelength of 633 nm .

RESULTS AND DISCUSSION

Consolute of CS and HOBt in Aqueous Solution

To investigate the co-existing condition, CS and HOBt were mixed together in 20 mL deionized water at various ratios. The structural unit of CS is $\text{C}_6\text{H}_{11}\text{O}_4\text{N}$ and its molar mass is 161 g/mol . Therefore, 0.10 g CS is nearly equal to 0.6 mmol . It can be seen from Fig. 1, when the molar ratio of CS and HOBt was in the range of $3:2.5$ to $3:3$, both of them could be full dissolved in deionized water and the obtained solution was transparent. However, once CS/HOBt ratio was lower than $3:3$, most CS could be dissolved, but HOBt could not. On the contrary, if the ratio was higher than $3:2.5$, most HOBt could be dissolved, but CS could not. Thinking that the extra HOBt was favorable for the subsequent coupling reaction, CS/HOBt ratio at $3:3$ was used in the following experiments.

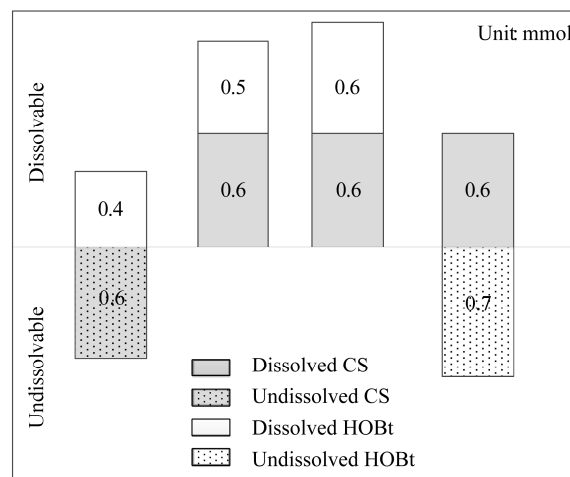


Fig. 1 Solubility of CS and HOBt mixture in deionized water
The amount of CS is 0.6 mmol and the corresponding amounts of HOBt were 0.4 , 0.5 , 0.6 and 0.7 mmol , respectively.

Usually, to synthesize CS-g-PLA copolymer through solution polymerization, CS needs to be dissolved in suitable solvent. Although carboxylic acids, such as formic and acetic acids, are good solvents for CS, these kinds of organic acids always associate by side reactions due to the active carboxyl groups, especially in the cases that carbodiimide is used as coupling agent^[21]. Therefore, non-carboxylic acid CS aqueous system was prepared in the present research. Normally, HOBt was also hard to be solubilized in aqueous environment and only served as a carboxyl activating agent in carbodiimide-mediated coupling reactions. Once CS and HOBt

were simultaneously added into deionized water at proper ratios, the transparent solution was formed. Moreover, the pH value of this solution was found to be 5.5, which was beneficial to the following self-assembly experiments.

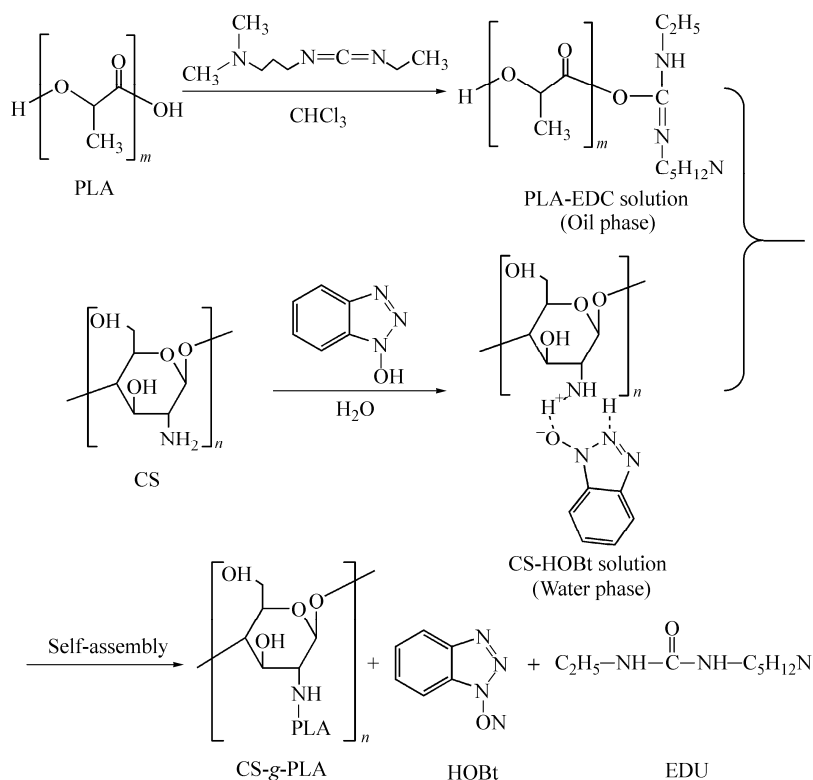
Emulsion Stability Determination

To maximize the amounts of PLA in CS-g-PLA copolymer, W/O microemulsion was developed and the outer phase contained excessive PLA component. The stability of CS and PLA-based emulsification system depended on a number of factors, and some crucial parameters are listed in Table 1. Primarily, the concentration of CS and PLA determined the droplets size and its distribution. Therefore, to acquire the stable and uniform microemulsion, the concentration of CS should be in the range of 5–10 g/L, while that of PLA was 100 g/L. Surfactant was another decisive factor which influenced emulsion stability. Thinking that low-dose of span-80 failed to induce sufficient effects of emulsification while high-dose was difficult to be removed in the subsequent purification process, the amount of 20 g/L was used in the present system. Besides, despite high-speed stirring and long-term dispersion was beneficial to the homogeneity, they also associated with the increased temperature and the accelerated movements of emulsion droplets and eventually resulted in an instable emulsion. As a result, the emulsifying speed of 10000 r/min and the time of 2–3 min were chosen, which could assure the microemulsion remained homogeneous and did not present apparent phase separation within 60 min at ambient temperature.

Table 1. Influence of experimental parameters on stability of CS and PLA-based microemulsion

Experimental parameter	Investigating range	Optimal value ^a
CS concentration (g/L)	5–30	5–10
PLA concentration (g/L)	50–200	100
Span-80 concentration (g/L)	5–50	20
Emulsifying speed (r/min)	5000–25000	10000
Emulsifying time (min)	1–10	2–3

^a The data were acquired from emulsion stability test (not less than 60 min) at ambient temperature.

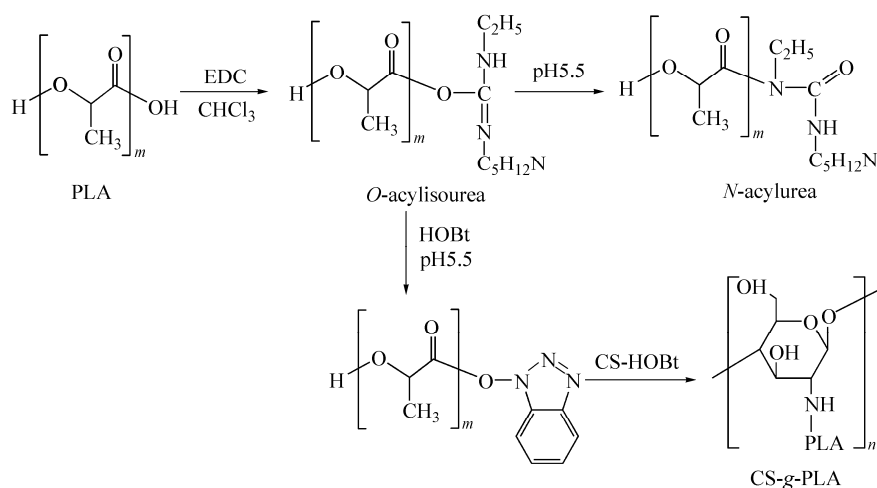


Scheme 1 Synthesis of CS-g-PLA copolymer by emulsion self-assembly strategy

Preparation of CS-g-PLA Copolymer via Emulsion Self-assembly

The emulsion self-assembly of CS-g-PLA copolymer is presented in Scheme 1. The carboxyl end groups of PLA were first activated by EDC to produce an intermediate *O*-acylisourea in chloroform solution. The amino groups of CS were also protonated by HOBt to form an organic complex in aqueous solution. With the emulsification between water and oil phase solution, the *O*-acylisourea was further reacted with the CS-HOBt complex and EDC was transferred to the hydrolyzed urea derivative EDU. After being thoroughly washed with ethanol to remove urea by-products, HOBt and span-80, CS-g-PLA copolymer was obtained.

According to the early report, direct carbodiimide-mediated coupling between carboxyl and amine groups in an aqueous environment could not effectively yield the predicted amide product^[22]. The unstable intermediate *O*-acylisourea was readily formed under mild acidic conditions, which rearranged rapidly by a cyclic electronic displacement to a stable *N*-acylurea in the absence of nucleophiles^[23]. This O→N migration of *O*-acylisourea also occurred when the nucleophile was a primary amine. In the present study, the use of chloroform, a solvent with low-dielectric constant, could minimize this side reaction. More importantly, the excess amounts of HOBt in emulsification system served to avoid this kind of rearrangement, since it was widely used in carbodiimide-mediated amide formation as an activating agent to increase yields and decrease side reactions. HOBt could convert unstable *O*-acylisourea to a more hydrolysis-resistant and non-rearrangeable active ester intermediate (Scheme 2). Nucleophilic addition to the ester formed from HOBt required the amine to be presented in an unprotonated form at acidic pH (about 5.5–7.0). Consequently, the CS-g-PLA copolymer was formed.



Scheme 2 The detail of HOBt-involved emulsion self-assembly strategy

FTIR Spectra

The FTIR spectra of CS-g-PLA copolymer and the corresponding CS and PLA components are shown in Fig. 2. The main bands of CS were as follows (Fig. 2a): a broad strong band around 3400 cm^{-1} ($-\text{OH}$ and $-\text{NH}$ stretch), a weak band at 2875 cm^{-1} ($-\text{CH}$ stretch), two middle strong bands at 1655 and 1597 cm^{-1} (amide I and II, respectively), 1153 and 896 cm^{-1} (saccharide structure), 1423 , 1381 , 1323 and 1259 cm^{-1} ($-\text{OH}$ and $-\text{CH}$ bend). Comparatively, two appreciable changes occurred in the spectrum of CS-g-PLA (Fig. 2b). A new characteristic peak appeared at 1759 cm^{-1} , which corresponded to the $\text{C}=\text{O}$ stretch in ester band of the PLA grafts (Fig. 2c). Meanwhile, the peak intensity around 3400 cm^{-1} for hydroxyl and amino groups of CS component decreased pronouncedly. These registered events suggested that PLA was grafted onto the backbone of CS through the interactions between end carboxyl and amino groups of the two components.

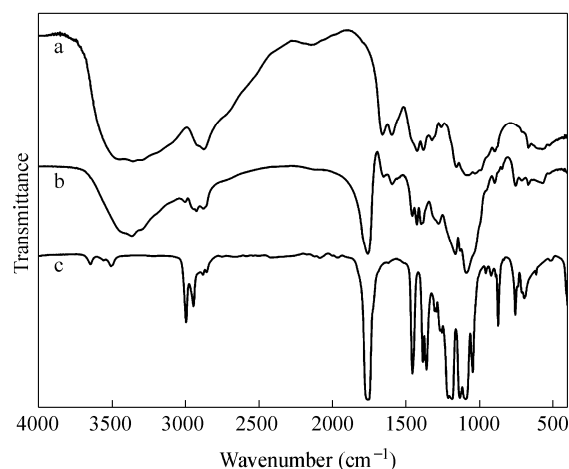


Fig. 2 FTIR spectra of CS (a), CS-g-PLA (b) and PLA (c)

¹H-NMR Spectra

The ¹H-NMR spectra of CS and CS-g-PLA copolymer are shown in Fig. 3. CS showed a singlet at $\delta = 3.01$ (H-2), multiplets between $\delta = 3.40$ and 3.74 (H-3, H-4, H-5 and H-6) and a small singlet at $\delta = 4.69$ (H-1). The signal at $\delta = 1.91$ (H-7) was due to the existence of *N*-acetylglucosamine units of chitin. Comparatively, the ¹H-NMR spectrum of CS-g-PLA graft copolymer showed the chemical shifts centered at $\delta = 1.29$ (H-a) and 1.04 (H-a'), which were corresponded to methyl (CH₃) in PLA main chain and adjacent to end hydroxyl groups, respectively^[24]. Meanwhile, the new peak at $\delta = 2.76$ (H-2') revealed the appearance of amide linkage, which further confirmed the successful self-assembly reaction between CS and PLA components. Regrettably, chemical shift corresponding to methine (CH) in PLA backbone was overlapped by D₂O. Here, the degree of substitution (DS) of PLA onto CS chain was also determined based on the ¹H-NMR results. The integral peaks for H-2 and H-2' of glucopyranose ring were the peaks of interest. In the case of Fig. 3, the integral ratio of H-2' to the sum of H-2' and H-2 was $0.19/(0.19 + 0.98)$. This confirmed that the PLA substitution was 16%.

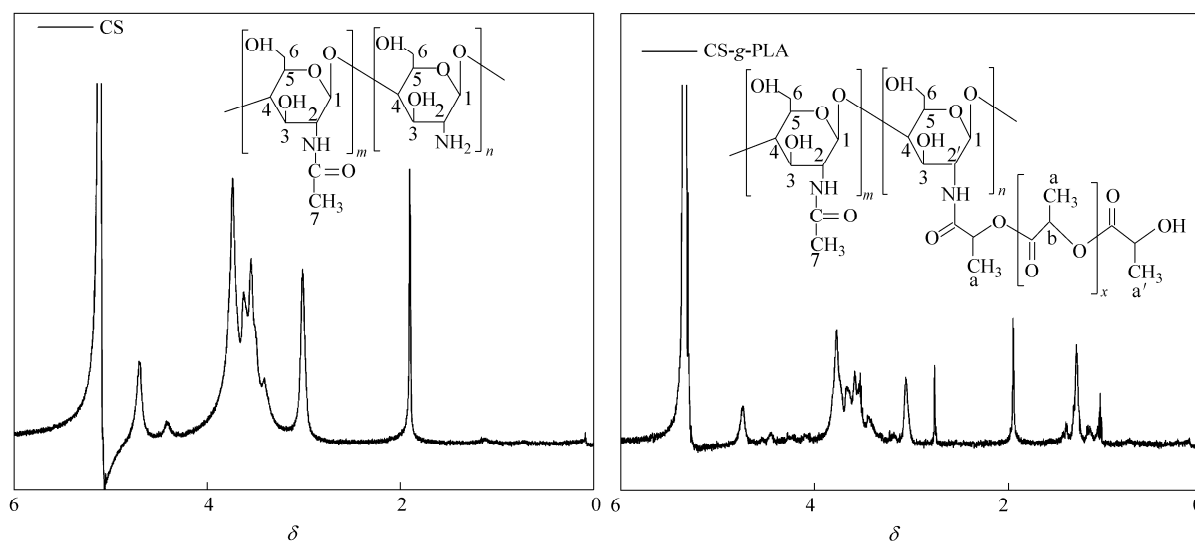


Fig. 3 ¹H-NMR spectra of CS and CS-g-PLA copolymer in CF₃COOD/D₂O

Aggregation of CS-g-PLA Copolymer

The aggregating behavior of CS-g-PLA graft copolymer in aqueous media and its size distribution are presented in Fig. 4. Since CS main chain was hydrophilic while PLA side chain was hydrophobic in nature, the obtained

copolymer tended to form micelles in appropriate solvents. In a typical procedure, CS-g-PLA was dissolved in DMF at a concentration of 2.5 g/L and the resultant solution was dialyzed against deionized water to remove most of the DMF. The size of obtained micelles distributed in the range of 125–375 nm with average diameter of 142 nm, which was determined by the dynamic light scattering measurements (Fig. 4).

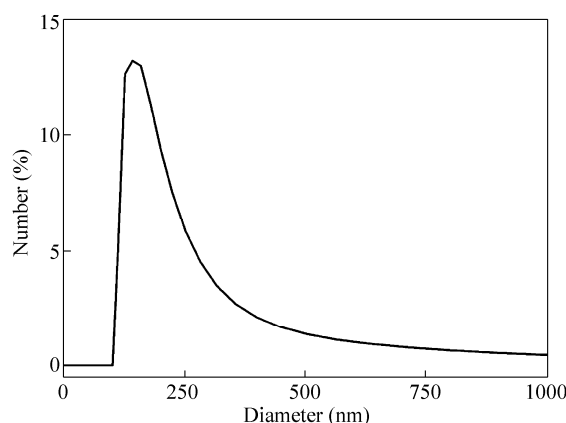


Fig. 4 The size distribution of CS-g-PLA copolymer in deionized water

In addition, compared with the insoluble nature of the original CS in common organic solvents, CS-g-PLA exhibited an improved solubility in a series of nonpolar and polar solvents (data not shown). This revealed that the PLA side chains had significant effects on the hydrophilic/hydrophobic balance of CS-g-PLA copolymer. Moreover, it could reduce the intermolecular and intramolecular hydrogen bonds of CS backbone. The improved solubility facilitated CS-g-PLA to be fabricated into various delivery systems for biomedical application.

CONCLUSIONS

In summary, hydrophobic PLA side chain has been grafted successfully onto hydrophilic CS backbone through an emulsion self-assembly method. The grafting reaction is carried out in W/O microemulsion. The appearance of HOBt makes CS dissolved easily in deionized water and further facilitates the interaction between end carboxyl in PLA and amino groups in CS under the effect of coupling agent EDC. The content of PLA in CS-g-PLA copolymer is 16%. The present design combines the advance in emulsion polymerization with the new progress in supramolecular self-assembly technology. It can effectively avoid the undesirable heterogeneous reaction and unstable composite structure, while integrating the favorable biological properties of CS and the excellent mechanical properties of PLA, which makes CS-g-PLA a competitive candidate for a broad array of drug delivery application.

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