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A Facile Approach for Controlled Modification of Chitosan under γ-Ray Irradiation for Drug Delivery

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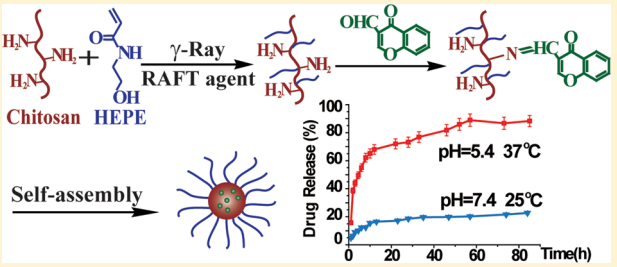
Supporting Information

ABSTRACT: (1→4)-2-Amino-2-deoxy-β-D-glucan (i.e., chitosan) is an abundant natural polysaccharide with huge availability and potential for biomedical applications due to its biocompatibility, biodegradability, and bioactivity, but its utilization in pharmaceutical formulations has been greatly limited by its intractability. We report here a novel, facile method of controlled modification of chitosan under γ-ray irradiation for drug delivery. Specifically, *N*-(2-hydroxyethyl)prop-2-enamide (HEPE) was grafted onto chitosan via a "one-pot" reversible addition−fragmentation chain transfer process under γ-ray irradiation, and then the unprotected amino group on chitosan was straightly used for the conjugation of chromone-3-carboxaldehyde. Importantly, there are no traditional protection−deprotection processes for amino groups of chitosan in this method. The conjugated graft copolymer can self-assembly into the micelles with the size of ∼170 nm in distilled water, and the chromone release profile shows the graft copolymer could be used as a pH- and thermo-responsive carrier for drug delivery.



1. INTRODUCTION

(1→4)-2-Amino-2-deoxy-β-D-glucan (i.e., chitosan) is deacetylated chitin, a natural polysaccharide with enormous availability in the biosphere.[1] It has many admirable properties for drug delivery, such as biocompatibility, biodegradability, and bioactivity.[2] However, owing to its semicrystalline nature and multiple H-bond forming groups, chitosan is insoluble in water (when pH > 6.2) and all common organic solvents,[3] which limited adopting chitosan for drug delivery, particularly for the delivery of hydrophobic therapeutic agents. Therefore, many works are dedicated to chitosan modification to improve its properties for the applications. Among them, grafting modification with synthetic polymer has been developed on chitosan for drug delivery.[4,5] For example, Gao et al.[6] synthesized temperature- and pH-responsive chitosan material by grafting polymerization of maleic anhydride and *N*-isopropylacrylamide (NIPAM) onto chitosan. Tsibouklis et al.[7] prepared the hybrid polymer networks for ophthalmic drug delivery by the free-radical-induced copolymerization of acrylic acid-functionalized chitosan with NIPAM. However, the structures of the graft copolymers could not be well controlled for drug delivery by the reported methods.

On the other hand, great progress has been made on controlled/living free radical polymerizations in the past few decades,[8−10] which would enable a wide variety of molecular designs to afford novel types of tailored hybrid materials composed of natural polysaccharides and synthetic polymers. In the previous work, we reported the controlled graft modification of chitosan via reversible addition−fragmentation chain transfer (RAFT) polymerization using chitosan-RAFT agent[11−13] and via nitroxide-mediated polymerization using chitosan-TEMPO macroinitiator.[14] And recently, Liu et al. synthesized comb-shaped chitosan-graft-poly(*N*-isopropylacrylamide) copolymer by atom transfer radical polymerization.[15] However, the amino groups of chitosan should be reacted for improving the solubility with phthalic anhydride or tosylic acid in these procedures, and it is tedious that the subsequent deprotection was always needed for the drug conjugation after the polymerization.[16] Therefore, recently we made the hydrophobic payloads conjugate with chitosan in ionic liquid,[17] which renders chitosan easily dissolvable in common organic solvents and amenable to further functional modifications, while we noticed dextran was modified at its hydroxyls with acetal moieties[18] or arylboronic esters[19] to make it soluble in common organic solvents, allowing for the facile preparation of stimuli-sensitive carriers.

In this study, we report a novel, facile method for controlled modification of chitosan via RAFT polymerization under γ-ray irradiation. Specifically, a good biocompatible polymer poly(*N*-(2-hydroxyethyl)prop-2-enamide) (PHEPE)[20,21] was grafted onto pure chitosan under γ-ray irradiation at room temperature. During the process, graft polymerization could be well

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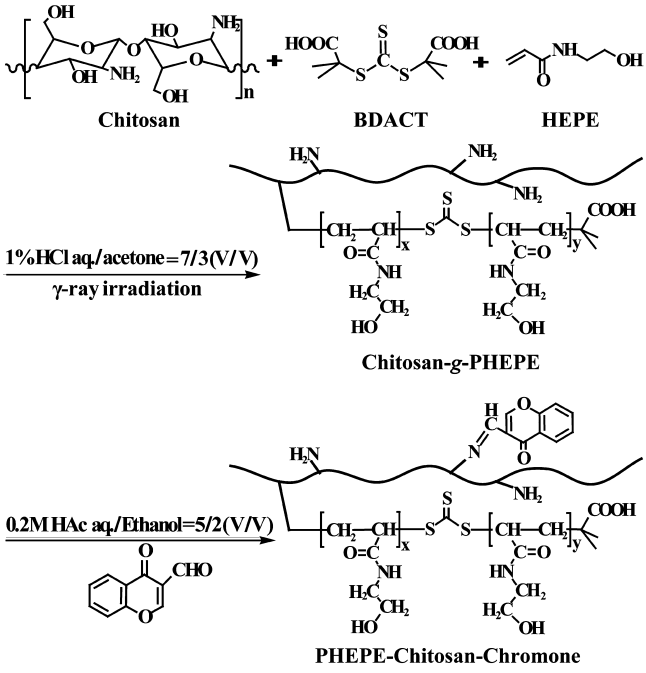
controlled with *S*,*S*′-bis(R,R′-dimethyl-R″-acetic acid)-trithiocarbonate (BDACT), and the unprotected amino group of chitosan could be straightly used for the conjugation of hydrophobic drugs with aldehyde group. In this study, an anticancer agent chromone-3-carboxaldehyde[22] was selected as the model drug to conjugate with graft copolymer by reacting with the amino group of chitosan to form Schiff base bond (Scheme 1). The Schiff base bond is cleavable via hydrolysis,

Scheme 1. Schematic for the Synthesis of PHEPE−Chitosan−Chromone



BDACT: S,S'-Bis(R, R'-dimethyl-R''-acetic acid)-trithiocarbonate
HEPE: *N*-(2-hydroxyethyl)prop-2-enamide

and the process can be accelerated at low pH conditions.[23] Therefore, the graft copolymer was expected to be used as a pH- or thermo-responsive carrier for drug delivery.

2. EXPERIMENTAL SECTION

2.1. Materials and Reagents. Chitosan (degree of decetylation = 95.2%, determined by elemental analysis, average molecular weight = 50 000 g/mol) was purchased from Golden-Shell Biochemical Co. Ltd., Zhejiang, China. *N*-(2-Hydroxyethyl)prop-2-enamide (HEPE) was purchased from TCI (Shanghai) Development Co., Ltd., and was purified by dissolving with acetone then passing through a column with base aluminum oxide and stored at −20 °C prior to use. Chromone-3-carboxaldehyde (98%) was purchased from Alfa Aesar China (Tianjin) Co., Ltd., and used as received. *S*,*S*′-Bis(R,R′-dimethyl-R″-acetic acid)trithiocarbonate (BDACT) was synthesized according to a related reference[24] (Figure S1, Supporting Information). All other chemical agents were used as received.

2.2. Characterization Methods. ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Varian INVOA-400 instrument working at 400 MHz. Fourier transform infrared (FT-IR) spectra were recorded on a Varian-1000 spectrometer; the samples were ground with KBr crystals, and the mixture was then pressed into a pellet for IR measurement. The viscosity of chitosan was measured by a viscometric method in the literature[25] using a Julabo visco 170 semiautomatic viscometer with a capillary of 0.8 mm inner diameter. The molecular weights and polydispersities of the free homopolymers were determined with a Waters 1515 gel permeation chromatograph (GPC) equipped with a differential refractometer and temperature control, using 120, 250, and 1000 Ultrahydrogel columns with molecular weight range 100−500 000 calibrated with PEO standard samples and 0.02 M $NaNO_3$ aqueous solution as the eluent at the flow rate of 0.5 mL/min. Field-emitting scanning electron microscopy (SEM) images were taken by a Hitachi S-4700 microscope operated at an accelerating voltage of 15 kV. The chromone concentrations were determined by UV−vis spectrophotometer (UV-3150, Shimadzu, Japan).

2.3. Synthesis of Chitosan-*g*-PHEPE Copolymer. A typical recipe for the graft polymerization can be described as follows: Chitosan (0.24 g, 0.03 g/mL), BDACT (0.0212 g, 0.08 mmol), and HEPE (0.8 g, 0.1 g/mL) were dissolved with 8 mL mixture of 1% HCl aqueous solution and acetone (v:v = 7:3) in a 10 mL ampule. After the contents were purged with argon for 20 min to eliminate the oxygen, the ampules were flame-sealed. Then the ampules were placed in an insulated room with a ^{60}Co source at the dose rate of 10 Gy/min. Samples were taken after different time intervals. The total radiation dose was determined by Fricke dosimeter accurately. After the graft polymerization, the reaction mixture was neutralized by aqueous ammonia and was precipitated in 10-fold acetone. The crude copolymer was collected on a filter and dried in vacuum oven at 40 °C.

The crude copolymer was then made free from homopolymer by Soxhlet's extraction with methanol for 72 h. Finally, the graft copolymers were dried in a vacuum oven at 40 °C to constant weight. The extraction solution was condensed by evaporation and then precipitated by adding into 10-fold acetone. The PHEPE homopolymers were collected by filtration and dried in a vacuum oven at 40 °C.

In addition, the polymerizations were also conducted with different chitosan and monomer concentrations to investigate their influence on the graft content (Figure S2).

In order to investigate the influence of γ-ray irradiation on chitosan chain, the control experiments using chitosan (0.24 g) with and without RAFT agent (0.0212 g) were performed in 8 mL mixture of 1% HCl aqueous solution and acetone (v:v = 7:3) under γ-ray irradiation. The reaction mixture was neutralized by aqueous ammonia and was precipitated in 10-fold acetone. Then the solid was collected by filter and rinsed thoroughly with acetone to remove unreacted RAFT agent. The resultants were characterized with viscometer and ^{1}H NMR spectrum (Table S1 and Figure S3).

2.4. Loading of Chromone onto Graft Copolymer. The chitosan-*g*-PHEPE copolymer was reacted with chromone-3-carboxaldehyde to form Schiff base bond according to the literature method:[26] Chitosan-*g*-PHEPE (0.050 g, 0.26 mmol chitosan unit) and chromone-3-carboxaldehyde (0.068 g, 0.39 mmol) were disslolved in a 7 mL mixture solution of 0.2 M acetic acid aqueous solution and ethanol (v:v = 5:2). After the mixture was placed at 55 °C for 18 h, it was precipitated in 10-fold acetone. Then it was filtrated and rinsed with acetone thoroughly to remove unreacted chromone-3-carboxaldehyde. The product was collected by filtration and dried in a vacuum oven at 40 °C. The percentage of chromone conjugation was inferred by ^{1}H NMR spectrum according to the integrated area of the characteristic peaks of chromone and chitosan. On the basis of the percentage of chromone conjugation, the weight of chromone ($W_{chromone}$) and chitosan ($W_{chitosan}$) in the conjugated graft copolymer was calculated, and the chromone loading efficiency was estimated as $LE_{chromone} = W_{chromone}/(W_{chromone} + W_{chitosan}) \times 100\%$.

2.5. Release of Chromone from the Conjugated Graft Copolymer Nanocarrier. The property of chromone release from the nanoparticles were determined in vitro as follows: 1 mL of PHEPE−chitosan−chromone conjugated copolymer solution (2 mg/mL) was put into dialysis bag (MWCO = 3500), and each of them was immersed into 30 mL of different buffers containing 20% ethanol. At a definite time interval, 5 mL of the solution outside the dialysis bag was sampled, and then 5 mL of the pure buffer solution with 20% ethanol was infused into the upper system. The buffers with different pH values were used (PBS buffer, pH = 7.4; acetate buffer, pH = 5.4).

The chromone concentration at time *i* ($C^i_{chromone}$, in mol/L) was determined by UV−vis measurement (slit = 4 nm) according to the

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Table 1. Results of Graft Polymerizations of HEPE on Chitosan under γ-Ray Irradiation

entry[a]	radiation dose (kGy)	monomer concn (g/mL)	conv[b] (%)	graft content[c] (%)	M_n[d]	M_w[d]	PDI[d]
1	4.84	0.1	83.2	46.7	7 700	9 200	1.20
2	10.79	0.1	91.0	52.9	8 000	10 100	1.26
3	15.91	0.1	92.4	59.7	8 300	10 600	1.28
4	10.79	0.15	96.9	80.9	11 800	15 600	1.32
5	10.79	0.2	98.8	88.4	16 700	22 800	1.37

[a]Entries 1−6 were performed in a 8 mL mixed solvent ($V_{1\%\ HCl}$:$V_{acetone}$ = 7:3) using 0.08 mmol of BDACT, 0.24 g of chitosan, and corresponding quantitative monomer. [b]The monomer conversion (conv, %) was calculated according to the equation conv (%) = $(W_p - W_0)/W_m \times 100\%$, where W_p and W_m stand for the weight of the crude polymer and monomer, respectively, and W_0 stands for the weight of chitosan and BDACT. [c]The graft content (G, %) was determined according to the equation G (%) = $(I_{3.4}/2)/I_{3.0} \times M_{PHEPE}/M_{chitosan}$, where $I_{3.4}$ and $I_{3.0}$ are the integration values of the peaks at δ = 3.15−3.45 ppm and 2.85−3.15 ppm, respectively, in ^{1}H NMR spectroscopy, and M_{PHEPE} and $M_{chitosan}$ are the molecular weights of PHEPE and chitosan, respectively. [d]Determined by GPC.

calibration curve (absorbance $A = 0.1088c + 0.0244$, c (mol/L), λ_{max} = 229 nm, for pH = 5.4; $A = 0.0667c - 0.0016$, c (mol/L), λ_{max} = 250 nm, for pH = 7.4) (Figure S4). The chromone release (%) at time i was calculated as $M_{chromone}(VC^i_{chromone} + \sum_{j=1}^{i-1} v_j C^j_{chromone})/W_{chromone}$, where $M_{chromone}$ is the molecular weight of vanillin, $W_{chromone}$ is the weight of chromone loaded into the tested nanocarriers initially, and V and v_j are the volume of the buffer solution and sampled solution (at time j), respectively.

3. RESULTS AND DISCUSSION

It is known that γ-ray can produce high energy, which can initiate the free radical polymerization of the monomer. Using this technique, Bai et al.[27] and Davis et al.[28] almost simultaneously realized the controlled radical polymerization of vinyl monomers with thiocarbonylthio compounds in 2001, and Davis and co-workers achieved the controlled graft polymerization of styrene onto a polypropylene solid phase[29] and from cellulose substrates[30] in the presence of cumyl phenyldithioacetate. In this study, to investigate the possibility for controlled modification of chitosan for drug delivery, the graft polymerization of PHEPE was performed on chitosan with BDACT at room temperature under γ-ray irradiation at the dose rate of 10 Gy/min.

The results of graft polymerizations are list in Table 1. The graft content could be well controlled in the polymerization and was related with radiation dose, monomer concentration, and chitosan concentration: the higher radiation dose or monomer or chitosan concentration, the larger the graft content (Figure S2). According to RAFT mechanism, the growing tethered chains generated on the surface are in a dynamic equilibrium with untethered chains in the solution phase.[31] Therefore, free polymers formed in the solution could be used as an indicator for the molecular weight and molecular weight distribution of grafted polymers.[31,32] It can be seen that the graft polymer showed the controlled molecular weight and narrow polydispersity (Table 1), suggesting that the polymerizations were controlled well by the RAFT agent.

However, it is noticed that chitosan chain may be degraded under γ-ray irradiation.[33,34] To clarify this point, the control experiments using chitosan with and without RAFT agent were performed under γ-ray irradiation. The results are listed in Table S1. The viscosity-average molecular weight of irradiated chitosan decreased with increasing radiation dose, which is consistent with the result reported by Tahtat et al.[34] Importantly, it was found that the viscosity-average molecular weight of chitosan irradiated with RAFT agent could reduce the degradation in comparison with that of chitosan without RAFT agent, suggesting that RAFT agent can prevent chitosan from degradation under γ-ray irradiation to some content. This result may be attributed that the trithio group of RAFT agent can capture the radicals on chitosan under γ-ray irradiation, which may lead to grafting RAFT agent onto chitosan. The ^{1}H NMR spectrum of the resultant further demonstrates this point (Figure S3).

The structures of graft copolymers were characterized by ^{1}H NMR spectra, and a typical ^{1}H NMR spectrum of chitosan-*g*-PHEPE is shown in Figure 1A. Besides the characteristic peaks

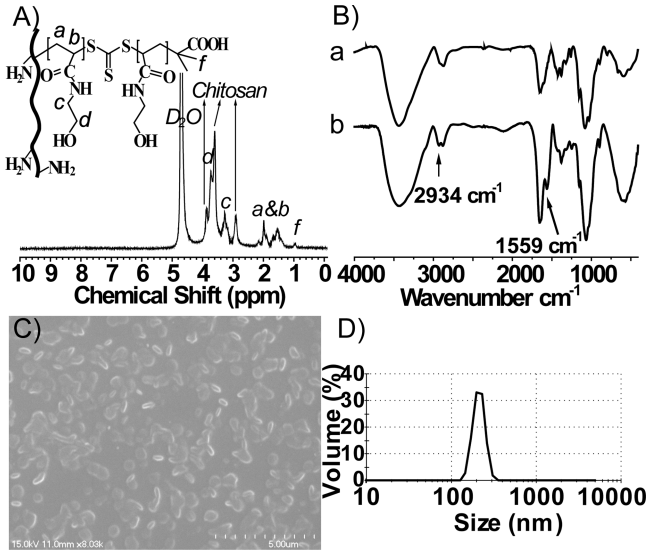

Figure 1. (A) ^{1}H NMR spectrum (400M, D_2O) of chitosan-*g*-PHEPE (M_{PHEPE} = 8000, G% = 52.9%. (B) FT-IR spectra of (a) chitosan and (b) chitosan-*g*-PHEPE (M_{PHEPE} = 8000, G% = 52.9%). (C) SEM images and (D) the particle size distribution of chitosan-*g*-PHEPE (M_{PHEPE} = 8000, G% = 52.9%) in distilled water: Z-average size = 198 nm, PDI = 0.437.

for chitosan, the characteristic resonances of PHEPE were detected at δ = 1.34−2.36 and 3.15−3.45 ppm, suggesting that PHEPE had been successfully grafted onto chitosan by RAFT polymerization under γ-ray irradiation. The structures of chitosan-*g*-PHEPE copolymer can be further confirmed by FT-IR spectra (Figure 1B). In comparison with chitosan (Figure 1B, trace a), the characteristic peaks occur for the chitosan-*g*-PHEPE copolymer (Figure 1B, trace b) at 2934 and 1559 cm^{-1} corresponding to $-CH_2$ stretching and C−N bending vibration, respectively. Because of its amphiphilic structure, chitosan-*g*-PHEPE can self-assemble into the micelles in the distilled water at room temperature, where the hydrophobic chitosan segments may collapse as the core and

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the hydrophilic PHEPE segments form the corona shell. The typical morphology of the micelles is shown in Figure 1C, and they have a narrow size distribution (Figure 1D). The sizes of the micelles are closely related with graft contents and molecular weights of PHEPE: for the similar molecular weight, the more graft content, the larger sizes of the micelles (Figure S5A–C); if the graft content is high (e.g., 80.9%) with a large molecular weight (e.g., M_{PHEPE} = 11 800 g/mol), the multiple distributions would occur (Figure S5D). These results should be attributed to the hydration of the hydrophilic shell: the more hydrophilic PHEPE, the stronger hydration, thereby leading to larger sizes. The multiple distributions for the graft copolymer with high graft content and large molecular weight might be related with the interaction of PHEPE chains between the micelles.

In order to investigate the possibility of the copolymer as drug carrier, the anticancer drug chromone-3-carboxaldehyde was conjugated with chitosan-*g*-PHEPE via Schiff base bond. The structure of the conjugated copolymer was confirmed by the ^{1}H NMR spectrum (Figure 2A). The characteristic peaks

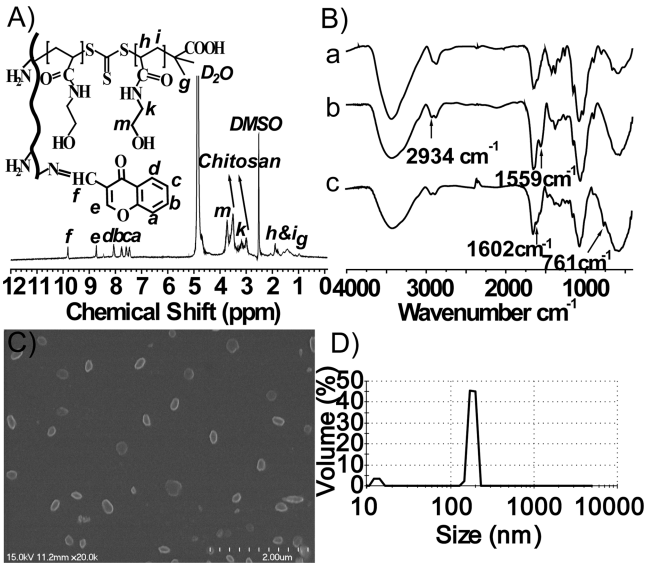

Figure 2. (A) ^{1}H NMR spectrum of PHEPE–chitosan–chromone (M_{PHEPE} = 8000, G_{PHEPE}% = 52.9%, $LE_{chromone}$ = 178.9 mg/g) in D_2O/DMSO-d_6 (v:v = 9:1). (B) FT-IR spectra of (a) chitosan, (b) chitosan-*g*-PHEPE (M_{PHEPE} = 8000, *G*% = 52.9%), and (c) PHEPE–chitosan–chromone (M_{PHEPE} = 8000, G_{PHEPE} % = 52.9%, $LE_{chromone}$ = 178.9 mg/g). (C) SEM images and (D) particle size distribution of PHEPE–chitosan–chromone (M_{PHEPE} = 8000, G_{PHEPE} % = 52.9%, $LE_{chromone}$ = 178.9 mg/g) in distilled water: *Z*-average size = 171 nm, PDI = 0.779.

were assigned for the phenyl group of chromone at from δ = 7.4 ppm to 8.2 ppm and the CH═N group at δ = 9.76–9.84 ppm. According to the integration area of characteristic peaks of chitosan (δ = 2.80–3.15 ppm) and CH═N group (δ = 9.76–9.84 ppm) of chromone in the ^{1}H NMR spectrum, the loading efficiency of chromone could be determined to be 178.9 mg/g. Figure 2B shows FT-IR spectra of HEPE–chitosan–chromone conjugated copolymer. In comparison with chitosan (Figure 2B, trace a) and chitosan-*g*-PHEPE copolymer (Figure 2B, trace b), the characteristic peaks occur for the PHEPE–chitosan–chromone conjugated copolymer (Figure 2B, trace c) at 1602 and 761 cm^{-1} corresponding to C═N stretching and phenyl ring bending vibration, respectively. The results further demonstrate chromone-3-carboxaldehyde was successfully loaded onto the graft copolymer.

The conjugated graft copolymer may self-assembly into the nanoparticles in distilled water. The morphology was characterized by SEM images and particle size distributions. The conjugated polymer nanoparticles showed the distinct core–shell structures with a diameter of ~200 nm (Figure 2C) and the narrow size distributions (Figure 2D). This property may be good for the graft copolymer as drug nanocarrier.

To examine the release behavior, we incubated the conjugated graft copolymers in buffers at different temperatures and pH. The released chromone was quantified by UV–vis measurement according to the calibration curve. The percentage of chromone release from the conjugated graft copolymer was monitored as a function of time (Figure 3).

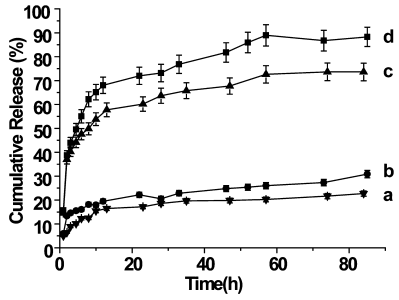

Figure 3. Chromone release profile in buffer solution (*I* = 0.15 M): (a) 25 °C, pH = 7.4; (b) 37 °C, pH = 7.4; (c) 25 °C, pH = 7.4; (d) 37 °C, pH = 5.4.

Chromone was released with a relatively slow pace at 25 °C and pH = 7.4 (Figure 3a), and the release rate was slightly increased if the temperature was raised to 37 °C (Figure 3b). In contrast, the amount of released chromone was markedly increased at pH = 5.4 (Figure 3c,d), especially at 37 °C the cumulative release could reach almost 70% within 20 h (Figure 3d). The results may be attributed that the hydrolysis of Schiff base bond can be accelerated at low pH condition or higher temperature[22,23] and that chitosan in the core may be dissolved and degraded[35] at low pH.

The morphology of the copolymer was characterized by SEM images and particle size distribution after chromone release (Figure S6). It can be seen that the aggregation of nanoparticles (Figure S6A) and multiple peaks appeared in the particle size distribution (Figure S6B), suggesting that the micelle structure was destroyed after drug release due to chitosan solubility at low pH. The results showed that the conjugated graft copolymer could be used as a pH- and thermo-sensitive carrier for drug delivery.

4. CONCLUSION

In summary, we demonstrate here a novel, facile method for controlled modification of chitosan under γ-ray irradiation at room temperature for drug delivery. Specifically, PHEPE was grafted onto chitosan with BDACT at room temperature under γ-ray irradiation, and the unprotected amino group of chitosan was straightly used for the conjugation of chromone-3-carboxaldehyde. During the polymerization, RAFT agent can prevent chitosan from degradation under γ-ray irradiation to some content. The structure of chitosan-*g*-PHEPE copolymer is confirmed by the ^{1}H NMR spectrum, and graft content can be influenced by radiation dose, monomer concentration, and

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chitosan concentration. Then chromone-3-carboxaldehyde was successfully loaded by Schiff base bond with $-NH_2$ groups on chitosan. Importantly, there are no traditional protection−deprotection processes in this method. The chromone release profile shows the graft copolymer can be used as a pH- and thermo-responsive carrier for drug delivery. To our knowledge, this is the first report for controlled modification of chitosan under γ-ray irradiation. This work indicates that RAFT polymerization under γ-ray irradiation can be a facile approach for controlled modification of chitosan for drug delivery.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis of BDACT; ^{1}H NMR spectrum (400M, $CDCl_3$) of BDACT; the influence of radiation dose and monomer and chitosan concentration on graft content; results of chitosan irradiated with and without RAFT agent under γ-ray irradiation; ^{1}H NMR spectrum of the resultant from chitosan irradiated with RAFT agent under γ-ray irradiation at 10.79 Gy; particle size distributions of chitosan-*g*-PHEPE with different graft contents and molecular weights of PHEPE; the absorbance curves and standard curves of chromone in different buffer solutions; SEM image and particle size distribution of PHEPE−chitosan−chromone in distilled water after chromone release. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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