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Amphiphilic chitosan nanosphere: Studies on formation, toxicity, and guest molecule incorporation

Rangrong Yoksan[a,b,*] and Suwabun Chirachanchai[c,d,*]

[a]*Division of Physico-Chemical Processing Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand*
[b]*Department of Packaging Technology and Materials, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand*
[c]*The Petroleum and Petrochemical College, Chulalongkorn University, Bangkok 10330, Thailand*
[d]*Center of Chitin-Chitosan Biomaterial, Chulalongkorn University, Bangkok 10330, Thailand*

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Abstract—Amphiphilic chitosan developed by conjugating hydrophobic phthalimido groups and hydrophilic poly (ethylene glycol) chains gives a well-dispersed colloidal solution in polar solvents and shows a regular nano-sized spherical structure (~200 nm) with negatively charged surface (−31.24 ± 4.85 mV). An in vivo acute oral toxicity test confirms the non-toxicity of the chitosan nanosphere with LD_{50} higher than 2000 mg/kg body weight. A success of incorporating amine molecules into the nanosphere declares that the heterogeneous incorporation system is more effective than the homogeneous ones. The study on guest molecule incorporation using alkylamines and carboxylic acid as models suggests requirements that the guest be hydrophobic and positively charged. TGA study clarifies that the weight loss at 250–300 °C relating to the amount of guest incorporation via heterogeneous system is as high as 42.3%, whereas those of homogeneous systems are about 16.86–28.27%. The nanosphere size is significantly changed after guest incorporation, that is, from 200 nm to approximately 500–1000 nm.

1. Introduction

In recent years, the delivery of macromolecules, e.g., genes,[1] vaccines,[2] proteins,[3] DNA,[4] and drugs[1] via carriers has received much attention due to the potential for novel clinical treatment. Either synthetic polymers, such as polymethyl methacrylate,[5] polyalkylcyanoacrylate,[6] and polystyrene,[7] or biopolymers, such as albumin,[8] gelatin,[8] and cellulose derivatives,[9] are accepted as the polymeric carriers. It should be noted that chitosan (Chart 1) is one of the most promising materials due to its bio-based properties of compatibility,[10] degradability,[11] activity,[12] and non-toxicity.[13]

As chitosan dissolves only in most aqueous carboxylic acids and mineral acids, its materialization has been reported using solution-based processes, such as bead[14] and gel formation,[15,16] film[17] and membrane casting,[18,19] fiber spinning,[20] and microparticle induction.[21]

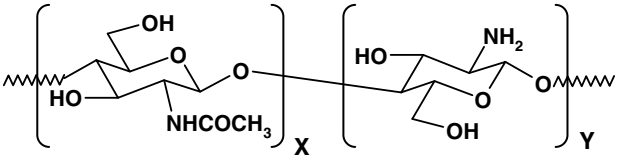

Chart 1. Copolymer of β-(1-4)-2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine, chitin unit) and β-(1-4)-2-amino-2-deoxy-D-glucose (D-glucosamine, chitosan unit).

Nanotechnology is an approach to bring specific properties close to the molecular level, and, as a consequence, the materialization of various polymers has been a challenge at the nano-meter level for the past few years. In the case of chitosan, a number of methods to induce nanoparticles, such as ionic gelation of chitosan-poly(ethylene oxide) containing polyanion sodium tripolyphosphate (CS/PEO-PPO nanoparticles),[22] grafting of chitosan with poly(ethylene glycol) (PEG-grafted chitosan nanoparticles),[23,24] self-aggregation of deoxycholic acid-modified chitosan (self-aggregated DAMC

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* Corresponding authors. Tel.: +66 2 562 5091x5384; fax: +66 2 562 5092 (R.Y.); tel.: +66 2 218 4134; fax: +66 2 215 4459 (S.C.); e-mail addresses: rangrong.y@ku.ac.th; csuwabun@chula.ac.th

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nanoparticles),[25,26] reverse micelle formation of ultrafine cross-linked chitosan nanoparticles in AOT/*n*-hexane,[27] and micro-emulsion of nano-sized heparin/chitosan complexes,[28] have been reported. It should be noted that although those studies are basically successful, size and shape regularity are points to be overcome in the subsequent stage. Previously, we report the preparation and characterization of a novel chitosan nanosphere (*N*-phthaloyl chitosan grafted mPEG),[29,30] however, the questions related to the substitution degrees of *N*-phthalimido group and mPEG chain, surface charge of particle, toxicity based on phthalimido group, and model drug incorporation efficacy have not yet been investigated. Herein, we reported the further work, which gives very important information not only about the specific properties of chitosan nanosphere, but also the model drug incorporation.

2. Results and discussion

2.1. Physico-chemical properties of *N*-phthaloylchitosan grafted mPEG

In our previous work, *N*-phthaloylchitosan grafted mPEG was successfully prepared as confirmed from FT-IR and NMR techniques.[29,30] The degree of phthaloylation and mPEG (Mn = 5000) grafting percentage were 0.89 and 7.83 as evaluated from EA, respectively. The calculations were based on C/N ratio. For example, the C/N ratio of the reference for *N*-phthaloylchitosan grafted mPEG, that is, a complete substitution repeat unit, was 105.23, whereas that of the *N*-phthaloylchitosan grafted mPEG obtained from EA experiment was 8.24. This results 7.83% mPEG grafting.

Although chitosan has specific properties for being a drug carrier, some problems regarding (i) the preparation of chitosan nanoparticles with uniform size and shape for effective circulation in the human body and (ii) the solubility and/or dispersion ability of the chitosan particles in aqueous systems are the points to be challenged. It is also reported that, as chitosan is highly swollen and/or dissolved in the acidic conditions of the stomach, the utilization of chitosan as a drug carrier is hardly satisfactory for specific targets, for example, the colon and large intestine.[31]

In our case, it should be pointed out that the *N*-phthaloylchitosan grafted mPEG shows a stable colloidal solution in polar solvents including 1% aqueous acetic acid (Fig. 1) as a consequence of modifying the amino group to *N*-phthalimido group. The colloidal particles form a well-defined sphere in regular size of ~200 nm as observed from SEM (Fig. 2b). The micrograph from TEM clearly shows the nanosphere for ~85 nm.[29,30] However, it was found that the size of colloidal particles depended on the dispersion media. For example, the size was 252.00 ± 81.02 nm when *N*-phthaloylchitosan grafted mPEG was dispersed in water, whereas 908.33 ± 54.50 nm in *iso*-propanol.

Figure 1. Physical appearance of *N*-phthaloylchitosan grafted mPEG in 1% aqueous acetic acid.

The comparative studies of *N*-phthaloylchitosan grafted mPEG in polar protic solvents (water, 1% aqueous acetic acid, methanol, ethanol, and 2-propanol), polar aprotic solvents (DMF and DMSO), and non-polar solvents (toluene, *n*-hexane, and chloroform) implied that this colloidal phenomenon is related to the hydrogen bonding between mPEG chains and polar solvent molecules.[29,30] As *N*-phthaloylchitosan grafted mPEG is soluble only in DMF and DMSO, we used polar solvents as the poor solvents to carry out the reprecipitation for guest incorporation (see Section 2.3).

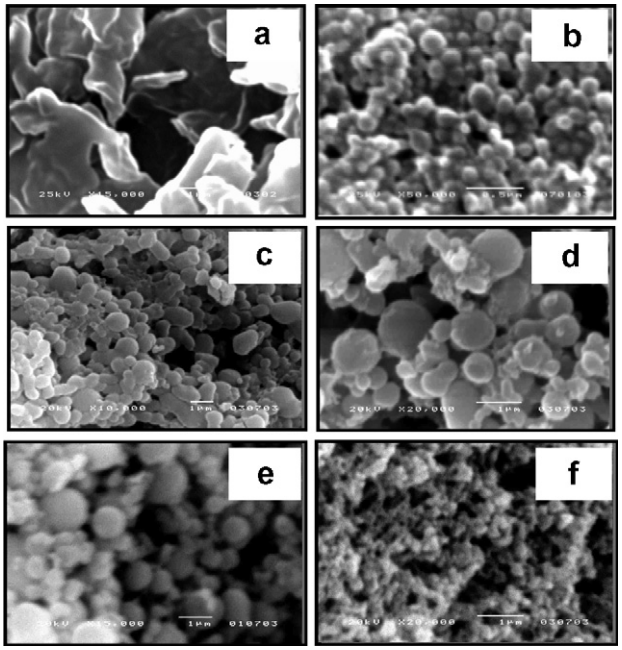
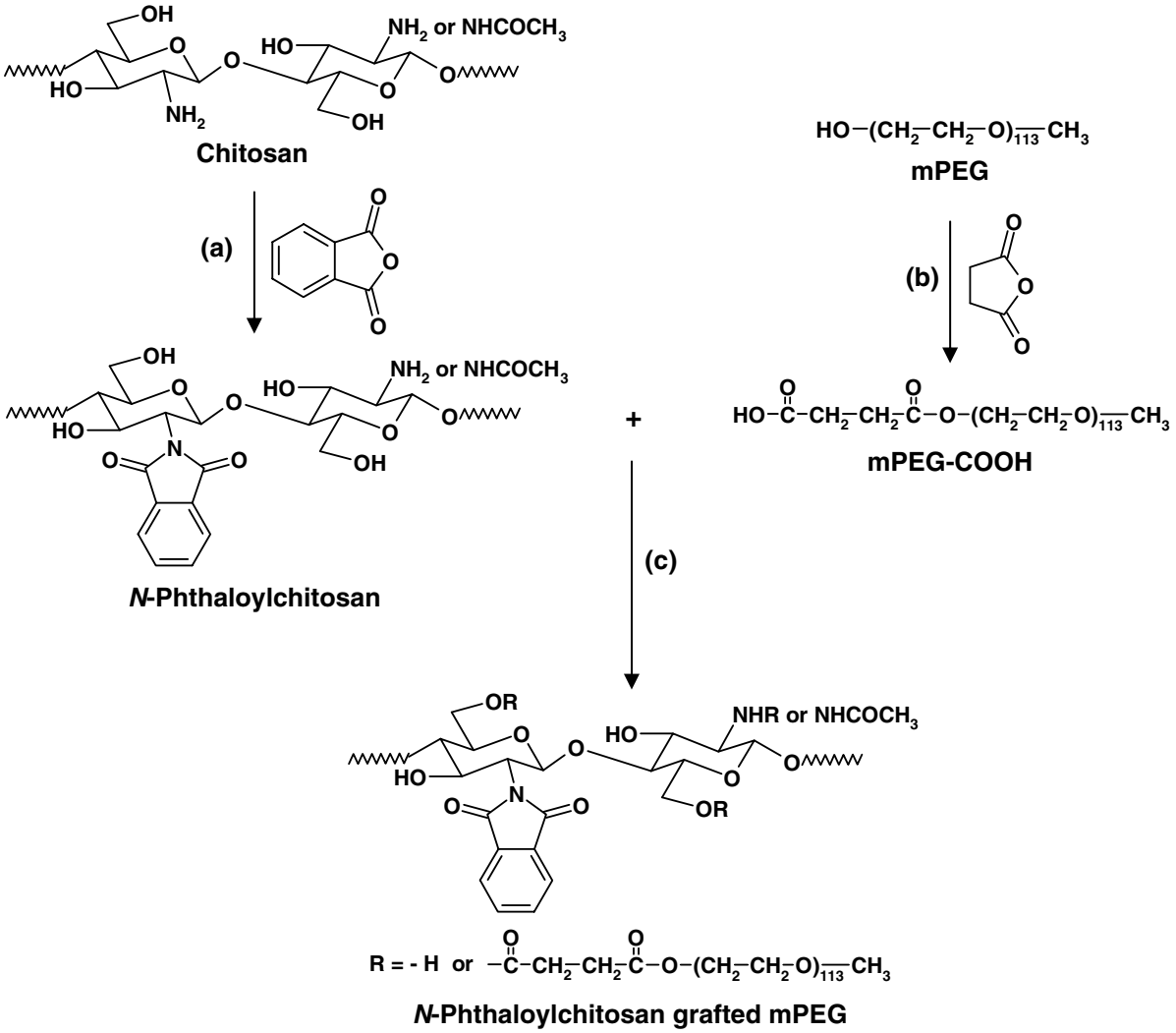


Figure 2. SEM photographs at 25 kV of (a) chitosan (15,000×), (b) *N*-phthaloylchitosan grafted mPEG (50,000×), (c) *N*-phthaloylchitosan grafted mPEG after hexylamine incorporation (10,000×), (d) *N*-phthaloylchitosan grafted mPEG after pentadecylamine incorporation (20,000×), (e) *N*-phthaloylchitosan grafted mPEG after stearylamine incorporation (15,000×), and (f) *N*-phthaloylchitosan grafted mPEG after stearic acid incorporation (20,000×).

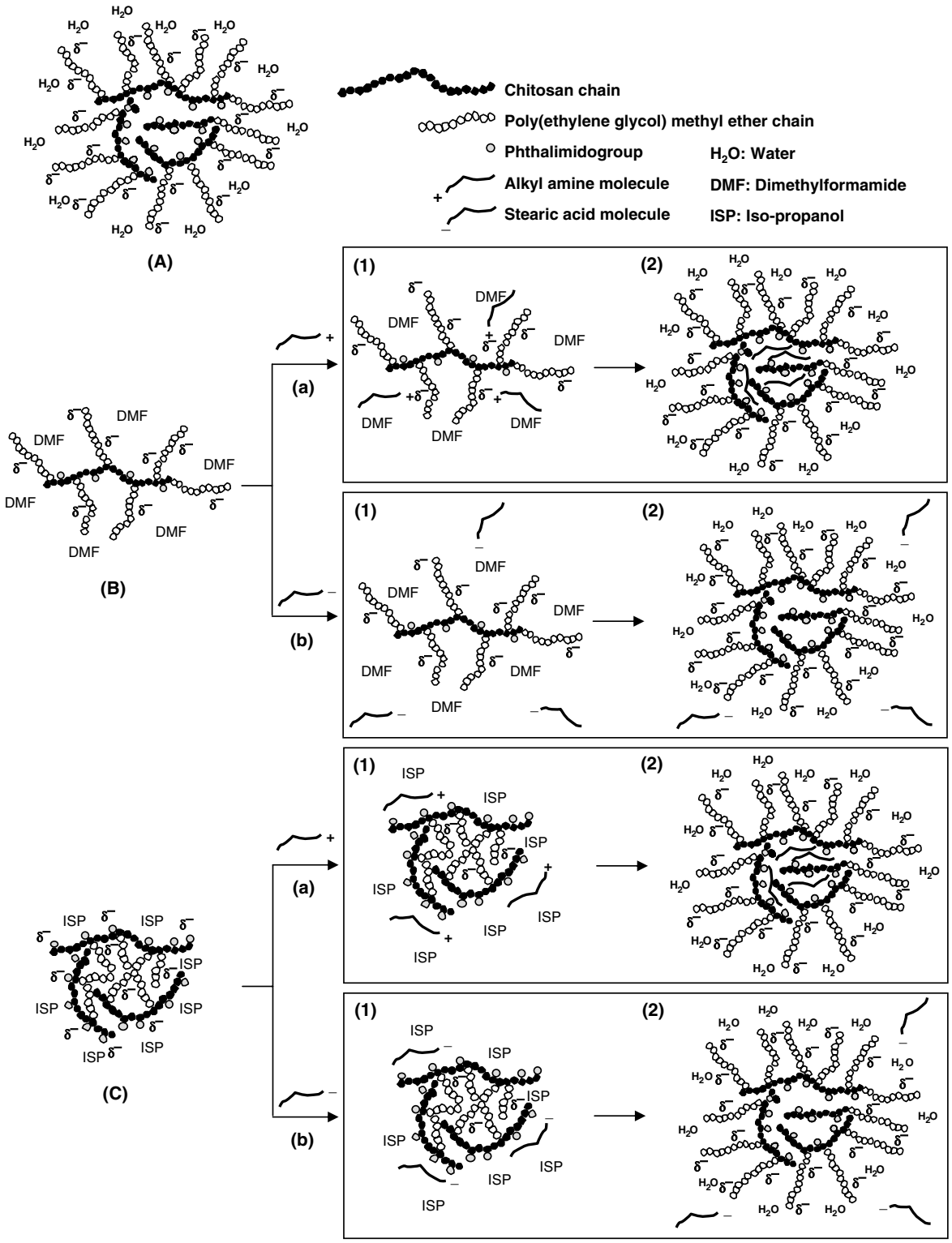
The charge of the nanoparticles is another factor related to the interaction with the guest molecules (e.g., drugs, proteins, etc.) and the target organs, and to the release mechanism.[32] The zeta potential of the chitosan nanosphere dispersed in water was -31.24 ± 4.85 mV implying that the nanosphere was covered with the negatively electrostatic charge. Considering the self-assembly nanosphere, we suspect that the negative charge might come from the high electron density of oxygen atoms along the mPEG chains, as illustrated in Scheme 2A. It is important to point out that the zeta potential of the particles was significantly affected by the media in which they were dispersed, for example, -31.24 ± 4.85 mV in water and 24.27 ± 2.45 mV in *iso*-propanol. Although the studies on the charges of the amphiphilic chitosan are in progress, the information about size and zeta potential suggested that the micelle structures of amphiphilic chitosan were different in those media. It is important to mention that, as reported previously, *N*-phthaloylchitosan grafted mPEG forms nanoparticles in polar solvents, that is, water, methanol, ethanol, and *iso*-propanol.[29,30] However, it dissolves in DMF and DMSO which prevents us from determining the sphere size and shape including zeta potential.

2.2. Acute oral toxicity studies

The acute oral toxicity test (Limit test, OECD 2001)[33] of the chitosan nanosphere was carried out in vivo to clarify its applicability in a bio-system. Table 1 gives the pertinent data of the two groups of rats with (experimental group) and without (control group) dosing of the aqueous chitosan nanosphere solution for 10 and 6 rats, respectively. The body weights observed for days 1, 8, and 15, including the rat survival, are for the evaluation of chitosan nanosphere toxicity. The gross pathological findings are also an indicator for toxicity. It should be noted that the body weights of all rats increase as a function of time and the observation on necropsy shows no gross pathological changes, implying the normal growth of rats. The mean body weight gain measured on day 8 and 15 was for determining how the chitosan nanosphere obstructs the growth of the rat. The treated female rats show an increase in the mean body weight



Scheme 1. Synthesis pathways of (a) *N*-phthaloylchitosan, (b) mPEG-COOH, and (c) *N*-phthaloylchitosan grafted mPEG.



Scheme 2. Schematic representation of (A) negatively charged chitosan nanosphere in water, (B) mechanisms of guest incorporation via homogeneous system, and (C) mechanisms of guest incorporation via heterogeneous system.

gain (~2 g on day 8 and ~18 g on day 15), while the males show little reduction (~0.5 g on day 8 and ~5 g on day 15) compared to those in the control group. This might also be related to the eating behavior of the female rats. It should be noted that the mortality rate is zero, implying that the nanosphere is non-toxic. It is known that the test substance concentration of 2000 mg/kg body weight is high enough to identify the toxicity on the dosing animals.[34,35] Here, we concluded that the LD_{50} in rats of *N*-phthaloylchitosan grafted mPEG (or chitosan nanosphere) is higher than 2000 mg/kg body weight.

Table 1. Body weights during the observation period, necropsy findings at termination, mean body weight gain, and mortality data of rats in experimental and control groups

Group/treatment/dose	Rat No.	Sex	Body weight[a] (g)			Gross pathological findings	Mean body weight gain (g)		Mortality rate
			Day 1	Day 8	Day 15		Day 8	Day 15	
Control group/distilled water/equi-volume to the experimental group	1	Male	190	223	234	Normal	27.33 ± 2.93	43.67 ± 0.33	0/3
	2		204	230	248	Normal			
	3		184	207	227	Normal			
	4	Female	156	178	201	Normal	21.00 ± 0.58	45.00 ± 0.58	0/3
	5		158	178	202	Normal			
	6		155	176	201	Normal			
Experimental group/ chitosan nanosphere/ 2000 mg/kg body weight	7	Male	188	214	224	Normal	26.80 ± 1.07	39.00 ± 2.14	0/5
	8		188	213	223	Normal			
	9		202	228	248	Normal			
	10		185	216	227	Normal			
	11		205	231	241	Normal			
	12	Female	164	189	228	Normal	22.80 ± 1.02	62.60 ± 0.93	0/5
	13		155	176	216	Normal			
	14		158	178	218	Normal			
	15		153	176	216	Normal			
	16		172	197	237	Normal			

[a] Fasted body weight.

2.3. Guest incorporation

The approaches to incorporate active molecules into the self-assembled nanospheres were studied. From the nanosphere phenomena, we suspected that the self-assembly of *N*-phthaloylchitosan grafted mPEG is formed in water due to the hydrophobic phthalimido groups gathered in the core and the hydrophilic mPEG chains interacting with water molecules outside the core (Scheme 2A). Two types of guests with different charges and hydrophobicity, that is, alkylamines and carboxylic acid, were studied. This will not only be a guideline for drug incorporation but will also be information for the incorporation mechanism.

2.3.1. Homogeneous and heterogeneous guest incorporation systems. As chitosan nanospheres are soluble in DMF or DMSO, the homogeneous incorporation system was prepared by dissolving both chitosan nanospheres and guests in DMF. For the heterogeneous incorporation system, the chitosan nanospheres were dispersed in the solution of *iso*-propanol containing guests. The nanospheres were then recovered by dialyzing against water. The possible guest incorporation mechanisms are illustrated in Scheme 2B and C.

For amines, the incorporation via homogeneous or heterogeneous systems was successful as qualitatively analyzed by FT-IR based on the C–H stretching peak at 2853 and 2924 cm^{-1} (Fig. 3c and d) and ^{13}C CP/MAS NMR for the CH_3 and CH_2 chemical shifts at 18.8 and 32.1 ppm, respectively (Fig. 4b).

In order to evaluate whether the homogeneous or the heterogeneous system gives more effective guest incorporation, a comparative study using a stearylamine guest was carried out. The quantitative FT-IR using the peak intensity ratio of the peaks at 2924 (C–H stretching) and 967 cm^{-1}(pyranose peak) declared the I_{2924}/I_{967} of the chitosan nanosphere without guest at 1.15 (Fig. 5a),

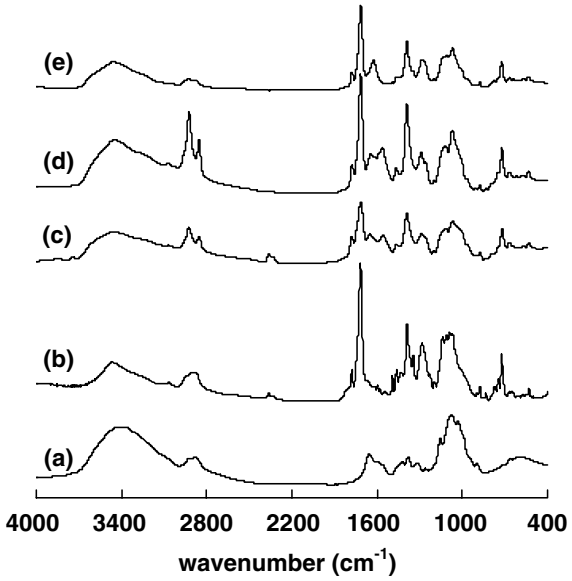


Figure 3. FT-IR spectra of (a) chitosan, (b) *N*-phthaloylchitosan grafted mPEG, (c) *N*-phthaloylchitosan grafted mPEG after stearylamine incorporation via homogeneous system/dialysis against water, (d) *N*-phthaloylchitosan grafted mPEG after stearylamine incorporation via heterogeneous system, and (e) *N*-phthaloylchitosan grafted mPEG after stearic acid incorporation via heterogeneous system.

whereas that of the chitosan nanosphere after stearylamine incorporation via the homogeneous system/dialysis against water was 2.15, implying the successful incorporation (Fig. 5b).

The incorporation mechanism of stearylamine via the homogeneous system is suspected to be as shown in Scheme 2B(a). Both *N*-phthaloylchitosan grafted mPEG and alkylamines are soluble in DMF, and the charge-charge interaction might be formed between the positively charged amine molecules and the negatively charged *N*-phthaloylchitosan grafted mPEG (step (1)). During dialysis against poor solvents (water), it was

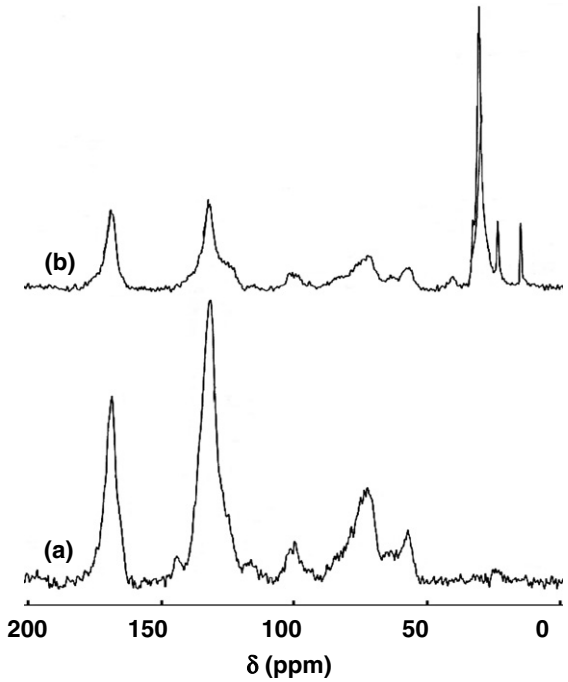


Figure 4. ^{13}C CP/MAS NMR patterns of (a) *N*-phthaloylchitosan grafted mPEG and (b) *N*-phthaloylchitosan grafted mPEG after stearylamine incorporation via heterogeneous system.

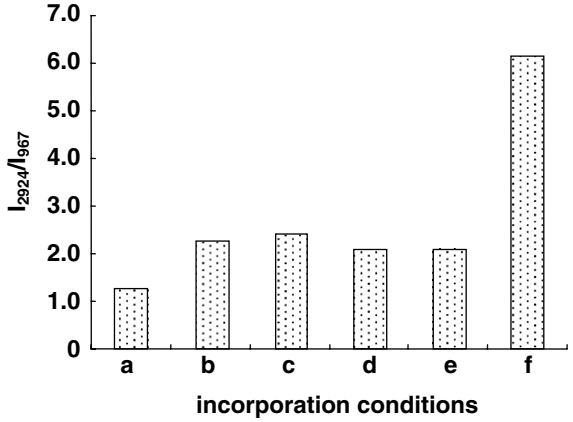


Figure 5. Intensity ratios I_{2924}/I_{967} of (a) *N*-phthaloylchitosan grafted mPEG5000 and (b)–(f) *N*-phthaloylchitosan grafted mPEG5000 after stearylamine incorporation via various conditions: (b) homogeneous system/dialysis against water, (c) homogeneous system/dialysis against methanol, (d) homogeneous system/dialysis against *iso*-propanol, (e) homogeneous system/dialysis against ethanol, and (f) heterogeneous system.

expected that the phthalimido groups gather to form the core, whereas mPEG chains interact with water to initiate the core-shell nanosphere, which simultaneously incorporates the vicinity amines into the core via the hydrophobic–hydrophobic interaction (step (2)).

Surprisingly, the heterogeneous incorporation system gives the I_{2924}/I_{967} as high as 6.4 (Fig. 5f). This implies that the heterogeneous system gave more stearylamine incorporation than the homogeneous system. Scheme 2C(a) is a schematic representing the incorporation of alkylamine carried out in the *iso*-propanol-based colloidal solution of nanospheres, where the amines are well dissolved but the chitosan nanospheres are dispersed. In *iso*-propanol, the alkylamines might form the hydrophobic–hydrophobic interaction with phthalimido groups of the chitosan nanospheres (step (1)). The size in nano-scale might allow the effective interaction on the nanosphere surface. The core-shell inversion occurring soon after the addition into water (step (2)) might be a main factor to offer the significant amine incorporation (I_{2924}/I_{967} was as high as 6.4). Another reason might relate to the fact that *iso*-propanol diffuses into the hydrophobic core resulting in the swelling and at the same time brings along some of the guest molecules, hence the incorporation of the alkylamines is favorable.

An attempt to obtain the better guest incorporation via the homogeneous system was carried out by changing the dialysis solvents, for example, methanol, ethanol, and *iso*-propanol. However, the obtained particles give a similar level of stearylamine incorporation to that of the one from dialysis against water (I_{2924}/I_{967} = 2.0–2.4), which is about 3 times less than that of the heterogeneous system (Fig. 5c–e). A simple reason for the poor incorporation of stearylamine by homogeneous incorporation system is that stearylamine is not soluble in water; thus, it may precipitate out from the medium during dialysis step.

Figure 6 gives information about the thermal stability of the chitosan nanospheres before and after amine incorporation using stearylamine as a case study. In the case of the chitosan nanosphere, there are three peaks observed, which are at 60 (demoisturization), 220 (loss of phthalimido group), and 360 °C (degradation of chitosan) (Fig. 6a). However, for the chitosan nanosphere incorporated with stearylamine (Fig. 6b), the peaks at

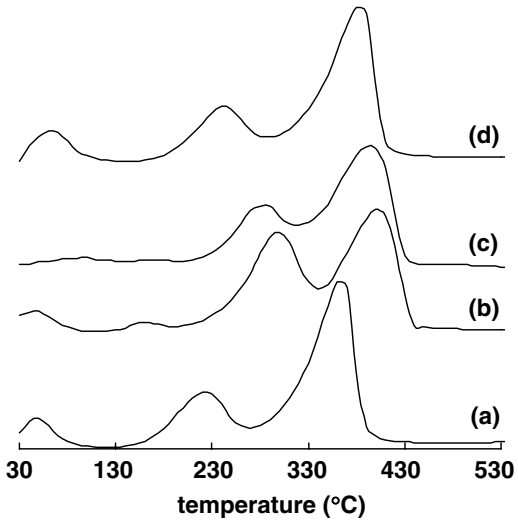


Figure 6. TGA thermograms of (a) *N*-phthaloylchitosan grafted mPEG and (b)–(d) *N*-phthaloylchitosan grafted mPEG after model drug incorporation via various conditions: (b) stearylamine incorporation via heterogeneous system, (c) stearylamine incorporation via homogeneous system/dialysis against water, and (d) stearic acid incorporation via heterogeneous system.

220 and 360 °C are shifted to 300 and 400 °C, respectively. Considering the interaction of the nanospheres with the stearylamine, we suspect that the peak shifts might be due to the hydrophobic interaction between the phthalimido group and the stearylamine, resulting in the higher degradation temperature. This supports our speculation given in Scheme 2B(a) and C(a). It should be noted that in the case of the homogeneous system (Fig. 6c), the peak shift is less significant than that of the heterogeneous one, implying that the heterogeneous system provides more thermally stable guest incorporation.

In general, stearylamine shows a degradation temperature (T_d) at ~233 °C, however T_d might increase when stearylamine interacts with other molecules. Here, it was speculated that the peak at ~250–300 °C representing the T_d of stearylamine bound to phthalimido group. Table 2 summarizes the percent weight loss at ~250–300 °C from TGA technique. The value relates to the amount of incorporated guest, that is, the higher the percent weight loss, the larger the amount of incorporated guest. The percent weight loss of stearylamine incorporated via the heterogeneous system (42.3%) is almost twofold higher than that via the homogeneous one dialyzed against water (28.3%). Methanol, *iso*-propanol, and ethanol were used as dialysis solvents, to find that the guest incorporations were less than that of water. This implies that water gives us the most effective guest incorporation via the homogeneous system. The results also support our quantitative FT-IR that the guest incorporation in the nanosphere prefers the heterogeneous system.

2.3.2. Guest hydrophobicity. The effect of the guest hydrophobicity was also studied by varying the chain length of alkylamines. A rough estimation to quantify the amount of alkylamine incorporated in chitosan nanosphere was carried out by quantitative FT-IR. It was found that all alkylamines used in this study were successfully incorporated into the chitosan nanosphere; however, significant incorporations for cyclohexylamine, nonylamine, pentadecylamine, and stearylamine guests were achieved (Fig. 7). This implies that the guest hydrophobicity is a factor to enhance the incorporation. It is important to mention that as the guests are different in terms of alkyl chain length; the quantitative analysis by ^{1}H NMR technique is in progress. It should be noted

Table 2. Weight loss percentage at ~250–300 °C evaluated from TGA technique

Incorporation systems	Weight loss at ~250–300 °C (%)
Heterogeneous system	42.32
Homogeneous system/ dialysis against water	28.27
Homogeneous system/ dialysis against methanol	19.71
Homogeneous system/ dialysis against *iso*-propanol	18.73
Homogeneous system/ dialysis against ethanol	16.86

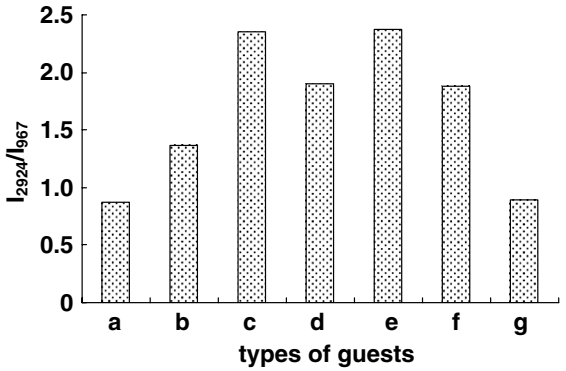


Figure 7. Intensity ratios I_{2924}/I_{967} of (a) *N*-phthaloylchitosan grafted mPEG2000 and (b)–(g) *N*-phthaloylchitosan grafted mPEG2000 after various model guests' incorporation via heterogeneous system: (b) hexylamine, (c) cyclohexylamine, (d) nonylamine, (e) pentadecylamine, (f) stearylamine, and (g) stearic acid.

that Figs 5f and 7f represent chitosan nanospheres incorporating stearylamine via heterogeneous system. As both derivatives are different in terms of molecular weight of mPEG, that is, Mn of 5000 (Fig. 5f) and 2000 (Fig. 7f), we can see the effect of mPEG molecular weight of which the higher the mPEG molecular weight is, the higher the guest incorporation efficiency will be.

The shape and size of the chitosan nanospheres after guest incorporation were further studied by SEM. It should be noted that as chitosan nanospheres were aggregated, the images obtained from SEM represented the aggregated particles. Fig. 2c–e shows that the aggregated particles still retained their spherical shape, whereas the size varied with the chain length of the amines, that is, stearylamine and pentadecylamine (~1000 nm) > nonylamine(~900 nm) > hexylamine (~800 nm). The particle size observed by SEM also reflected the effect of guest structure. For example, the chitosan nanosphere incorporated with cyclohexylamine showed a particle size of ~500–600 nm, whereas the one with hexylamine was ~800 nm. This smaller particle size might come from the cyclic structure of the cyclohexylamine molecules.

2.3.3. Types of guests. As stearic acid was a negatively charged molecule, a comparative study of the types of guests was carried out. In either homogeneous or heterogeneous incorporation system, FT-IR shows no peaks referring to stearic acid (2800–3000 and 1700 cm^{-1}) (Fig. 3e). The quantitative FT-IR (I_{2924}/I_{967}) confirms that stearic acid is hardly incorporated, as observed from the similar value to that of chitosan (I_{2924}/I_{967} = 0.9) (Fig. 7g and a). Fig. 6d shows the degradation steps of the chitosan nanosphere after treating with stearic acid to support that stearic acid incorporation was not successful. The fact that there are no changes in shape and size of the sphere, as observed by SEM, further confirms the unsuccessful stearic acid incorporation (Fig. 2f).

Scheme 2B(b) and C(b) shows the mechanisms speculated for an unsuccessful stearic acid incorporation both

in homogeneous and in heterogeneous systems. For the homogeneous system (Scheme 2B(b)), although stearic acid and *N*-phthaloylchitosan grafted mPEG are dissolved well in DMF, the negatively electrostatic condition of the stearic acid molecules and mPEG chains might induce the repulsion (step (1)). As a result, the hydrophobic–hydrophobic interaction of stearic acids and phthalimido groups inside the nanosphere core was difficultly formed during the dialysis step (step (2)). In addition, stearic acid is insoluble in water, it may, thus, precipitate out in the dialysis approach.

For the heterogeneous system (Scheme 2C(b)), stearic acid, which is soluble in *iso*-propanol, might form the hydrophobic–hydrophobic interaction with the phthalimido groups (step (1)). However, by dispersing those particles in water, the negatively electrostatic repulsion between stearic acid molecules and mPEG chains is dominant; as a result, the stearic acid is expelled and consequently not incorporated in the core of the nanosphere (step (2)). We also suspect that the particles retain their original structure in water (i.e., hydrophobic core inside and hydrophilic shell outside), carboxyl groups may form hydrogen bonding with PEG and block the diffusion of the guest through the core of the particle, hence a poor loading.

3. Conclusions

An amphiphilic chitosan nanosphere was successfully prepared via a grafting reaction of hydrophilic mPEG onto hydrophobic *N*-phthaloylchitosan. The product gave a stable colloidal solution in water at room temperature for months. The TEM and SEM confirmed the individual particles of the chitosan nanospheres to be 85 nm and the nanosphere aggregates to be 200 nm. The zeta potential confirmed that the surface of the chitosan nanosphere is negatively charged. An in vivo acute oral toxicity test in rats (Limit test, OECD 2001) showed that chitosan nanospheres have an LD_{50} higher than 2000 mg/kg body weight. Effective guest incorporation was achieved by dispersing the chitosan nanosphere in a guest-*iso*-propanol solution, or so-called heterogeneous system. The favorable conditions for the guest molecules to incorporate into the chitosan nanosphere were the high hydrophobicity of the guest itself and the positive charge. The chitosan nanospheres might be a novel drug carrier, especially for the positively charged hydrophobic drug.

4. Materials and methods

4.1. Materials

Chitosan (degree of deacetylation (DD) = 0.9 and $Mv = 1.7 \times 10^5$ g/mole) was provided by the Seafresh Chitosan (Lab) Company Limited, Thailand. Phthalic and succinic anhydrides, pentadecylamine, nonylamine, hexylamine, and cyclohexylamine were purchased from Fluka Chemika, Switzerland. Poly(ethylene glycol) methyl ether (mPEG5000, Mn = 5000 and mPEG2000, Mn = 2000) and stearic acid were obtained from Aldrich Chemical Company, Inc., USA. Water-soluble 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (WSCI) and stearylamine were purchased from TCI, Japan, and 1-hydroxy-1*H*-benzotriazole, monohydrate (HOBt) was obtained from BDH Laboratory Supplies, England. *N*,*N*-Dimethylformamide (DMF) was supplied by UNIVAR, Australia. All chemicals were used without further purification.

Healthy Wistar rats with body weight ranges of 196–220 g for males and 165–187 g for females were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. The rat food was from Pokphand Animal Feed Co., Ltd., Thailand.

4.2. Instruments and equipment

FT-IR spectra were recorded on a VECTOR 3.0 BRUKER spectrometer with 64 scans at a resolution of $4\ cm^{-1}$ using a deuterated triglycinesulfate detector (DTGS) with a specific detectivity, D^*, of 1×10^9 cm $Hz^{1/2}$ w^{-1}. ^{13}C CP/MAS NMR spectra were recorded at 300 MHz with a BRUKER DPX-300 at 23 ± 1 °C. ^1H NMR spectra were obtained from a JEOL GSX 400 (400 MHz) at 70 ± 1 °C for chitosan and at 25 ± 1 °C for its derivatives. Elemental analysis (EA) results were obtained using a YANAKO CHN CORDER MT-3, MT-5 Analyzer with a combustion temperature at 950 °C under air with O_2 as a combustion gas (flow rate of 20 mL/min) and He as a carrier (flow rate of 200 mL/min). A Dupont thermogravimetric analyzer was used for thermal gravimetry analysis (TGA) using a N_2 flow rate of 20 mL/min and a heating rate of 20 °C/min from 30 to 600 °C. Scanning electron microscopy (SEM) analysis of the products was carried out using a JEOL JSM-5200 at an operating voltage of 25 kV. Transmission electron microscopy (TEM) analysis was carried out using a Hitachi H700 at an accelerate voltage of 200 kV. The samples after dialysis against water were dropped on a copper mesh and dried under reduced pressure and subsequently sputtered with carbon until the layer was about 20–50 nm thick. The size of dispersing particles was determined at 20 °C by a Malvern Zetasizer Nano Series equipped with a He-Ne laser operating at 4.0 mW and 633 nm with a fixed scattering angle of 90°. Zeta potential was measured for 30 times at a DC voltage of 300 using a ZETA-METER 3.0+ microscope module.

4.3. Preparation of *N*-phthaloylchitosan grafted mPEG (chitosan nanospheres)

N-Phthaloylchitosan grafted mPEG was prepared as reported previously (Scheme 1).[29,30] In brief, chitosan (1 g) was reacted with phthalic anhydride (4.48 g, 5 mol equiv to pyranose ring) in DMF (20 mL) at 100 °C under reduced pressure for 6 h to give *N*-phthaloylchitosan. The reaction of mPEG (Mn = 5000, 3 g, 6×10^{-4} mol) with succinic anhydride (0.06 g, 1 mol equiv to mPEG) was carried out in DMF (2 mL) at 60 °C overnight to yield mPEG-COOH. To a solution of *N*-phthaloylchito-

san (1 g, 3.71×10^{-3} mol) in DMF (20 mL), mPEG-COOH (7.58 g, 0.40 mol equiv to *N*-phthaloylchitosan), HOBt (0.68 g, 3 mol equiv to mPEG-COOH), and WSCI (0.85 g, 3 mol equiv to mPEG-COOH) were added. The mixture was stirred at room temperature overnight before dialyzing against water to remove impurities from the coupling reaction and at the same time to obtain white particles of *N*-phthaloylchitosan grafted mPEG.

N-phthaloylchitosan; EA $(C_{14}H_{13}O_6N)_{0.8}$ $(C_6H_{11}O_4N)_{0.1}$ $(C_8H_{13}O_5N)_{0.1}$, Anal. calcd (%) C, 56.17; H, 4.75; and N, 5.20, found (%) C, 56.18; H, 4.45; and N, 4.35: FT-IR (KBr, cm^{-1}) 3472 (OH), 1776 and 1714 (C=O anhydride), and 721 (aromatic ring): ^{13}C CP/MAS NMR (δd, ppm) 23.3 (CH_3), 57.0 (C-2), 64.7 (C-6), 73.2 (C-3, C-5), 80.5 (C-4), 100.4 (C-1), 131.1 (C_6H_5), and 169.1 (C=O in acetamide and anhydride): ^1H NMR (DMSO-d_6, 25 °C) (δd, ppm) 1.7 (CH_3 in acetamide), 3.4–5.0 (pyranose ring), and 7.6–7.7 (C_6H_5).

mPEG-COOH; FT-IR (KBr, cm^{-1}) 3472 (OH), 2875 (C–H stretching), 1736 (C=O), and 1105 (C–O–C): ^1H NMR (D2O, 25 °C) (δd, ppm) 2.4 (CH_2 in succinic anhydride), 3.2 (O-CH_3), and 3.5 (CH_2 in PEG).

N-Phthaloylchitosan grafted mPEG; EA $(C_{14}H_{13}O_6N)_{0.509}(C_{245}H_{471}O_{122}N)_{0.291}(C_6H_{11}O_4N)_{0.027}(C_{468}H_{927}O_{236}N)_{0.073}(C_8H_{13}O_5N)_{0.064}(C_{239}H_{471}O_{12}N)_{0.036}$, Anal. calcd (%) C, 55.47; H, 6.51; and N, 3.05, found (%) C, 56.22; H, 4.82; and N, 6.82: FT-IR (KBr, cm^{-1}) 3464 (OH), 2882 (C–H stretching), 1776 and 1714 (C=O anhydride), 1714 (C=O ester), and 721 (aromatic ring): ^{13}C CP/MAS NMR (δd, ppm) 23.5 (CH_3 in acetamide and mPEG), 57.2 (C-2), 64.8 (C-6), 72.7 (C-3, C-5), 80.3 (C-4), 100.0 (C-1), 132.2 (C_6H_5), and 171.5 (C=O in acetamide, anhydride, and ester): ^1H NMR (DMSO-d_6, 25 °C) (δd, ppm) 2.4 (CH_2 in succinic anhydride), 3.2 (O-CH_3), 3.5 (CH_2 in PEG), 2.8–4.7 (pyranose ring), and 7.6–7.8 (C_6H_5).

4.4. Acute oral toxicity test of *N*-phthaloylchitosan grafted mPEG

The acute oral toxicity test was performed according to the OECD (Organization for Economic Co-operation and Development) Guidelines for Testing of Chemicals (2001) No. 401.[33] Ten rats (five of each sex) in an experimental group and six rats (three of each sex) in a control group were acclimatized to the laboratory environment for a week prior to experimentation. All rats were fasted for 16 h before dosing with the *N*-phthaloylchitosan grafted mPEG solution, while drinking water was available ad libitum.

The *N*-phthaloylchitosan grafted mPEG solution (20% w/v) was prepared by using distilled water. The mixture was orally dosed to the rats of the experimental group at 2000 mg/kg body weight based on the weight after fasting. The rats of the control group were fed with distilled water at the equi-volume as the experimental rats.

After dosing, all rats were further fasted for 4 h, but water was provided ad libitum. Any toxic signs were immediately observed at 1/2, 1, and 3 hour(s), and once a day for 14 days. The body weights of rats were recorded on day 1 (prior to dosing), day 8, and day 15 (at termination) or after death during the experimental period. On day 15, euthanasia of all survivors was carried out by CO_2 asphyxiation, and gross pathology was performed. The mean body weight gain of the rats in the experimental group was calculated and compared to those of the rats in the control group using Student's *t*-test ($P = 0.05$). The in vivo experiment was carried out under the animal testing laws of Thailand.

4.5. Guest incorporation into *N*-phthaloylchitosan grafted mPEG

A series of alkylamines, that is, hexylamine, cyclohexylamine, nonylamine, pentadecylamine, and stearylamine, as well as stearic acid, were used as model guests. Each guest was incorporated into the chitosan nanospheres via two preparation systems: (i) a homogeneous system in DMF and (ii) a heterogeneous system in *iso*-propanol. For the first system, *N*-phthaloylchitosan grafted mPEG (0.04 g) and the guest (0.01 g) were mixed in DMF (4 mL) at room temperature for a day. The homogeneous mixture was dialyzed against various poor solvents, that is, water, methanol, ethanol, and *iso*-propanol, to give a white precipitate. The precipitate was collected and washed thoroughly by the good solvents for each guest several times to remove the physisorbed guests on the nanosphere surface and was then dried under reduced pressure. For the latter system, *N*-phthaloylchitosan grafted mPEG (0.04 g) was dispersed in the solution of guest-*iso*-propanol (2.5×10^{-3} g/mL, 4 mL) and was slowly stirred at room temperature for a day. The particles were collected from the solution and re-dispersed in water. The collected particles were washed thoroughly with the good solvents for each guest several times, rinsed with methanol, and consecutively dried under reduced pressure.

N-phthaloylchitosan grafted mPEG after stearylamine incorporation; FT-IR (KBr, cm^{-1}) 3447 (OH), 2853 and 2924 (C–H stretching), 1777 and 1716 (C=O anhydride), 1716 (C=O ester), 1643 (amide I), 1562 (amide II), 962 (pyranose ring), and 721 (aromatic ring): ^{13}C CP/MAS NMR (δd, ppm) 24.3 (CH_3 in acetamide and mPEG), 57.1 (C-2), 63.7 (C-6), 71.8 (C-3, C-5), 80.7 (C-4), 102.1 (C-1), 132.1 (C_6H_5), 168.1 (C=O in acetamide, anhydride, and ester), 18.8 (CH_3 in alkyl amine), and 32.1 (CH_2 in alkyl amine).

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Supplementary data

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