Deoxycholate-Chitosan Nanospheres Fabricated by γ-Irradiation and Chemical Modification: Nanoscale Synthesis and Controlled Studies

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ABSTRACT: A systematic protocol to fabricate self-assembly deoxycholate-chitosan nanospheres (DC-CsNS) by γ-ray pre-irradiation and chemical modification was studied. Hydrophobic deoxycholic acid moieties were chemically conjugated to pre-irradiated chitosan. The influences of chitosan physical forms (i.e., colloid and flake) during irradiation, radiation doses, and the reaction system (heterogeneous or homogeneous) on the chemical modification and the particle shape and size were investigated. Pre-irradiation of chitosan in colloidal form produced smaller DC-CsNS particle size than that of flake form. In the heterogeneous reaction, the pre-irradiated dose influenced the DC-CsNS particle size, whereas in the homogeneous reaction all pre-irradiation doses gave an identical average size range of 30–50 nm. By pre-irradiation (10 kGy) of chitosan in colloidal form before heterogeneous chemical conjugation, it is possible to obtain DC-CsNS with an average size of 46 nm. DC-CsNS of about 50 nm in size could also be synthesized using homogeneous chemical conjugation onto non-irradiated chitosan with the addition of *N*-hydroxysuccinimide (NHS). © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 3309–3320, 2012

Key words: chitosan nanoparticles; gamma rays; chemical modification; deoxycholic acid

INTRODUCTION

A molecular self-assembly process has been proposed as a method of bottom-up fabrication, and has become an important concept in nanotechnology. Polymeric self-assemblies and micelles of amphiphilic graft copolymers are presently recognized as among the most effective formulations for application in drug delivery systems. Their properties are influenced by the parent hydrophilic polymer and the hydrophobic groups. It is well-known that polymer micelles have a core-shell structural design composed of a hydrophobic portion as the internal core and a hydrophilic portion as the surrounding corona in an aqueous medium.[1] The hydrophobic core provides a water-insoluble drug loading space, whereas the modified hydrophilic shell affects pharmacokinetic behavior.[2,3] This self-assembly-based core-shell structure plays an important role in the adsorption, transfer, and slow release of a drug, and is applicable for human treatment.

Several formulations of polymeric self-assembly nanoparticles have been intensively studied in clinical trials.[4,5] Naturally occurring polymers, especially polysaccharides such as chitosan, have been researched as a primary material in forming self-assembly nanoparticles. Chitosan is the second most naturally abundant polysaccharide next to cellulose.[6] It is found in the shells of crustaceans, insects, and fungi. The polymer skeleton consists of copolysaccharides of β-(1-4)-2-acetamido-2-deoxy-β-D-glucose (chitin) and β-(1-4)-2-amino-2-deoxy-β-D-glucose (chitosan) with a glycosidic linkage. Chitosan has gained considerable attention in the pharmaceutical and biomedical fields due to its favorable biological properties, such as biological activity, biocompatibility,[7] and biodegradability,[8] in combination with its low toxicity.[9,10] The reactive amino and hydroxyl groups at the C-2 and C-6 positions of chitosan have been the focus of chemical modification to develop several derivatives and value-added products.[11,12] Based on its properties, chitosan has received much attention in the development of polymeric nanoparticles for drug-delivery systems. Several chitosan formulations—such as nanobeads,[13] nanohydrogel,[14] nanoparticles,[15] and nanospheres,[16] as well as self-assembled micelles[1,17]—have been reported. Non-modified chitosan also has been investigated for its

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ability to form a fine, self-aggregating substance in an aqueous solution.[18] Hydrophobically modified chitosan (with hydrophobic groups such as bulky cholesteryl groups, i.e., long alkyl or acyl chains) has been widely used in the study of micelles and self-aggregation. For example, deoxycholate-modified chitosan,[19,20] poly(ethylene glycol)-chitosan,[21,22] and phosphonic and alkyl-modified chitosan[23] have been the subject of recent research. Self-assembled particles or micelles formed by hydrophobically modified chitosan derivatives have successfully served as nanocarriers for certain materials such as water-insoluble drugs,[24,25] proteins,[26] genes, and DNA.[27,28]

Many chitosan derivatives, based on self-assembly formulation, with various particle sizes have been synthesized and fabricated successfully by several protocols—for example: poly(ethylene glycol)-modified phthaloyl-chitosan nanospheres with a size of 1500 nm[29]; self-aggregating hydrophobically modified glycol chitosan nanoparticles in a range of 280–330 nm[20]; hydrophilic glycol chitosan shells and hydrophobic inner cores of cholanic acid, with an average diameter of 200 nm[30]; and deoxycholate-chitosan micelles with diameters of 200–600 nm[31] and 130–300 nm.[32]

Since the controlled fabrication of nanometer-scale objects is one of the foremost aspects in the growth of nanoscience and technology, the study of nanosized polymeric micelles for use as carriers is of considerable importance. Nanosized polymeric micelles not only enhance the drug carrier solubility and stability, but also exhibit many other advantages as drug-delivery carriers, such as prolonged circulation, and tumor localization by the enhanced permeability and retention (EPR) effect.[33] Therefore, it is believed that the use of drug-encapsulated nanosized polymeric carriers to solid tumors can enhance the drug's therapeutic efficacy and potentially decrease severe toxic effects. From the current viewpoint of nanoscience and technology, nanoscale material is defined as having a size range of 10–100 nm.[34] Nanoscale-size materials have been recognized as possessing many superior advantages, as described above. To our knowledge, although many chitosan derivatives in self-assembly forms have been reported, the development of a systematic protocol for reducing and controlling the particle size of hydrophobically modified chitosan micelles using γ-irradiation has not yet been studied. Recently, our group proposed a systematic protocol for preparing and reducing the particle size of self-aggregating non-modified chitosan nanoparticles in an aqueous solution, using a γ-irradiation process.[35] Radiolytic methodology using γ irradiation has been extensively used for polymer modification[36–41] due to the simplicity of the process, which involves single-step preparation without purification. Since ionizing radiation is concerned with very small objects such as electrons, photons, or ions, it may prove to be a very important tool for future technological developments in nanoscience. Therefore, we have investigated the systematic synthesis and controlled fabrication of nanoscale hydrophobically modified chitosan micelles, i.e., deoxycholate-chitosan nanospheres (DC-CsNP), using γ-ray irradiation and chemical modification. Also examined were the effects of chitosan physical forms during pre-irradiation, pre-irradiation doses, and chemical modification systems on the degree of chemical modification and particle formulation, i.e., particle shape and size.

EXPERIMENT

Chemicals

Chitosan, with percent degree of deacetylation (%DD) of 95 ($M_v = 7 \times 10^5$ Da), was supplied by Seafresh Chitosan (Lab; Thailand). Chemicals purchased included: deoxycholic acid (DA) from Nacalai Tesque (Japan); 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC) from TCI (Japan); *N*-hydroxysuccinimide (NHS) from TKK (Japan); sodium hydroxide (NaOH) from Carlo Erba Reagents (Italy); and methanol and acetic acid (AR grade) from RCI Labscan (Thailand). All chemicals were used without further purification.

Instruments and equipment

γ-Ray irradiation from a ^{60}Co source was carried out using a Gammacell 220 irradiator with a dose rate of 10 kGy/h. Fourier transform infrared spectroscopy (FTIR) was performed using a Bruker Tensor 27, with 32 scans at 2 cm^{-1} resolution in a frequency range of 4000–400 cm^{-1}. Elemental analysis was carried out using a LECO CHNS-932 with a combustion temperature of 950°C under air atmosphere with O_2 as a combustion gas (flow rate 20 mL/min) and He as a carrier (flow rate 200 mL/min). Transmission electron micrographs were taken using a Hitachi H7650. Aqueous colloidal chitosan was diluted to suitable concentrations (1×10^{-4}% w/v). Vigorous stirring and sonication were performed before dropping the solution onto a copper grid and then drying in a desiccator for 24 h before analysis. A NanoWorld NCHR-***50*** atomic force microscope (AFM) was used to confirm nanoparticle shapes and sizes. Samples were dispersed in water at a concentration of 1×10^{-4}% w/v. Five microliters of the solution was pipetted onto a fresh glass slide and air-dried before analysis using a Pointprobe Silicon-SPM-Sensor with a resonance frequency of ***320*** kHz and a force constant of ***42*** N/m.

Preparation of γ-ray irradiated chitosan

Chitosan flakes (0.5 g) were dissolved in 1% v/v acetic acid (100 mL) to obtain a 0.5% w/v chitosan solution. Chitosan was reprecipitated in 1% w/v sodium hydroxide aqueous solution to obtain white colloidal particles. The colloidal gel was washed with water five times to obtain chitosan in aqueous colloidal form (Cs-colloid). Cs-colloid (0.5% w/v) was irradiated with γ-ray doses of 0, 5, 10, 20, 40, and 100 kGy. Chitosan in solid formulation, or chitosan flake (Cs-flake), was originally irradiated with varying doses of 0, 5, 10, 20, and 40 kGy before reforming by reprecipitation, as mentioned previously, to obtain a colloidal chitosan formulation for the conjugation process in the next step.

Synthesis of deoxycholate-chitosan nanospheres by chemical modification

The starting irradiated chitosan (Cs-colloid and Cs-flake) for chemical conjugation was in colloidal form, which was prepared according to Pasanphan et al.[35] For the heterogeneous system, deoxycholic acid (DA 0.7851 g, 1 mol equivalent to chitosan) in methanolic solution (50 mL) was added to colloidal chitosan (0.1631 g, 1 mol). EDC, 0.3834 g, 1 mol equivalent to chitosan, was then added to the mixture of chitosan and DA. Heterogeneous reactions were carried out at ambient temperature for 1–24 h to obtain intermediate **1**, followed by **2** (DC-CsNS **2**). Compound **2** was rigorously washed with methanol and then dried. In the case of the homogeneous reaction, colloidal chitosan (0.1631 g, 1 mol) was predissolved in NHS (0.2303 g, 1 mol equivalent to chitosan) with distilled water (30 ml) and stirred for 0.5 h. Then DA (0.7851 g, 1 mol equivalent to chitosan) and EDC (0.3834 g, 1 mol equivalent to chitosan) in methanol solution were added to NHS-dissolved chitosan to obtain intermediate **1** and intermediate **3,** followed by product **4** (DC-CsNS **4**). Product **4** was also rigorously washed with methanol and then dried. Cs-colloid and Cs-flake, irradiated with varying doses of 0, 5, 10, 20, 40, and 100 kGy, were used as the starting chitosan for chemical modification.

RESULTS AND DISCUSSION

Chemical modification: Conjugation of deoxycholic acid onto chitosan

Chitosan is reportedly able to bind compounds possessing a hydrophobic group such as bile acid,[42,43] for example chitosan-conjugated DA.[27,41] *In vitro* assay has been widely accepted for estimating the potential bile acid-binding capacity of polysaccharides including chitosan, ready-to-eat breakfast cereals, and other dietary fibers.[44,45] Conjugation of bile acid with chitosan to form self-assembly for drug delivery systems has been widely reported, as previously mentioned. In the present work, the chemical conjugation efficacy of DA on non-irradiated and pre-irradiated chitosan was studied. The conjugation was carried out in both heterogeneous and homogeneous reactions. The reactive amino at C-2 and the hydroxyl group at C-6 were presumably conjugated with the carboxylic groups of DA molecules to form amide and ester linkages, respectively.

In the heterogeneous reaction, EDC reacts with the carboxyl group of DA to form an intermediate active ester, **1**. This intermediate **1** can react with a primary amine at C-2 of chitosan to form an amide bond, producing a DC-CsNS **2**. The non-conjugated DA and a by-product of isourea can be easily removed by washing with methanol and water. The formation of an amide bond is confirmed from the FTIR spectrum. Figure 1(A) shows the FTIR spectra of chitosan (a) and DC-CsNS **2** (a and b). The peak assignments of chitosan [Fig. 1(A:a)] are as follows (cm^{-1}): 3464 (O—H stretch overlapped with N—H stretch); 2952 and 2864 (C—H stretching); 1653 (amide I band, C=O stretching of acetyl group); 1580 (amide II band, N—H stretching); 1430–1370 (C—H bending); and 1322 (C—N stretching). Absorption bands at 1156 cm^{-1} (asymmetric stretching of the C—O—C bridge), 1075 and 1033 cm^{-1} (skeletal vibration involving the C—O stretching of C3 and C6, primary and secondary OH, respectively) are the characteristics of its saccharine structure. Compared with chitosan, peaks of DC-CsNS [Fig. 1(A:b)] appear at 2952 and 2864 cm^{-1} (stretching of $-CH_2-$), and the peak at 1653 cm^{-1} (Amide I) is observably increased. This confirms the formation of an amide linkage between the primary amino groups at C-2 of chitosan and the carboxylic groups of DA (Scheme 1).

For the homogeneous system, NHS was additionally used as a coupling agent with EDC. In many previous experiments, the chemical modification of chitosan by homogeneous reaction has commonly been carried out in acetic acid,[27,31] which is well-known as a good solvent of chitosan. In addition, chitosan in the presence of 1-hydroxy-benzyltriazole (HOBt) has recently been proposed as a novel system for chemical modification of chitosan in aqueous media.[46] The mixture of chitosan and HOBt was found to be a unique but simple way to dissolve chitosan in water. Based on our previous work,[47] it has been shown that NHS not only promotes the esterification reaction but also possibly dissolves chitosan in water as well as HOBt. This creates the possibility of conjugating DA onto chitosan in the homogeneous system. Therefore, the challenge was to demonstrate the chemical modification of chitosan in the presence of NHS. In this way, in the homogeneous system NHS was not only to be used as a coupling

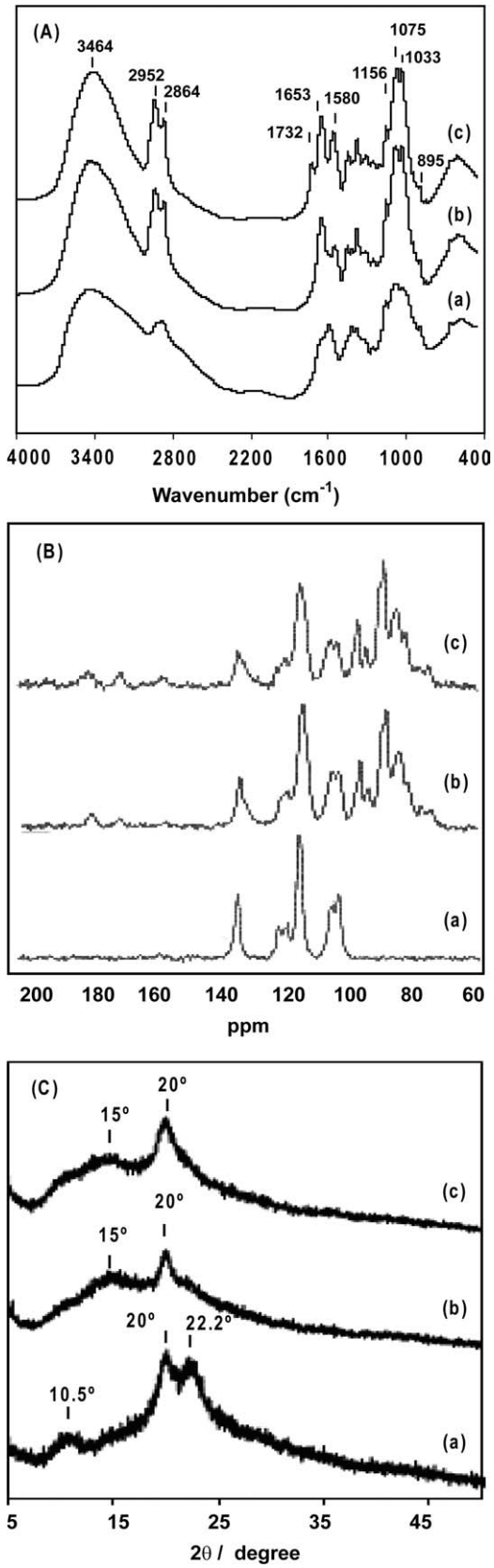


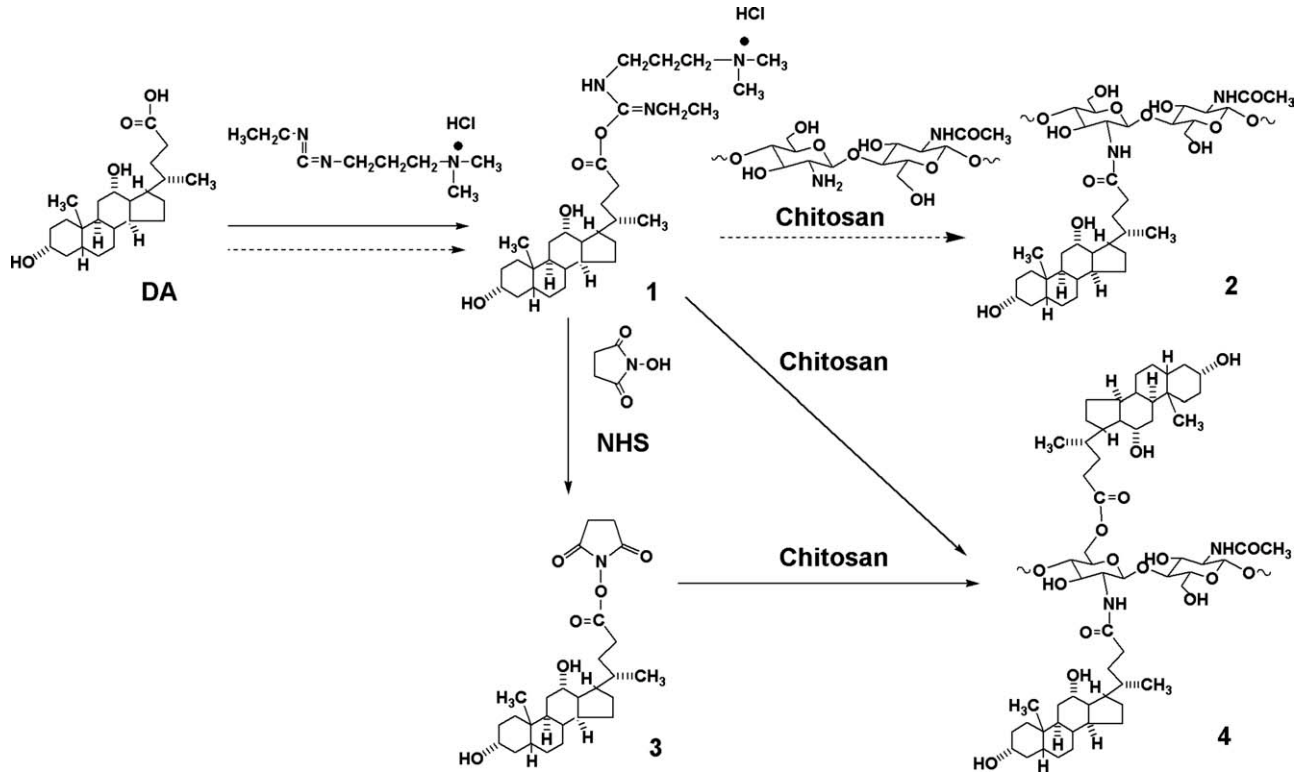
Figure 1 (A) FTIR spectra, (B) ^{13}C-NMR spectra, and (C) XRD patterns of: (a) chitosan, (b) DC-CsNS **2**, and (c) DC-CsNS **4**.

agent but was also proposed as a solvent of chitosan.

To facilitate the coupling reaction with primary amine groups in chitosan, the carboxylic group of DA was activated by converting the carboxylic acid group into NHS-ester form[28,47] (Scheme 1). In this fashion, EDC reacted with the carboxylic acid group of DA to form *O*-acylisourea, or intermediate **1**. Because this intermediate is unstable, it is often the case that NHS is added to carboxylic acid together with carbodiimide in order to form an NHS-ester species of succinimido deoxycholate (intermediate **3**), which is a much more stable compound that remains reactive with both amino (C-3) and hydroxyl (C-6) groups. The possible chemical structure of DC-CsNS is shown as compound **4** (Scheme 1). Figure 1(A:c) shows the FTIR spectra of DC-CsNS obtained from the homogeneous reaction. Compared with DC-CsNS from the heterogeneous reaction [Fig. 1(A:b)] a new absorption band at 1732 cm^{-1}, which is an indicator of the ester groups, was observed in addition to the amide bond at the peak of 1653 cm^{-1}.

The ^{13}C-CP/MAS NMR spectra of DC-CsNS are presented in Figure 1(B), which further confirmed its molecular structure. Typical peaks at 105.2 ppm (C_1 and C_3), 75.0–81.7 ppm (C_5), and 57.9–60.8 ppm (C_2 and C_6) are assigned to the pyranose ring in chitosan saccharine units [Fig. 1(B:a)].[47] The spectra in Figure 1(B:b,c) implied the chemical structures of DC-CSNS **2** and **4**. The peaks at 18.8–57.0 ppm are attributed to C of cyclohexyl groups from conjugated DC. The bond between chitosan and DC, which resulted from the conjugation of the amino (-NH_2) and hydroxyl (-OH) groups of chitosan and the carboxylic acid (-COOH) group of DA, caused the peaks of ester and amide linkage at 160.2–176.1 ppm. In addition, obvious peaks at 160.2–176.1 ppm were also observed in the homogeneous reaction [Fig. 1(B:c)]. The ^{13}C-CP/MAS NMR result confirms the FTIR result.

It has been proposed that chitosan exhibits six polymorphs: tendon, annealed, l-2, L-2, form-I, and form-II.[48] The crystallographic structures of chitosan and DC-CsNS were determined by X-ray diffraction (XRD). As presented in Figure 1(C:a), chitosan exhibited an XRD pattern at about 10.5°, 19.8°, and 21.5° 2θ, which are the characteristic XRD peaks of chitosan.[47,49] Such a pattern characterizes a chitosan crystal, which is referred to as the "tendon" polymorph[50] of the water-insoluble chitosan. As is well known, chitosan with a high degree of deacetylation is not soluble in water due to its strong intermolecular hydrogen bond. For DC-CsNS [Fig. 1(C:b,c)], the peak associated with chitosan at 10.5° was absent, and the reflection at about 21.5° also decreased significantly. In addition, a broad peak appeared in the



Scheme 1 Reaction pathway of chitosan conjugated with DA via (·····) heterogeneous system and (—) homogeneous system.

region around 15°. This result indicated that the crystalline structure had been disrupted by the chemical bond between chitosan and conjugated DC in the prepared DC-CsNS. The XRD pattern of DC-CsNS implied looseness in chain packing after conjugation of the bulky groups of DC onto the chitosan chains. The intermolecular hydrogen bond in DC-CsNS has decreased obviously in comparison with that of chitosan, and as a result the hydrophobic side groups were significantly conjugated onto chitosan.

Chemical modification: Effect of reaction time and reaction system

The relationships between the reaction time and the DC conjugation amount on chitosan were observed in heterogeneous and homogeneous reactions. Here, a non-irradiated Cs-colloid sample was used. To investigate the amount of conjugated DC moiety, the intensity ratios between a summation of amide-peak and ester-peak intensities ($I_{1653} + I_{1732}$) and the pyranose ring intensity (I_{895}), which is an internal standard peak, were determined. Figure 2 shows the relative intensity ratios ($[I_{1653} + I_{1732}]/I_{895}$) against the reaction time plot of DC-CsNS **2** and **4** from heterogeneous and homogeneous reactions, respectively. The results indicated that the relative intensity ratios increased when the reaction times increased from 1 to 24 h. The higher relative intensity ratio implied higher conjugated DC moieties. It was also found that at the same reaction time, the conjugation carried out in the homogeneous system exhibited a higher relative intensity ratio than that in the heterogeneous one. This demonstrated the higher DC

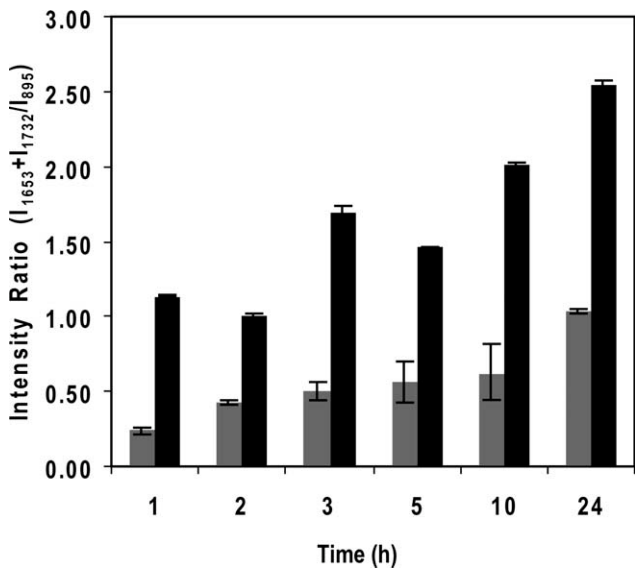


Figure 2 Intensity ratio ($[I_{1653} + I_{1732}]/I_{895}$) of DC-CsNS from DA conjugated onto non-irradiated Cs-colloid in heterogeneous (■) and homogeneous (■) reactions at various reaction times.

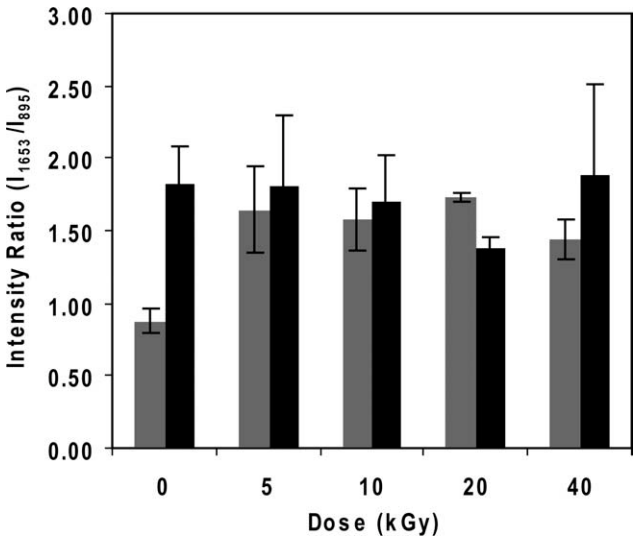


Figure 3 Intensity ratio (I_{1653}/I_{895}) of DC-CsNS from DA conjugated onto pre-irradiated Cs-flake (■) and Cs-colloid (■) in heterogeneous reactions for 24 h.

moieties of chitosan when the conjugation was carried out in the presence of NHS or in the homogeneous reaction.

Chemical modification: Effect of chitosan physical forms and γ-ray doses

To study the effect of chitosan physical forms and γ-ray doses on chemical modification efficiency, chitosan in two physical forms—that is, flake (Cs-flake) and colloid (Cs-colloid)—were pre-irradiated with doses of 5, 10, 20, 40, and 100 kGy before conjugating with DA under the heterogeneous system. The FTIR spectra of DC-CsNS from all studied conditions exhibited an amide linkage only at 1653 cm^{-1} (data not shown). Therefore, the FTIR intensity ratios of I_{1653}/I_{895} were determined in order to investigate the relative amount of chemical conjugation. The result indicated that the intensity ratios for all conditions were insignificantly different, even when changing the pre-irradiated doses as well as the physical form of chitosan (Fig. 3).

Elemental analysis was also performed to further characterize the amount of conjugation (Table I). The ideal structure of DC-CsNS from the heterogeneous reaction (as shown in Scheme 1) was found to be: C, 70.44%; H, 9.76%; and N, 1.62%. Based on the elemental composition, it was assumed that the ideal substitution degree (%DS) of DC on the chitosan chain at the C-2 position is 100%. The %DS produced a C/N ratio of 43.55. Compared with the ideal substitution, the %DS of DC-CsNS obtained from Cs-colloid pre-irradiated at 0, 5, 10, 20, 40, and 100 kGy were 30.7, 26.5, 26.8, 25.8, 24.9, and 30.8%, respectively. It was found that the %DS were also not different, as in the case of the relative intensity ratios observed from the FTIR spectra. It was suspected that the irradiated dose may not be important influence on the amount of chemical conjugation. Although higher γ-ray doses resulted in shorter chitosan chain lengths (as reported in our previous work[35]), the amount of the amino groups, which are the conjugating sites, was suspected to be unchanged for pre-irradiation doses in the given range of 5–100 kGy. In other words, γ-ray irradiation did not affect the amino groups (at C-2) of aqueous Cs-colloid.[35] It has been reported that the reactive species or free radicals, e.g., e^-_{aq}, $H^•$, $HO^•$, obtained from the radiolytic process attack the β-1-4 glycosidic bonds and create the degradation of chitosan.[49,51] As a result, the increase of the peak at 1375 cm^{-1} indicated the formation of methyl after a ring-opening reaction due to chain scission at the C-1 and C-4 positions. However, the unchanged peak at 1580 cm^{-1} (N—H stretching) confirmed the remaining amino groups after γ-irradiation of chitosan in colloidal form.[35]

Formation of DC-CsNS: Effect of chitosan physical forms and γ-ray doses

Information about particle shape and size of DC-CsNS was investigated by transmission electron microscope (TEM). Figure 4 shows TEM images of DC-CsNS obtained from the conjugation of DA onto 10 kGy-irradiated Cs-flake [Fig. 4(a)] and Cs-colloid [Fig. 4(b)]. It was found that the DC-CsNS from Cs-colloid produced particles with individual spherical shape [Fig. 4(b)]. Compared with the DC-CsNS from Cs-colloid, the particles from Cs-flake exhibited irregular shape with no distinct particle edge [Fig. 4(a)]. An attempt to clarify the particle size was performed. It was found that the average particle diameters of DC-CsNS from pre-irradiated Cs-flake and Cs-colloid were approximately 72 and 47 nm, respectively. Considering the non-modified chitosan nanoparticles initiated by γ-ray irradiation,[35] the molecular weights of Cs-flake and Cs-colloid after 10 kGy irradiation were reduced to 567 and 184 kDa,

TABLE I
Elements' Content of DC-CsNS from Heterogeneous Conjugation of DA onto Preirradiated Cs-Colloid

	Element composition					
Conditions	C	H	O	N	C/N	%DS
Ideal	70.44	9.76	18.07	1.62	43.55	100.00
0 kGy	54.31	8.75	32.81	4.06	10.66	30.70
5 kGy	53.92	8.64	32.73	4.68	11.15	26.47
10 kGy	52.81	8.61	34.03	4.52	10.74	26.81
20 kGy	52.15	8.60,	34.57	4.64	9.18	25.80
40 kGy	51.81	8.48	35.70	4.72	11.85	24.86
100 kGy	55.17	8.65	32.03	4.11	13.41	30.80

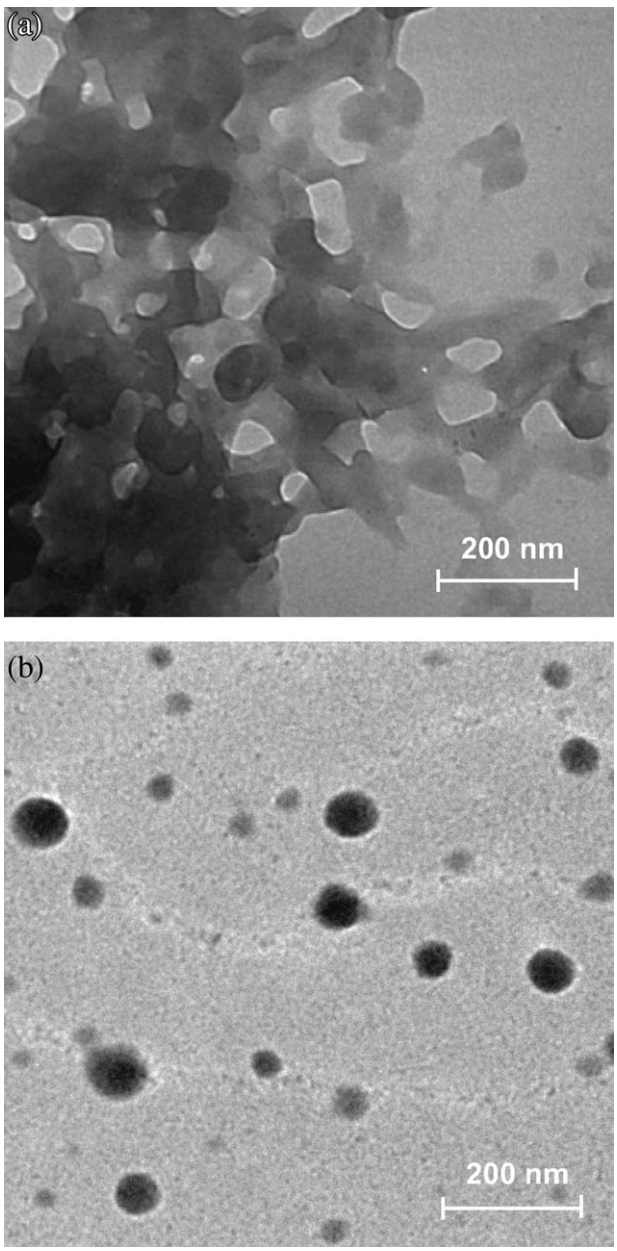
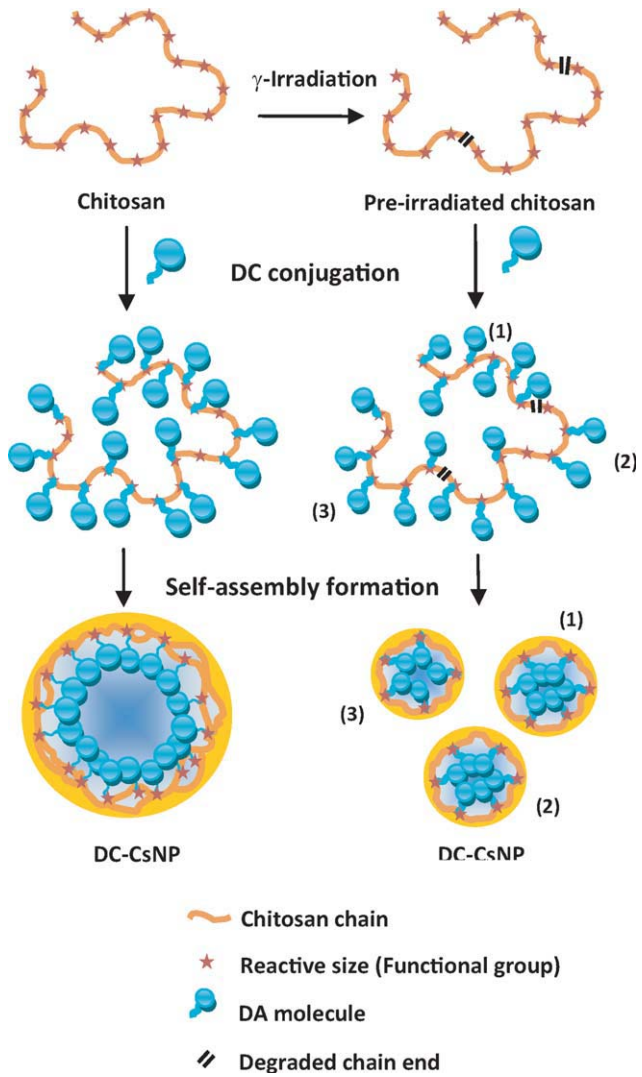


Figure 4 TEM images and size distribution of DC-CsNS from DA conjugated onto 10 kGy pre-irradiated (a) Cs-flake and (b) Cs-colloid in heterogeneous reactions for 24 h.

respectively. It was suspected that the shorter chain length of the pre-irradiated chitosan resulted in the smaller particle size of the DC-CsNS, as suggested in Scheme 2. Irradiation of aqueous Cs-colloid reduces the molecular weight by 3–4 times compared to that of solid-state Cs-flake, based on a radiolysis mechanism.[35,49] In an aqueous system, many types of water radiolysis species (i.e., e^-_{aq}, $H^\bullet$, $HO^\bullet$, H_2, H_2O_2) were produced after the sample was exposed to radiation. These reactive species abstract hydrogen from the main chain of chitosan, and the radicals on the chitosan chain subsequently rearrange. Degradation occurs, producing degradation fragments.[49] In the solid state, there are no reactive species produced by the solvent; the radiation directly interacts with molecules by ejecting electrons on the polymer chain. The radicals are produced and subsequently promote the degradation. Our observations proved that the physical form of chitosan during irradiation was also a parameter that affected DC-CsNS formation, due to the original chitosan chain length or its molecular weight. In addition, although the molecular weight of chitosan due to the pre-irradiated dose did not influence the total amount of conjugation, as shown in Figure 3, it possibly affected the self-assembly formulation. Unsuitable chain length of chitosan may play an important role in the spherical formulation as well as the particle size of DC-CsNS.

Figure 5 shows TEM images and particle size distribution of DC-CsNS from the conjugation of DA onto pre-irradiated Cs-colloid in the heterogeneous system. It is seen that spherical-like shapes could



Scheme 2 Possible formation of DC-CsNP from non-irradiated chitosan and pre-irradiated chitosan. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

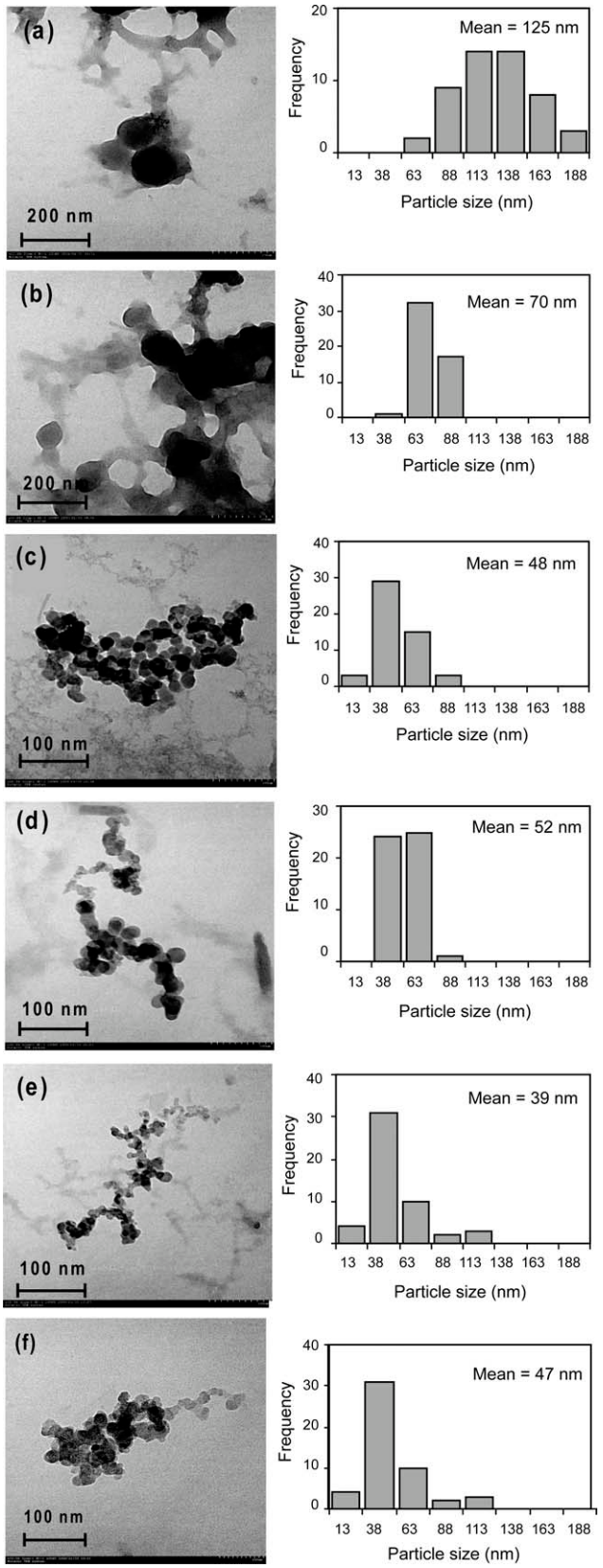


Figure 5 TEM images and size distribution plots of DC-CsNS from heterogeneous conjugation of DA onto Cs-colloid with γ-ray pre-irradiation doses of (a) 0, (b) 5, (c) 10, (d) 20, (e) 40, and (f) 100 kGy.

clearly be observed when DA was conjugated onto 10, 20, and 40 kGy pre-irradiated Cs-colloid [Fig. 5(c–f)]. Even though the particles observed in TEM images seem agglomerated, distinct particle edges confirmed the formation of DC-CsNS. The particle size distribution plot indicated that γ-ray pre-irradiation produced DC-CsNS with a narrow size distribution when compared with non-irradiated samples [Fig. 5(a)]. The γ-ray dose affects the particle size since a left-shift of the distribution plots was observed. Based on our investigation, it is supposed that γ-ray irradiation can be used for DC-CsNS synthesis and its nanoscale-size control. It has been reported that γ-rays not only initiated aqueous Cs-colloid to form non-modified chitosan nanoparticles, but also caused reduced particle size with a narrower size distribution than in the case of non-irradiation.[35]

Formation of DC-CsNS: Effect of chemical modification

To indicate the effect of chemical modification on the particle size of DC-CsNS, the particle sizes of non-modified chitosan nanospheres and DC-modified chitosan nanospheres (DC-CsNS) were plotted in Figure 6. The particle sizes of DC-CsNS were insignificantly smaller than non-modified chitosan nanospheres, for both pre-irradiated Cs-flake [Fig. 6(A)] and Cs-colloid [Fig. 6(B)]. This may be due to self-assembly formation by packing of the hydrophobic group in a water system. In a hydrophilic surrounding solution, i.e., water, the hydrophobic side groups of conjugated DC molecules turned and closely packed inside the particle to serve as the core, while the chitosan chains were wrapped around outside to serve as the particle surface. One can see in Figure 6 that γ-ray pre-irradiation doses also had a pronounced effect on particle size. The relative plot between particle size and pre-irradiation dose implies that the particle size of DC-CsNS showed a tendency to decrease when Cs-flake and Cs-colloid were pre-irradiated with gradually increasing γ-ray doses. This supported our speculation based on the size distribution plots in Figure 5. It is also interesting to note that the plot between particle size and pre-irradiation dose indicates a reduction with an exponential relationship. The tendencies of DC-CsNS from both Cs-flake [Fig. 6(A)] and Cs-colloid [Fig. 6(B)] were consistent with their molecular weight reduction trends induced by γ-irradiation, as in our previous research.[35] The phenomenon of radiation-induced molecular weight reduction of chitosan has also been reported to show an exponential relationship. This can be explained by the fact that the amorphous region of the chitosan structure is easily degraded. Therefore, in a dose range of 5–10 kGy the molecular weight is markedly

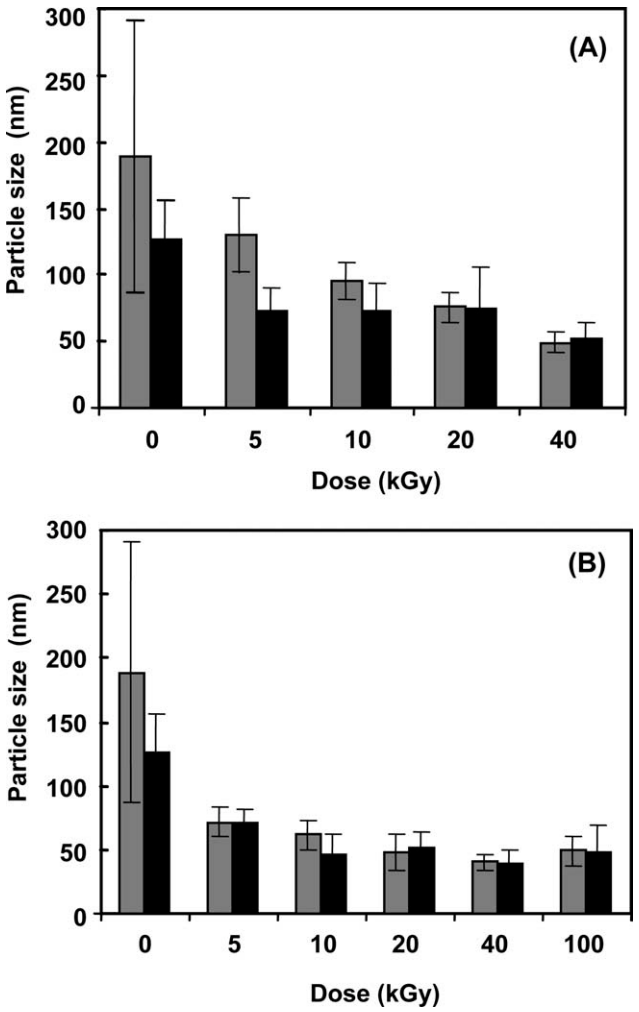


Figure 6 Particle sizes of non-modified chitosan (■) and DC-CsNS (■) from heterogeneous conjugation of DA onto pre-irradiated (A) Cs-flake and (B) Cs-colloid with various γ-ray doses.

reduced. With increasing radiation doses, the molecular weight steadily decreases due to degradation of the crystalline portion, which has a dense packing structure and a low amount of oxygen molecules. Moreover, oxygen molecules can barely penetrate through this region. This supported our finding that DC-CsNS obtained from 5 to 10 kGy pre-irradiation exhibited significant particle size reduction in comparison to 0 kGy. On the other hand, when using higher radiation doses (10–100 kGy) the particle sizes were mostly identical in size—approximately 50 nm in the case of Cs-colloid. This phenomenon is due to the fact that particle sizes are significantly reduced with a decrease in molecular weight.

Formation of DC-CsNS: Effect of reaction system

The heterogeneous and homogeneous systems were carried out in order to study the effect of the system-

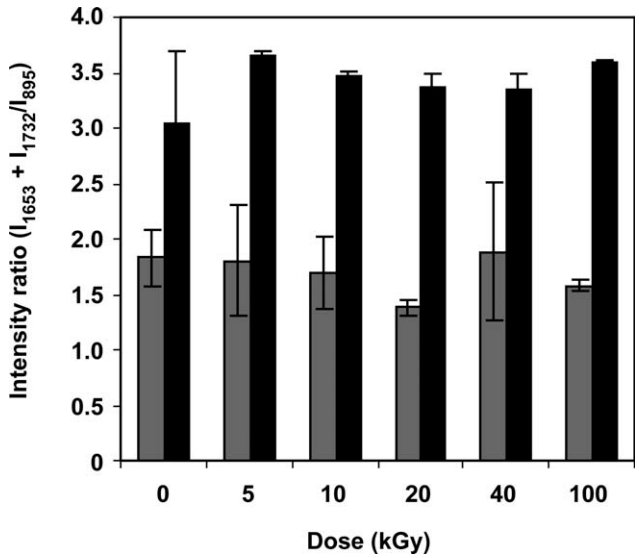


Figure 7 Intensity ratio ($I_{1653} + I_{1732}/I_{895}$) of DC-CsNS from DA conjugated onto pre-irradiated Cs-colloid in heterogeneous (■) and homogeneous (■) reactions.

atic condition on DC-CsNS formulation, as well as the chemical modification efficiency. Pre-irradiated Cs-flake and Cs-colloid were further functionalized with DA under the heterogeneous and homogeneous systems, as described above. Figure 7 indicates that the homogeneous conjugation resulted in higher chemical modification due to the intensity ratios $(I_{1653} + I_{1732})/I_{895}$. Compared with the heterogeneous system, the intensity ratios of the homogeneous reaction were increased by a factor of approximately 2. This result confirmed that the homogeneous reaction should promote greater conjugating efficiency because the reaction can be performed completely.

The particle shapes and sizes of DC-CsNS synthesized by the heterogeneous and homogeneous systems were also investigated. In TEM images it was

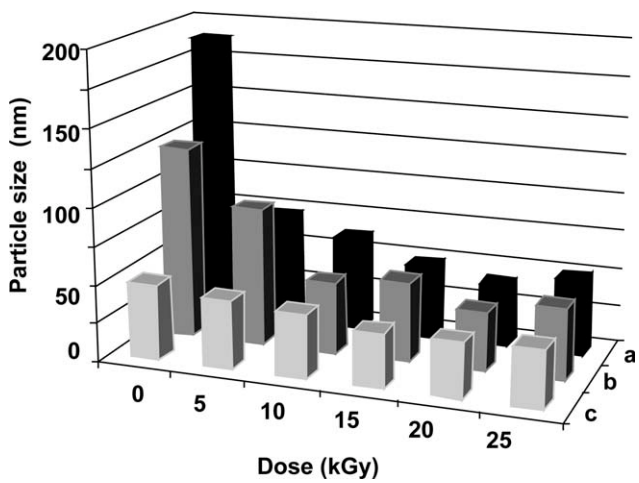


Figure 8 Particle sizes of (a) non-modified Cs-colloid, (b) DC-CsNS from the heterogeneous system, and (c) DC-CsNS from the homogeneous system.

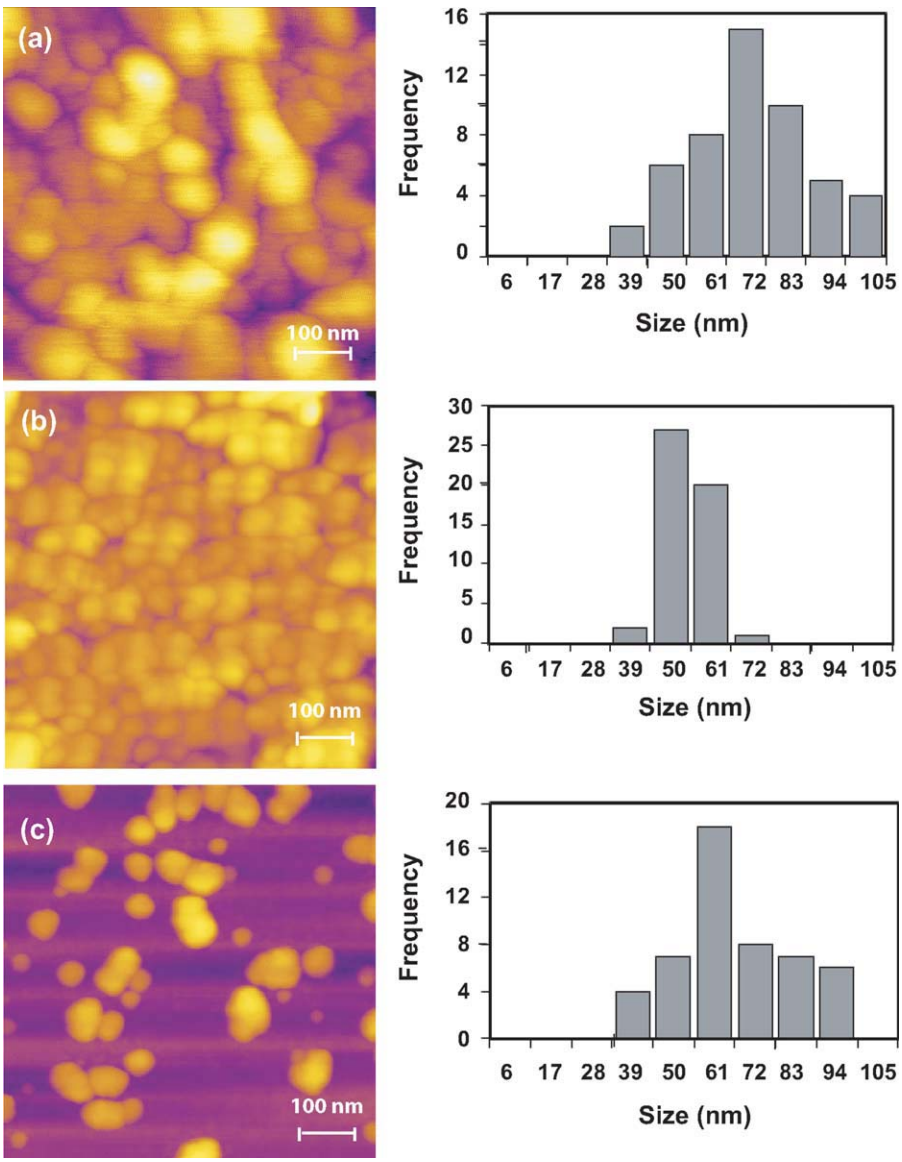


Figure 9 AFM images and size distribution plots of (a) 10 kGy pre-irradiated Cs-colloid; (b) DC-CsNS from conjugation of DA onto (a) in the heterogeneous system; and (c) DC-CsNS from conjugation of DA onto (a) in the homogeneous system. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

found to be more difficult to observe the spherical-like shapes of DC-CsNS from the homogeneous system than from the heterogeneous one (data not shown). Nevertheless, their mean diameters were determined. The relative mean particle sizes of DC-CsNS against the γ-ray dose curve are given in Figure 8. In the homogeneous reaction, the particle sizes of DC-CsNS from all pre-irradiated doses as well as non-irradiated were insignificantly different [Fig. 8(c)], averaging approximately 30–50 nm. In the case of the heterogeneous reaction [Fig 8(b)], a noticeable decrease in particle sizes with increasing γ-ray pre-irradiation doses was exhibited. It can be seen that the mean diameters of DC-CsNS decreased exponentially (due to molecular weight reduction, as mentioned previously). Compared with the non-irradiated particle size (125 nm), particle sizes from pre-irradiation with 5 and 10 kGy were reduced to 90 and 46, respectively; whereas the average particle sizes from pre-irradiation with >10 kGy (20, 40, and 100 kGy) were indistinguishable from those of 10 kGy. We suggest that under the heterogeneous reaction a γ-ray pre-irradiation dose of 5–10 kGy may be sufficient to decrease and control the particle size of DC-CsNS to nanoscale range (10–100 nm). Even at pre-irradiation doses higher than 10 kGy, the particle sizes were insignificantly reduced and showed little difference. We suspect that DC-CsNS of nanoscale size could be easily prepared by conjugating DA onto 10 kGy pre-irradiated Cs-colloid in the heterogeneous system. Unlike the homogeneous reaction, the heterogeneous reaction can be carried out without NHS.

It is important to note that using the heterogeneous system with pre-irradiation would serve as a promising protocol when the chemical, preparation, and purification steps need to be reduced.

The morphologies of DC-CsNS were also observed by AFM. Figure 9 shows AFM images of non-modified Cs-colloid [Fig. 9(a)] and DC-CsNS from the heterogeneous [Fig. 9(b)] and homogeneous [Fig. 9(c)] reactions. The morphologies of DC-CsNS were confirmed as having a spherical-like shape, as seen in TEM images. Compared with the non-modified Cs-colloid, the particle sizes of DC-CsNS from the heterogeneous and homogeneous systems were reduced 25% and 10%, respectively. It is suspected that the amount of conjugated DC moieties and the chitosan chain length may play important roles in particle size. The appropriate amount of conjugated molecules as well as the chitosan chain length may lead to desirable particle shape and size.

Our experiment proved that three parameters—the physical form of chitosan during irradiation, the γ-ray doses, and the reaction system—influenced the chemical modification efficiency and the particle formulation and size of DC-CsNS. It was found that γ-ray irradiation could possibly serve as a simple and effective tool in controlling and reducing the particle formation and size of DC-CsNS, as well as the molecular weight, under the appropriate systemic condition. Unlike many types of chitosan nanoparticles from previous reports,[29,31,32,52] the chitosan particles obtained in this study are possible to prepare at nanoscale size (10–100 nm). DC-CsNS sizes as small as 40–50 nm can be achieved. Currently, nanoscale size is one of the most important features to be considered for enhancing the potential of several advanced applications. The production of nanoscale material of a controlled size is also extremely desirable. Janes et al.[25] observed that the use of nanoscale particles as drug carriers in a drug delivery system may increase the efficiency of drug penetration of the cell membrane. Additionally, the use of nanoscale particles is capable of optimizing the properties of many products, e.g., nanoparticle-reinforced polymer films for food packaging and medical devices. Moura et al.[53] reported that chitosan-triphosphate nanoparticles improved the mechanical and thermal properties of hydroxypropyl methylcellulose edible film. Compared with the film without nanoparticles, the tensile strength of films containing modified chitosan nanoparticles with sizes of 221 and 85 nm increased from 26 MPa to 38 MPa and 63 MPa, respectively.

CONCLUSIONS

DC-CsNS could be fabricated by systematic controlled preparation, with nanoparticles forming via self-aggregation in water. The degree of chemical modification and DC-CsNS shape and size depend on the chitosan physical form during irradiation, the pre-irradiation dose, and the reaction system. It is possible to obtain DC-CsNS particles as small as 50 nm via a pre-irradiation process using heterogeneous reaction. The mean sizes of DC-CsNS obtained from the homogeneous reaction were nearly identical (~ 30–50 nm), even with increased pre-irradiation doses. In the heterogeneous system, pre-irradiation with a dose of at least 5–10 kGy is required; on the other hand, in the homogeneous system the addition of NHS is necessary to achieve reduced particle size.

The present work proposes that the self-assembly of DC-CsNS of nanoscale size could be systematically controlled, based on three parameters: chitosan physical form during pre-irradiation, pre-irradiation dose, and conjugation system. A simple yet effective protocol to fabricate self-assembly DC-CsNS with a particle size of approximately 50 nm can be achieved by the heterogeneous conjugation of DC onto 10 kGy pre-irradiated Cs-colloid.

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