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Development of ¹⁵³Sm-folatepolyethyleneimine-conjugated chitosan nanoparticles for targeted therapy

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The aim of this study was to develop biocompatible, water-soluble ¹⁵³Sm-labeled chitosan nanoparticles (NPs) containing folate and polyethyleneimine functionalities i.e. chitosan-graft-PEI-folate (CHI-DTPA-g-PEI-FA), suitable for targeted therapy. The physicochemical properties of the obtained NPs were characterized by dynamic light-scattering analysis for their mean size, size distribution, and zeta potential; scanning electron microscopy for surface morphology; and ¹H-NMR, FT-IR analyses for molecular dispersity of folate in the NPs. NPs were spherical with mean diameter below 250 nm, polydispersity of below 0.15, and positive zeta potential values. The NP complex (¹⁵³Sm-CHI-DTPA-g-PEI-FA) was stable at 25 °C (6–8 h, >90% radiochemical purity, instant thin layer chromatography (ITLC)). Binding studies using fluorescent NPs for internalization also demonstrated significant uptake in MCF-7 cells. MCF-7 cell internalization was significantly greater for 4T1. In blocking studies, both MCF-7 and 4T1 cell lines demonstrated specific folate receptor (FR) binding (decreasing 45%). *In vivo* biodistribution studies indicated major excretion of NPs metabolites and/or free ¹⁵³Sm through the kidneys. The preliminary imaging studies in 4T1 tumor-bearing mice showed minor uptake up to 96 h. The present folic acid that functionalized chitosan NP is a candidate material for folate receptor therapy.

Keywords: chitosan nanoparticles; ¹⁵³Sm; biodistribution; folate receptor; MCF-7; 4T1; breast cancer; SPECT

Introduction

The advances in nanoparticle (NP) science application in molecular medicine have led to the development of many nanoconstructs for therapy and diagnosis in human diseases. Radiolabeled NPs based on various inorganic nanocores including paramagnetic metal oxides,¹ and organic/biological polymers such as chitosan, ² aggregated albumin,³ and so on have been reported for targeted therapy/diagnosis based on the size and molecular surface decorations.

Because of its excellent effects, internal radiation therapy is in widespread use for the treatment of a variety of diseases. Such diseases include cancer⁴ and rheumatoid arthritis.⁵ ¹⁵³Sm, which has a suitable half-life (46.7 h) and β -emitting property along with γ -ray for imaging, represents an interesting candidate for developing therapeutic molecules.

For many years, complexes combining radioactive metals with diethylene triamine pentaacetic acid (DTPA) have been widely used in molecular imaging⁶ and radiotherapy.^{7–9} Moreover, many DTPA lanthanides analogs have proved stable enough for use in a physiological medium as radiopharmaceuticals.¹⁰ So immobilization of these auxiliary agents on polymeric carrier matrix is essential.¹¹

Chitosans, a family of linear binary polysaccharides, comprised of beta (1–4)-linked 2-amino-2-deoxy- β -D-glucose (GlcN; D-unit) and the *N*-acetylated analog (GlcNAc; A-unit), have been proposed as biocompatible alternative cationic polymers that are suitable for drug delivery.

Conjugation of chitosan with polyethyleneimine (PEI) has been shown to effectively increase cell uptake rely on excess cationic charge to mediate cellular entry due to its buffering capacity.¹² PEI exists as a branched polymer, as well as in linear form. The branched form of PEI shows a theoretical ratio of primary to secondary to tertiary amine groups of 1:2:1. These amines have pKa values spanning the physiological pH range, resulting in buffering capacity. ¹³ On the other hand, many studies have addressed concerns about the toxicity of conventional PEI. The cytotoxicity of PEI is dependent on its molecular weight. A lower molecular weight PEI has a lower cytotoxicity.^{14,15} Because of negatively charged plasma components, cellular entry mechanism based on charge

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*Correspondence to: Amir R. Jalilian, Radiation Application Research School, Nuclear Science and Technology Research Institute (NSTRI), Tehran, 14155-1339, Iran. E-mail: ajalili@aeoi.org.ir interaction is likely to be ineffective for *in vivo* drug delivery of vectors administered intravenously.

The folate receptor is a high-affinity membrane folate-binding protein, overexpressed in a wide variety of human tumors. Meanwhile, normal tissue distribution of folate receptor is highly restricted, making it a useful marker for targeted drug delivery to tumors.^{16,17} Folate conjugates have been shown to be taken into receptor-bearing tumor cells via folate receptor-mediated endocytosis. Folate conjugation, therefore, presents a useful method for receptor-mediated drug delivery into receptor-positive tumor cells.

In this study, in the first part, PEI-folate (PEI-FA) was synthesized according to the reported method using a simple conjugation step via a water elimination reaction (Figure 1).

In the next step, the chitosan-graft-PEI-folate (CHI-g-PEI-FA) NPs were prepared by an imine reaction between periodateoxidized chitosan and the aforementioned low molecular weight PEI to reduce cytotoxicity and enhance the cell nucleus membrane transfer efficiency (Figure 2).



Figure 1. Proposed reaction scheme for synthesis of PEI-FA.

Finally, samarium-153 chitosan-graft-PEI-folate (¹⁵³Sm-CHI-g-PEI-FA) NPs were prepared at optimized conditions followed by stability, human tumor cell binding, and biodistribution studies in normal and tumor-bearing animals using dissection and Single Photon Emission Computed Tomography (SPECT) studies.

Experimental

Chitosan (medium molecular weight; deacetylation degree, 87.7%), sodium periodate, sodium borohydride (granular, 10-40 meshes, 98%), branched PEI 1800 Da, folic acid dihydrate and diethylene triamine pentaacetic acid-dianhydride (DTPA-DA), dicyclohexylcarbodiimide (DCC), and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich Chemical Co. (Germany); (99%). All other reagents were commercially available and used as received. Production of ¹⁵³Sm was performed at the Tehran research reactor using ¹⁵²Sm (n, gamma) ¹⁵³Sm nuclear reaction. ¹⁵²Sm with purity of >98% was obtained from ISOTEC Inc., USA. Radiochromatography was performed by counting of Whatman no. 2 (GE, England) using a thin layer chromatography scanner, Bioscan AR2000 (Paris, France). Calculations were based on the 103-keV peak for $^{153}\mbox{Sm}.$ All values were expressed as mean \pm standard deviation, and the data were compared using student's t-test. Statistical significance was defined as P < 0.05. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edition. Cell lines were purchased from the Institute Pasteur of Iran.

Production and quality control of ¹⁵³SmCl₃ solution

The ^{153}Sm was produced by neutron irradiation of 100 μg of enriched $^{152}\text{Sm}_2\text{O}_3$ according to reported procedures 18 at a thermal neutron flux of 5×10^{13} n cm $^{-2}$ s $^{-1}$ for 5 days. Specific activity of the ^{153}Sm was 27.75 GBq/mg. The irradiated target was dissolved in 200 μL of 1.0 mol/ L HCl, to prepare $^{153}\text{SmCl}_3$ and diluted to the appropriate volume with



CHI-D TPA-PEI-FA

Figure 2. Proposed reaction scheme for synthesis CHI-DTPA-g-PEI-FA.

ultra pure water, to produce a stock solution. The mixture was filtered through a 0.22 μ m of biological filter and sent for use in the radiolabeling step. Radionuclidic purity of the solution was tested for the presence of other radionuclides using beta spectroscopy and High Purity Germanium (HPGe) spectroscopy to detect various interfering beta and gamma emitting radionuclides. The radiochemical purity was also checked by Whatman no. 1 chromatography paper, and developed in a mixture of 10 mmol/L DTPA solution and also 10% ammonium acetate/methanol (1:1) as mobile phases.

Preparation of low molecular weight chitosan

Chitosan (2 g, medium molecular weight, 400,000 g/mol) was dissolved in 100 mL of acetic acid (6%, v/v) and depolymerized at room temperature under stirring with 10 mL of NaNO₂ solutions in water at definite concentration (12 g/L), to obtain the desired final molecular weight of 9000 g/mol. Afterwards, chitosan was precipitated by the addition of 4 M of NaOH until pH 9 was reached. The resulting precipitate was filtered and washed with cold acetone. The white yellowish solid was filtrated, washed thoroughly with acetone, and re-dissolved in a minimum volume of acetic acid of 0.1 N (40 mL), and purification was carried out by subsequent dialysis against demineralized water. (Sigma dialysis tubes, molecular weight cutoff, 5-8 kDa). The dialyzed product was lyophilized using a LyoTrap plus Freeze dryer (LTE Scientific, Oldham, UK), and the yellowish lyophilized product was then stored at 4 °C until use. The average molecular weights of the prepared chitosans were determined by a gel permeation chromatography. The molecular weight of the product was checked according to the published procedure.19

Synthesis of CHI-DTPA conjugate

The conjugated chitosan-diethylene triamine pentaacetic acid (CHI-DTPA) was synthesized by the coupling reaction of free carboxyl group of DTPA-DA with amine group of chitosan in the presence of DCC and NHS. Briefly, to a 60 mg of DTPA-DA dissolved in a 5 mL of hot ethanol were added 25 mg of DCC and 13 mg of NHS, respectively, and mixed for 1 h. After elapsed time, they were then mixed together under mechanical stirring. The coupling reaction was carried out for 5 h at 70 °C under mechanical stirring with 300 rpm.

After the reaction, the reaction solution was dialyzed against deionized water. About 160 mg of low molecular weight chitosan, prepared as described earlier, was dissolved in 15 mL of acetic acid (1%, v/v). The mixture was dialyzed against water using a dialysis membrane (molecular weight cut-off (MWCO): 5–8 kDa) for 48 h with successive exchange of fresh deionized water to remove water-soluble by-products. The reaction solution was then lyophilized. Dialyzed product was freeze-dried and stored at -20 °C until use.

Synthesis of folate-PEI

Before reaction of PEI, an activated ester of folate, NHS-folate, was synthesized. Folic acid (240 mg) was dissolved in 10 mL of dimethyl sulfoxide (DMSO) along with addition of 200 mg excess of DCC and 120 mg excess of NHS into the solution. The reaction mixture was stirred for 2 h in darkness at room temperature. The insoluble by-product, dicyclohexylurea, was removed by filtration through glass wool. The filtrate containing the DMSO solution of the NHS-folate product was stored at -20 °C until use in further synthesis. Folate-PEI conjugate was synthesized by reacting 400 mg of PEI dissolved in 5 mL of DMSO with the NHS-folate synthesized earlier, as shown in Figure 1. The product was then diluted in two volumes of deionized water and then poured into over an excess amount of acetone.

The suspension was decanted, and the precipitate was re-dissolved in deionized water. The reaction solution was then lyophilized, and product was freeze-dried and stored at -20 °C until use.

Synthesis of copolymer

The CHI-DTPA-g-PEI-FA copolymer was synthesized in two steps. In the first step, periodate-oxidized chitosan was prepared by a modified method of Y. Jia et al. ²⁰ Briefly, 300 mg of CHI-DTPA was dissolved into 20 mL of acetic acid (1%, v/v), and the obtained clear solution was degassed by purging N₂ for 30 min, and then 1.5 mL of sodium periodate (NalO₄) solution (10 mg/mL) was added to the solution in a lightprotected glass vessel. After mixing the solutions, the reaction was performed for 30 min at 50 °C with occasional shaking. In order to quench the reaction, 0.1 mL of glycerine of reaction medium was added. Then the solution was dialyzed (MWCO = 3500 Da) against deionized water. In the second step, 200 mg of PEI-FA was reacted with the periodate-oxidized chitosan solution with magnetic stirring for 6 h at 70 °C. Subsequently, the solution was treated with sodium borohydride (0.4 g NaBH₄/g chitosan), dialyzed against deionized water (MWCO = 5-8 kDa), to remove unreacted PEI-FA. After dialysis, the copolymer was lyophilized.

Characterization of CHI-DTPA-g-PEI-FA conjugate

¹H-NMR spectrum of the prepared CHI-DTPA-g-PEI-FA was recorded on Avance[™] 500 spectrometer (Bruker, Germany) operating at 500 MHz using D₂O and DMSO-d₆ as solvents. Chemical shifts (δ) were given in parts per million using tetramethylsilane as an internal reference. The Fourier transform infrared spectrum of products was recorded using KBr pellet at room temperature.

Size distribution of NPs

The mean diameter and size distribution of the NPs were determined by dynamic light scattering using Zetasizer[®] (Nano-ZS, Malvern, Instruments, Malvern, UK). All dynamic light-scattering measurements were carried out at a wavelength of 633 nm at 25 °C with an angle detection of 90°. The samples were diluted in acetic acid (16 μ mol/L) in deionized water, three subsequent measurements were determined for each sample, and the result was expressed as mean size ± standard deviation.

Determination of zeta potential

The zeta potential measurements were performed by Laser Doppler Electrophoresis using Zetasizer (Nano-ZS). To maintain a constant ionic strength, the samples were diluted (1:50 v/v) in 1 mmol/L (pH 6.5) of NaCl. ²¹ Each sample was measured three times.

Radiolabeling of CHI-g-PEI-FA and the effect of concentration of the chitosan-conjugate solution and pH on the labeling yield

The $^{153}\text{Sm-CHI-g-PEI-FA}$ NP suspension was prepared by adding 16 μL of ¹⁵³SmCl₃ (1 mCi, obtained by neutron activation of ¹⁵²SmCl₃) solution to 20 μg of lyophilized modified chitosan (CHI-DTPA-PEI-FA) particles, which were dispersed in 0.5-1.0 mL of phosphate buffer saline (PBS) solution followed by thorough stirring in a lead-shielded sonicator. The resulting suspension was incubated for 1 h at 37 °C. The contents were transferred into an evacuated sterile sealed vial for subsequent use. In order to investigate the effects of various factors on the labeling yield of the complex, the pH of the reaction mixture was varied in the range of 4.5-7.5 by adding an appropriate volume of 0.5 N of HCl and 0.5 N of NaOH to the chitosan solution. The labeling yield was measured by instant thin layer chromatography using ITLC with a solvent system of MeOH/H₂O/HOAc (50:50:5). The R_f values of free ¹⁵³Sm and ¹⁵³Sm-CHIg-PEI-FA NP complex were 0.5 and 0.0, respectively. The stability of ¹⁵³Sm-CHI-g-PEI-FA derivative was examined by temporally measuring the radiochemical purity of the complex by ITLC at 1, 2, 4, and 24 h after preparation.

Radiolabeling of chitosan by ¹⁵³Sm for control biodistribution studies

The complex was prepared according to the recently published method. ²² Briefly, ¹⁵³Sm-chitosan complex was prepared by dissolving of chitosan (35 mg) in 3.5 mL of 1% acetic acid aqueous solution following the addition of ascorbic acid (15 mg), and the mixture was stirred at room temperature until a transparent solution was formed. To the aforementioned mixture, 296–370 MBq (in 0.5 mL) of ¹⁵³SmCl₃ was added followed by stirring for 5 min and standing for 30 min at room temperature. For measuring radiochemical purity and radiolabeling yield, 1 µL sample of the ¹⁵³Smchitosan complex was spotted on a chromatography paper (Whatman no. 1), and developed in a mixture of methanol/water/acetic acid (4:4:2) as the mobile phase. The $R_{\rm f}$ values of free ¹⁵³Sm and ¹⁵³Sm-chitosan complex were 0.45 and 0.0, respectively.

Biodistribution of ¹⁵³Sm-CHI-g-PEI-FA, ¹⁵³Sm-chitosan, and ¹⁵³SmCI₃ in Balb/c mice

Biodistribution of ¹⁵³Sm-conjugates and free ¹⁵³SmCl₃ was studied in 2– 3-month-old mice weighing 35–42 g. One hundred microliters of sterile radiolabeled copolymer (concentration: 2 mg/mL) was administered through the tail vein of each mouse, (injected dose: $100 \,\mu$ Ci). The animals were sacrificed by CO₂ gas at different time intervals, and different organs (bone, blood, liver, spleen, kidney, muscle, lung, heart, bladder gastric, and intestinal) were removed, washed with normal saline, and dried in paper folds. The radioactivity in each organ was counted using Nal(TI) well-type gamma counter, and the counts were recorded in the gamma spectrometer (Canberra, Meriden, USA), and expressed as percent injected dose per gram of the organs. Further, specific counts per gram of blood were calculated for each time interval.

Cell culture

The cell binding and internalization of ¹⁵³Sm conjugates into MCF-7 and 4T1 breast cancer cell lines as folate receptor positive (FR(+)) and CHO (Chinese hamster ovarian) cell lines as folate receptor negative (FR(-)) were studied. The cell lines were grown at 37 °C in a humidified atmosphere containing 5% CO₂ in a special Roswell Park Memorial Institute (RPMI) 1640 culture media without folic acid with 10% fetal bovine serum, L-glutamine, and antibiotic in tissue culture flasks. Twenty hours prior to each experiment, the cells were seeded in 24-well plates (10⁵ cells per well) to form confluent monolayers overnight.

In vitro cell binding and internalization

For cell binding experiments, the monolayers of cells were rinsed with ice-cold PBS pH7.4. Pure special RPMI medium (without fetal bovine serum/L-glutamine/antibiotic) only (1 mL), or medium (500 μ L), and a folic acid solution (for blocking study, 1 mM, 500 μ L) were added into the corresponding wells.

The well plates were pre-incubated at 37 °C for 40 min. The solutions of the complexes (8 μ Ci, 1 mCi/mL), purified by column chromatography using Sephadex G-25, were added in each well, and the well plates were incubated again at 37 °C for 1 h and then rinsed with ice-cold PBS pH 7.4 and $300 \,\mu\text{L}$ of stripping buffer (aqueous solution of 0.1 M of acetic acid and 0.15 M of NaCl) for 5 min, respectively, to remove bound complex from the FR on the cell surface.²³ The monolayers were dissolved in 1 N of NaOH (1000 $\mu\text{L})$ and internalized (acid-resistant), radioactivity was measured in a γ -well counter. Then the cells were pelleted by centrifugation at 800 g and resuspended in full media. The percentage of cell uptakes of the ¹⁵³Sm-NPs was calculated as percentage uptake = [pellet activity (cpm)]/[pellet activity (cpm) + supernatant activity (cpm)]×100, and internalizations of each sample were determined. The percent of internalized radioactivity was calculated by dividing the internalized radioactivity by the total radioactivity associated with the cells (surface-bound + internalized) and multiplying by 100.24,25

Internalization studies of fluorescent NPs using fluorescent imaging microscopy

For qualitative uptake studies, the MCF-7 cells were seeded in the chambered glass system (Lab-Tek; Nunc International Co., Naperville, IL, USA). Cells were washed four times after incubation with FA-loaded NPs for 2 h and then fixed by a cold mixture of methanol/acetone (50:50 v/v) for 15 min at room temperature. The cells were washed twice with PBS and mounted in mounting medium consisting of Na2 HPO4 and acetic acid (pH 5.5)/glycerol (50:50 v/v) to be observed by fluorescence microscope (λ ext: 540 nm and λ em: 580 nm; BX40; Olympus, Tokyo, Japan). The fluorescent images were taken by DP70 digital imaging system (Olympus, Tokyo, Japan) and analyzed by Olysia imaging software (Olympus, Tokyo, Japan).

Cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT)

MCF-7 cells were seeded in clear 96-well plates at a density of 4000 cells per well for 16 h to reach in suitable confluent monolayer. After time interval, the cells were subsequently incubated with 200 μ L/well of no or 1, 2, 5, and 10 μ g, μ Ci, and μ g/ μ Ci, respectively, of CHI-g-PEI-FA, ¹⁵³SmCl₃, and ¹⁵³Sm-CHI-g-PEI-FA complex, and the cells were kept in the incubator for 24, 72, and 96 h. After incubation for time intervals at 37 °C, the cells were incubated for 4 h with a 0.5 mg/mL solution of MTT, which was disposed afterwards and replaced with 200 μ L of equal parts of DMSO. Absorbance at 570 nm was measured. Data shown are based on five different experiments, and the results were expressed as mean ± standard deviation.

In vivo imaging of the radiolabeled NPs

The animal experiments were carried out using female Balb/c mice weighing 18–21 g each (5–7 weeks old) from Pasteur Institute, Tehran, Iran. The animals were inoculated subcutaneously with suspended 4T1 cells (1×10^{6} /0.1 mL) in the left loin region. The radiotracer studies were carried out 2 weeks after 4T1 cell injection. ¹⁵³Sm-CHI-g-PEI-FA NPs were administered intravenously via a tail vein to the animals. We then performed scintigraphy and recorded the 24-h, 48-h, and 96-h images by using gamma camera. The *in vivo* work was approved by the ethical committee of the Pharmaceutical Research Centre, Faculty of Pharmacy, Tehran University of Medical Sciences.

Results and discussion

Preparation of conjugate and characterization

The key steps in the synthesis were the functionalization of DTPA into chitosan and the conjugation of PEI-FA functions to chitosan by a rarely used method. PEI contains primary amino groups, which account for 25% of the nitrogen atoms, through which a desired targeting ligand may be attached, either directly or via a spacer. CHI-g-PEI is more positively charged; therefore, they are likely to interact more effectively with the negatively charged cell surface via nonspecific charge interaction.

After synthesis of CHI-DTPA and PEI-FA, grafting of this two polymers was carried out using periodate ion as an oxidizing agent, which splits the carbon–carbon bond of vicinal diols to give the dialdehyde group.²⁶ The study by Nicolet and Shinn suggested that this oxidation can be extended to cases in which hydroxyl is replaced by primary or secondary amines.²³ Figure 3 shows the Fourier transform infrared spectra of chitosan (a), CHI-DTPA (b), and CHI-DTPA-g-PEI-FA (c). From the chitosan spectrum, it was found that distinctive absorption bands appear at 1668 cm^{-1} (amide II), 1575 cm^{-1} (–NH₂ bending), and 1405 cm^{-1} (amide III). The absorption bands at 1154 cm^{-1}



Figure 3. FT-IR spectra for chitosan (a), CHI-DTPA (b), and CHI-DTPA-g-PEI-FA (c).

(asymmetric stretching of the C–O–C bridge), 1070 cm^{-1} , and 1033 cm^{-1} (skeletal vibration involving the C–O stretching) are the characteristics of its carbohydrate structure. Compared with that of chitosan, the peak at 1646 and 1404 cm⁻¹ (amide groups characteristic) in spectra (b) appears sharper compared with that of chitosan. Also, the peak at 1735 cm^{-1} is related to the carbonyl group of carboxyls in DTPA. These results indicated the derivation reaction of DTPA that took place at the *N*-position, and –NH–CO– groups have been formed. The peak at 1115 cm⁻¹ (vibration of C–N in PEI), strong peaks at 2873 and 2926 cm⁻¹ (related to methylene groups in PEI), and peaks at 2099–2182 (of R–N=C– bonds in pteridine moiety from the folate) show the conjugation of PEI-FA to chitosan derivative.

¹H-NMR spectra of chitosan before and after conjugation with DTPA and grafting with PEI-FA are shown in Figure 4. The ¹H-NMR assignments of chitosan resonances are as follows:



Figure 4. 1 H-NMR spectra of chitosan-DTPA (a), PEI (b), PEI-FA (c), and CHI-DTPA-g-PEI-FA conjugate in D₂O.

 $\delta = 4.69 \text{ ppm}$ (H₁), $\delta = 2.89 \text{ ppm}$ (H₂), $\delta = 3.63 - 3.89 \text{ ppm}$ (H₃, H₄, H₅, H₆), and $\delta = 1.87$ ppm (–NCOCH₃) (data not shown). ¹H-NMR of CHI-DTPA shown in Figure 4a was as follows: $\delta = 4.50 \text{ ppm}$ (H₁), $\delta = 3.54 - 3.70 \text{ ppm}$ (H₄, H₅, H₆), $\delta = 2.87 - 3.70 \text{ ppm}$ 2.98 ppm (protons of CH₂-CH₂), δ = 3.21 ppm, and δ = 3.41-3.46 ppm (related to protons of CH₂ near carbonyl group). The assignment of chemical shifts of PEI was shown in Figure 4b in which the proton peaks of PEI (-NHCH₂CH₂-) appeared at 2.44–2.58 ppm. In Figure 4c, the chemical shifts at $\delta = 2.40$ – 2.81 ppm (CH₂CH₂- of PEI) and δ = 6.75, 7.65 ppm (correspond to the para-aminobenzoic acid from the folate), and 8.58 ppm (corresponds to the pteridine moiety proton from the folate) obviously showed the conjugation of PEI-FA. In the ¹H-NMR spectra illustrated in Figure 4d, appearance of new proton signals at $\delta = 3.06 - 3.53$ ppm and the signals at 6.37, 7.39, and 8.58 ppm, all indicated that PEI-FA was grafted to the chitosan chain and confirmed the formation of CHI-DTPA-g-PEI-FA polyplex. FT-IR together with ¹H-NMR measurements pointed to the conclusion that the different groups have successfully conjugated onto the chitosan via a condensation and subsequent reduction reaction.

Size determination

The CHI-DTPA-g-PEI-FA NPs prepared in white, porous, lyophilized powder were used for material experiments. Figure 5 represents the particle size distribution of CHI-DTPA-g-PEI-FA particles, where the majority of particles (88.4%) have diameters in the range of 200–220 nm. Particle size distribution was measured by using Coulter Counter (Coulter Multisizer, Coulter



Figure 5. Zeta potential distribution of CHI-DTPA-g-PEI-FA nanoparticles recorded up to 200 mV.



Figure 6. Size distribution of CHI-DTPA-g-PEI-FA nanoparticles using Coulter Counter.

Electronics Inc., Hialeah, FL, USA). A degree of self-aggregation was also observed (Figure 6).

To investigate the shape of CHI-DTPA-g-PEI-FA NPs, scanning electron microscopy was performed using a Jeol T330A (Jeol USA Inc., Peabody, MA, USA). Scanning electron microscopy images reveal that these particles have a spherical shape, as shown in Figure 7.

Effect of various factors on the formation of ¹⁵³Sm-CHI-g-PEI-FA complex

The ¹⁵³Sm-CHI-g-PEI-FA complex solution was prepared by mixing the ¹⁵³SmCl₃ solution (1 mCi) and the chitosan solution, which were prepared by dissolving chitosan in normal saline. The pH of the reaction mixture also varied as described in the Experimental Section. The R_f values of ¹⁵³Sm-chitosan-conjugate and free ¹⁵³SmCl₃ in the solvent system were 0.1–0.3 and 0.8–1.0, respectively. Figure 4 shows the effect of various factors on the labeling yield (%) of ¹⁵³Sm-chitosan. Figure 4b shows the effect of chitosan (MW = 11 kD) concentration versus ¹⁵³Sm activity (1, 2, 4, and 8 mg: 1 mCi ¹⁵³Sm) on the labeling yield. The yield did not increase significantly with increasing the chitosan concentration range of 1–8 mg/mL; however, it shows that the labeling yield reached 96% when the concentration reached 16 mg/mL.

Figure 8A shows that the labeling yield reaches a maximum of 90% in the pH of 5–6 and chitosan concentration of 2 mg/mL. As a result, the pH of the reaction mixture was adjusted to 5.7 in the



Figure 7. Scanning electron microscopy image of CHI-DTPA-g-PEI-FA nanoparticles.

subsequent experiments. Based on the aforementioned results, it was concluded that the optimal procedure for the preparation of 153 Sm-CHI-g-PEI-FA with a high labeling yield (96%) is as follows: 16 mg of chitosan (MW = 11 kD) was dissolved in 1 mL of PBS pH 5.7 of the solution. The solution was then mixed with 1 mCi of the 153 SmCl₃. A high degree of stability of the 153 Sm-chitosan-conjugate was retained for at least 24 h after labeling, as shown in Figure 8.

The labeling yield was determined by instant thin layer chromatography. Each point represents mean \pm standard deviation (n = 3).

Biodistribution studies

Radioactivities in the major organs of mice at 2, 4, 72, and 96 h after the tail vein administration of $^{153}\text{Sm-CHI-g-PEI-FA}$, $^{153}\text{Sm-chitosan}$, and $^{153}\text{SmCI}_3$ at a dose of (0.1 mCi)/100 μL are shown in Figures 9, 10 as percent of the injected dose per gram of tissues (%ID/g).

For ¹⁵³Sm cation, the biodistribution was mainly in the liver, kidney, and bone. The free cation is mainly soluble in water and it can be excreted via urinary tract. Because the metallic ¹⁵³Sm is transferred in plasma in protein-bond form, the major final accumulation was showed to be liver. After 48 h, the metabolites and/or free cation were excreted from liver into intestines via hepatobiliary tract resulting significant activity in this tissue, but it was not significant at 96 h. Trace accumulation was also observed in spleen.



Figure 9. Percentage of injected dose per gram (%ID/g) of 153 Sm-chitosan in Balb/c mice tissues at 2–96-h post-injection (n = 3).



Figure 8. Effect of pH of the reaction mixture (A), concentration of conjugated chitosan solution (B) on the labeling yield of the ¹⁵³Sm-chitosan complex and stability of labeled compound in elapsed times (C).



Figure 10. Percentage of injected dose per gram (%ID/g) of 153 Sm-CHI-g-PEI-FA complex in normal Balb/c mice 2–96-h post-injection (n = 3).

For ¹⁵³Sm-chitosan, among the organs examined, liver and spleen, lung showed much higher levels of radioactivity counts than the other organs. The major route of excretion of ¹⁵³Sm-CHI-g-PEI-FA is urinary tract as shown in Figure 10, about 80% of the whole activity was excreted from the kidneys into the urine in 4 h. Because NPs with a diameter of 250 nm would not be excreted through the kidneys, it must be breakdown products. The high water solubility of the complex is a major cause of this behavior. Other minor accumulated tissues are liver and bone; however, it is believed that free ¹⁵³Sm cation is the dominant species accumulating in these organs.

The accumulation of radioactivity in reticuloendothelial system including liver (about 1% ID/g organ), spleen (less than 0.5% ID/g organ), and lung (averagely 0.5% ID/g organ) was low at all time points. A low uptake of radioactivity was observed in the stomach until 96 h (less than 0.2% ID/g), indicating a minimal *in vivo* decomposition of the radioligands to form free ¹⁵³Sm.

In vitvo discrimination between MCF-7, 4T1 FR(+) tumors, and CHO FR(-) cell lines

Folate receptor binding of the derivatives was performed using *in vitro* experiments with MCF-7 human breast cancer cell line (overexpressing the FRs) and 4T1, as another FR(+) and invasive breast cancer cell line. CHO cells were also seeded as FR(-) studies to confirm the preferential uptake of complex by folate receptor. Internalization into tumor cells via receptor binding is important for therapy/scintigraphy of folate receptor-expressing lesions. After the removal of surface-bound radioactivity with stripping acidic buffer, internalization studies were performed to determine the degree of internalization of ¹⁵³Sm-CHI-g-PEI-FA NPs in MCF-7, 4T1, and CHO cells (Figure 11).

Studies showed that the internalization of radioactive NPs in MCF-7 cells was significantly greater than 4T1 cells (due to less FR on the cell surface) at all time intervals (using approximately 200,000 cells) even better observed after 90 to 180 min. In contrast with FR(+) cells, CHO (FR(-)) cells showed also higher accumulation of ¹⁵³Sm-CHI-g-PEI-FA complex.

The higher efficiency of nonspecific uptake was probably because of high positive charge of polymer along with increasing internalization efficiency of related PEI immobilized onto chitosan. It seems that the type and nature of cells may also be responsible for different cellular internalization characteristics of ¹⁵³Sm-NPs.

Blocking study was carried out where excess FA was added into medium 40 min prior to incubation of ¹⁵³Sm-CHI-g-PEI-FA. The nonspecific binding in the presence of a molar excess of folic acid after 30 min showed no significant difference in the accumulation of ¹⁵³Sm-CHI-g-PEI-FA in MCF-7 and 4T1 cells (Figure 12). However, after 180 and 330 min, displacement of the superficial activity in MCF-7 and 4T1 (internalization up to 50%), in respect of their related blocked forms MCF-7 + folic acid



Figure 11. Percentage of cell internalization and uptakes of radioactivity in MCF-7, 4T1, and CHO cell lines after 30, 90-min, 180-min, and 330-min incubation with ¹⁵³Sm-CHI-g-PEI-FA complex.

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Figure 12. Percentage of cell internalization of radioactivity in unblocked MCF-7, 4T1, and related blocked cells after 30-min, 180-min, and 330-min incubation with ¹⁵³Sm-CHI-g-PEI-FA.

and 4T1 + folic acid, was observed (averagely to 45%), demonstrating that the internalization of the radiolabeled folate analog into the tumor cells was partially through FR-mediated endocytosis suggesting the involvement of folate receptor. It seems that CHI-g-PEI-FA copolymer is likely to interact more effectively with the negatively charged cell surface via nonspecific charge interaction. Combination of an increase in the positive charge of the corresponding copolymer and presence of PEI moiety leads to the elevated cellular uptake and more efficient endosome release of the internalized ¹⁵³Sm-CHI-g-PEI-FA, respectively.

Internalization results of the labeled Fluorescein isothiocyanate (FITC)-NPs were shown in Figure 13. The targeted NPs can readily enter the cells, and the NPs were localized inside endosome. On the other hand, the intensity of FITC was significantly increased (green clusters inside the cell).

Tumor accumulation of ¹⁵³Sm-CHI-g-PEI-FA NPs was estimated using gamma camera imaging by the injection of radiolabeled NPs (100μ Ci/ 100μ L) via tail vein after 24, 48, and 96 h, which demonstrated signal of radioactivity into tumor after 96 h (Figure 14).

Results suggest that (i) the main delivery pathway for ¹⁵³Sm-CHI-g-PEI-FA complex into the FR+ cells is via folate receptor, which mediated NPs endocytosis; (ii) NPs's uptake can be inhibited by folic acid in the case of MCF-7 and 4T1 cells (FR+),



Figure 14. Imaging of intravenous administration of $^{153}\mbox{Sm-NPs}$ into 4T1 tumorbearing mice.

and internalizations drop averagely to 45%; and (iii) the remaining uptake of NPs to MCF-7 and 4T1 cells and the lower uptake of NPs to the CHO cells can be attributed to the some nonspecific tumor affinity of NPs.

The comparison of ¹⁵³Sm-CHI-g-PEI-FA and ¹⁵³Sm-chitosan uptake among tissues demonstrated significant difference among the accumulating pattern, in case of ¹⁵³Sm-chitosan, the major accumulation is taking place in the liver and spleen due to the insolubility of the unprocessed chitosan at normal serum pH.

Recent studies on the production and quality control of ^{99m}Tcchitosan-folate as a possible diagnostic agent for noninvasive imaging of folate receptor-positive cells have been reported; however, no *in vivo* details were presented.²⁴

Recently, water-soluble derivatives of chitosan were labeled with ^{99m}Tc as possible targeted delivery for nuclear imaging; however, they mostly were accumulated in the liver and excreted via the kidneys because of the water-soluble nature of chitosan derivatives. The targeting was based on the particle size rather than any biological targeting.²⁵ On the other hand, in the present study, the labeled compound is almost exclusively excreted through the kidneys.

On the other hand, many efforts have been carried out on the development of folate-based radiotracers for malignancy imaging. In one case, radiolabeled super paramagnetic folate was developed and successfully used in the imaging; however, the water solubility remained a disadvantage.²⁷ Also, various folate-based conjugates have been developed using SPECT radionuclides; however, they lack suitable biodistribution properties such as stability and *in vivo* stability.²⁸ The present



Figure 13. Fluorescent images of MCF-7 cells incubated with FITC-Chi-PEI-FA nanoparticles for 4 h.

developed compound however potentially possesses the folate targeting properties, as well as rapid removal from the kidneys compared with other liver-accumulating agents already presented.

Conclusion

¹⁵³Sm-CHI-g-PEI-FA was radiolabeled using In summary, optimized conditions. The spectroscopic as well as the biodistribution studies emphasized on the development of a new chitosan complex with diverse biological properties and the complex high water solubility can be an advantage for a systemic targeted therapy candidate leading to the fast removal of the excess non-accumulated fraction imposing less unwanted dose rate to other organs. The cell internalization of radioactive NPs in MCF-7 cells was significantly greater than 4T1 cells as FR + cell lines up to 330 min. In blocking studies, both MCF-7 and 4T1 cell lines demonstrated specific FR binding after 180 and 330 min decreasing to 45% of initial binding. ¹⁵³Sm-chitosan and free ¹⁵³SmCl₃ were also administered for biodistribution comparison. In vivo biodistribution studies in wild-type rats indicated that the NP is majorly excreted through the kidneys with a rapid washout compared with other radiolabeled species. The preliminary imaging studies in 4T1 tumor-bearing mice showed minor uptake up to 96 h. Because the abdominal uptake dominates the image, further studies are essential to increase the tumor animal biodistribution with ¹⁵³Sm-CHI-g-PEI-FA. ¹⁵³Sm-CHI-g-PEI-FA potentially possesses the folate targeting properties, as well as rapid removal from the kidneys compared with other liver-accumulating agents already presented.

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Conflict of interest

The authors did not report any conflict of interest.

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