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Research paper

# Radiolabeling efficiency and cell incorporation of chitosan nanoparticles

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ABSTRACT

Cationic nanoparticles of CS were developed according to ionotropic gelation process as potential cancer cell targeting agent. CS nanoparticles (CSNP) (F1 and F2) diameters varied between ranges of 100–800 nm. Particle size, polydispersity index and zeta potential values of formulations were measured by photon correlation spectroscopy. The morphological analysis for CSNPs was provided with scanning electron microscopy. For cell incorporation study, F1 and F2 were directly labeled by Technetium-99m ($^{99m}$Tc), radiochemical purity and stability of the complex were analyzed by radioactive thin layer chromatography and radioactive high performance liquid chromatography studies. After that, incorporation of $^{99m}$Tc labeled F1 and F2 were evaluated in $U_2OS$ and NCI–H209 cell lines. The six well plates were used for all experiments and the integrity of each cell monolayer was checked by measuring its TEER values with an epithelial voltammeter. Results confirmed that F1 and F2 formulations were successfully radiolabeled with $^{99m}$Tc. The incorporation percentages of $^{99m}$Tc labeled F1 and F2 in NCI–H209 and $U_2OS$ cell lines were found different when they compared to $^{99m}$Tc solution. Since $^{99m}$Tc labeled F1 and F2 highly uptake in cancer cell line. The results demonstrated that radiolabeled CSNPs may be a promising agent for cancer diagnosis.



## 1. Introduction

Nanoparticles (NPs) can be defined as particle size of 1000 nm or less with easily penetrating cellular membranes due to their relative small size. They can be prepared stable, homogeneous and well characterized systems in size and shape [1–4]. NPs should be nontoxic and biocompatible in biological media with high selectivity for diagnosis or therapy [5,6].

Since NPs are taken up by the reticuloendothelial system (RES) the concentration of NPs in the targeted site is reduced [7]. Many investigations have been aimed at reducing the RES uptake and increasing the concentration of the particulate carriers at the desired sites in the body. The common strategy is achieved by reducing the particle size of drug delivery systems.

Recent studies have demonstrated that size of nanoparticles and type of polymer are important for avoiding RES uptake and obtaining more concentration in the targeted site [8,9]. Nanoparticles which contain hydrophilic materials such as chitosan (CS) can be used to investigate their cell binding on normal and cancer cells and to explore their potential for tissue targeting. CS is a cationic polysaccharide and pH sensitive polymer. It can adhere to epithelium surfaces of cells. CS nanoparticle (CSNPs) formation is based on the simple technique of ionotropic gelation where the CS protonated amino groups are cross-linked by inter- and intramolecular bonds by multivalent polyanions. Sodium tripolyphosphate (TPP) is a very popular polyanion because it is non-toxic and forms gels with desirable properties [10,11].

Although experimental animal models are significant for cancer diagnosis, breast cancer, bone cancer, lung cancer binding affinities were established with in vitro cell culture studies. Also radiolabeled drugs or formulations can be used for in vitro cell binding studies by measuring the radioactivity in different types of cells after administration of labeled formulations to the cells. In this study, NPs were prepared with CS. After preparation, CSNPs are labeled with a commonly used radioisotope Technetium-99m ($^{99m}$Tc). When CSNPs are labeled with $^{99m}$Tc, CS forms complex through its dextran moiety (Beta(1,4)-D-glucosamine) with reduced $^{99m}$Tc ions and the amine groups of polymer is also expected to bind to $^{99m}$Tc ions at pH > 7 [12,13].

The aim of this study was to determine the particles can be used in cancer targeting. For this purpose, CSNPs (F1 and F2 formulation)

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were radiolabeled by $^{99m}$Tc, quality controls of labeled compound was performed with radioactive thin layer chromatography (RTLC) and radioactive high performance liquid chromatography studies (RHPLC). In vitro incorporation study of $^{99m}$Tc-CSNPs was evaluated in $U_2OS$ (human bone osteosarcoma) and NCI–H209 (human bone carcinoma) cell lines.

## 2. Methods

### 2.1. Materials

CS was obtained from Sigma Aldrich. $^{99m}$Tc-sodium pertechnetate was obtained from Department of Nuclear Medicine of Ege University. Stannous chloride (Sigma Aldrich) was used as reducing agent in labeling studies. Cell culture reagents and supplies were obtained from Gibco Invitrogen (Grand Island, NY). The $U_2OS$ and NCI–H209 were obtained from American Type Culture Collection (ATCC).

### 2.2. Preparation of chitosan nanoparticles

CSNPs were prepared according to the ionotropic gelation process. The CSNPs were believed to form due to the interaction of positive amino groups of chitosan and negative groups of TPP [14,15]. CS was dissolved at a concentration of 0.25% (w/v) in 1% acetic acid solution. The solution of TPP at the concentration of 0.125% (w/v) was prepared with deionized water. 10 mL TPP aqueous solution (0.125% of w/v) was added to 10 mL CS aqueous solution (0.25% of w/v) under constant stirring at room temperature to obtain nanoparticles for formulation 1 (F1). To prepare formulation 2 (F2), 10 mL TPP aqueous solution (0.125% of w/v) was added to the 10 mL CS aqueous solution (0.75% of w/v) with same technique described for F1. Spontaneously formed nanoparticles were further separated by centrifugation at 4750 for 10 min and discarding of the supernatant [16]. CSNPs were washed with distilled water three times. Afterward, they were collected and lyophilized to dry powder for subsequent use.

### 2.3. Characterization and stability of nanoparticles

Mean diameter (nm ± SD) and polydispersity index values of CSNPs (F1 and F2) were measured by photon correlation spectroscopy (Nano ZS, Malvern Instruments, UK). The analyses were performed in triplicate at 25 °C and 173° angle.

Surface charge of the nanoparticle formulations were determined by zeta potential measurements using Malvern Nano ZS also in triplicate at 173° angle and 25 °C in ultrapure water.

After preparation of nanoparticles, F1 and F2 were stored at 25 ± 1 °C and 40 ± 1 °C in stability chamber for 3 months and the average particle size, PDI and zeta potential were measured again.

### 2.4. SEM analysis

SEM imaging of the nanoparticle formulations was performed with a Phillips XL-30S SEM FEG instrument. Nanoparticle samples were fixed on metal plates and sputtered with gold–palladium mixture at a thickness of 100 Å and observed at an accelerated voltage of 20 kV.

### 2.5. Radiolabeling of chitosan nanoparticles

The radiolabeling procedure for F1 and F2 was carried out with small modification as described before [12]. $^{99m}$Tc pertecnetate was eluted from $^{99}$Mo/$^{99m}$Tc generator in +7 oxidation state since $^{99m}$Tc pertecnetate is not able to label any compound on direct addition, it was eluted. So prior to labeling procedure, reduction of $^{99m}$Tc is required for converting $^{99m}$Tc from the +7 state to a desired lower oxidation state, which can complexes with the ligand to form the radiopharmaceuticals. To radiolabel chitosan nanoparticles, four hundred micrograms of lyophilized CSNPs (F1 and F2) was dissolved in 1 mL of 0.01% HCl-saline buffer. 1 mg stannous chloride was dissolved in 1 mL of 0.1% HCl solution. To this CSNPs stock solution, 50 uL of stannous chloride was added under atmosphere of bubbling nitrogen. Reduction of $^{99m}$Tc was performed at acidic pH (1 mg stannous chloride dissolved in 1 mL 0.1% HCl). Radiolabeling was performed with $^{99m}$Tc (1 mCi) in 0.9% sodium chloride solution (0.1 mL). The mixture was shaken for 30 s and incubated for 15 min at room temperature. The labeling efficiency of F1 and F2 was assessed by RTLC and RHPLC.

### 2.6. RTLC studies

Instant Thin Layer Chromatography Silica Gel coated fiber sheets (ITLC-SG) were used as stationary phases. Free $^{99m}$Tc was determined by using acetone as the mobile phase. 5 μL of samples were spotted on the chromatographic sheets, air dried and developed in acetone. The chromatography sheets were dried and scanned by using a TLC scanner. The percentage of radiochemical purity (RP %) of $^{99m}$Tc-CSNPs (F1 and F2) was calculated from the following equation by subtracting from 100 the sum of measured free $^{99m}$Tc percentage (Equation (1)).

$$\mathrm{RP}(\%) = 100 - \mathrm{Free}^{99m}\mathrm{Tc}(\%) \quad (1)$$

### 2.7. RHPLC studies

The radiolabeled F1 and F2 were analyzed by an Ultra-HPLC system equipped with a $C_{18}$ column connected to a photodiode array detector (PDA) and additional NaI gamma detector for the $^{99m}$Tc compounds (wavelength 300 nm). The flow rate was 1.0 mL/min for analytical runs. In all runs the eluent was 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile.

### 2.8. Cell culture studies

The $U_2OS$ and NCI–H209 cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum. Cell monolayers were prepared by seeding $2 \times 10^5$ cells on six wells. Cell culture was maintained at 37 °C under 90% humidity and 5% $CO_2$. 24 h after seeding, the integrity of each cell monolayer was checked by measuring its transepithelial electrical resistance (TEER) with an epithelial voltammeter (EVOM, World Precision Instrument, Sarasota, FL, USA) during test period. The TEER value was calculated from Equation (2):

$$\mathrm{TEER} = \left(R_{\mathrm{monolayer}} - R_{\mathrm{blank}}\right) \times A \quad (2)$$

$R_{\mathrm{monolayer}}$ is the resistance of the cell monolayer along with the filter membrane, $R_{\mathrm{blank}}$ is the resistance of the filter membrane and A is the surface area of the membrane [17,18].

### 2.9. Stability studies

After labeling F1 and F2 with $^{99m}$Tc, the preparation was left at room temperature for six hours. The labeling stability of the complex was evaluated by RTLC studies for every hour.

To test the stability of $^{99m}$Tc-CSNPs (F1 and F2) in cell medium at 37 °C, 1 mCi radiolabeled complex was added to cell medium. The