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Research paper

Methotrexate loaded chitosan nanoparticles: Preparation, radiolabeling and *in vitro* evaluation for breast cancer diagnosis

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ABSTRACT

This study aimed to developed radiolabeled methotrexate (MTX) loaded nanoparticulate systems for breast cancer imaging. For this aim, two formulations (F1 and F2) of MTX loaded chitosan nanoparticles (CSNPs) were prepared via ionic gelation process by using CS and tripolyphosphate (TPP). The obtained results showed that by using F1 and F2 formulations with mean diameter of 169.000 nm and 427.633 nm, zeta potential of 20.133 mV and 29.067 mV were successfully developed with ionotropic gelation process and the encapsulation efficiency (EE) of F1 and F2 was found nearly 35% and 64% respectively.

In addition, MTX-CSNPs were radiolabeled by Technetium-99m (^{99m}Tc) and radiochemical purity and stability of labeled compound were performed using a gamma counter up to 6 h. Results indicated that MTX-CSNPs were radiolabeled by ^{99m}Tc with high labeling efficiency (>90%) and stability.

For *in vitro* incorporation studies the uptake differences between ^{99m}Tc labeled MTX loaded and unloaded CSNPs, reduced/hydrolyzed (R/H) ^{99m}Tc were evaluated in human breast cancer (MCF-7) and human keratinocyte (HaCaT) cell lines. According to cell culture studies, the incorporation percentages of ^{99m}Tc-MTX-CSNPs were highly uptake in cancer cell line. The results demonstrated that radiolabeled MTX-CSNPs may be a promising agent for breast cancer diagnosis.

1. Introduction

Breast cancer is the most common invasive cancer in women worldwide [1]. In cancer medicine, nanotechnology holds great promise to revolutionize drug delivery, gene therapy, diagnostics and many areas of research, development and clinical application [2]. In general, nanocarriers may protect drug from degradation, enhance drug absorption by facilitating diffusion through epithelium, modify pharmacokinetic and tissue distribution profile, and/or improve intracellular penetration. Also nanosystems were found useful to improve the performance of imaging techniques applied for the *in vivo* diagnosis of tumors [3].

Nanoparticles (NPs) are materials with typically overall dimensions less than several hundred nanometers and about 2–4 orders of magnitude smaller than human cells. Because of this unique physical property, NPs demonstrate marvelous interactions with both on surface and inside the cancer cells. Therefore, NPs have wide applications as targeted delivery agents in cancer diagnosis and treatment [4,5]. Also unwanted effects of drugs on bystander cells and tissues can be minimizing by using NPs with appropriate particle size [6].

Polymeric NPs have shown preferential accumulation at tumor sites, their usage as carriers improves efficacy and reduces side effects. Chitosan (CS) has been widely used to prepare nanoparticulate drug delivery system since has many good bioproperties and physiochemical characteristics. CS is a natural polysaccharide which derived from chitin by deacetylation. This cationic polymer is regarded as biocompatible, biodegradable and non-toxic. The cationic properties of CS are particularly valuable for drug delivery systems. For example, ion complexes between CS and anionic drugs (i.e. methotrexate (MTX)) can be formed to NPs [7–9].

Recent studies showed that in tumor tissue folate receptors were found higher compared with healthy tissue. The folate receptors are overexpressed on a variety of human tumors, such as breast tumor, thus making folate receptors a potential molecular target for tumor imaging. MTX, is an analogue of folic acid, which exhibits not only a targeting role as folic acid but also a therapeutic effect to many types of cancer cells that overexpress folate receptors on their surfaces. So it has been utilized for the treatment of

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several forms of cancers for decades, including leukemias, breast cancer, head and neck cancer, lymphomas and carcinomas [9–15].

Theoretically, for cancer imaging an ideal target for radionuclide detection would be tumor-specific. All of these properties make MTX an ideal candidate for being used in drug delivery system for breast cancer diagnosis [16,17].

In recent years, targeted nanocarriers have been developed to improve the biodistribution, pharmacological, therapeutic and toxicity properties of agents used in cancer diagnosis and therapeutics. Because of this, nanocarriers have been extensively used in detect cancer at early stages and treat cancer with less harming on normal tissues [18–20].

Radiopharmaceuticals are drugs which include pharmaceutical and radioactive parts together. Technetium-99m (^{99m}Tc) is the most popular radionuclide to prepare radiopharmaceuticals with its versatile chemistry, near-ideal energy (140 keV), low radiation dose and short half-life (6 h). Radiolabeled NPs can be designed and used for cancer diagnostic purposes when tagged with appropriate radionuclides [21,22].

The aim of this study was to prepare a new radiopharmaceutical to use in breast cancer diagnosis. For this purpose MTX loaded CSNPs were prepared and radiolabeled with ^{99m}Tc. Labeling efficiency and stability of the labeled compound was investigated. Binding affinity of the newly developed formulation to cancer cells was evaluated by cell culture incorporation studies. As part of the cell culture studies, incorporation of ^{99m}Tc-MTX-CSNPs to normal and cancer cell lines was evaluated in human breast cancer (MCF-7) and human keratinocyte (HaCaT) cell lines.

2. Materials and methods

2.1. Materials

MTX was obtained from Kocak Pharma (Turkey). CS (low molecular weight), tripolyphospate (TPP), stannous tartrate and ascorbic acid were purchased from Sigma–Aldrich (Germany). ^{99m}Tc was eluted from the Molybdenum-99 (^{99}Mo)/^{99m}Tc generator from Nuclear Medicine Department of Ege University. All solvents were obtained from Merck (Germany). Cell culture reagents and supplies were obtained from Gibco Invitrogen (Grand Island, NY). The MCF-7 and HaCaT cells were obtained from American Type Culture Collection (ATCC). All chemicals and solvents were of either HPLC or analytical grade and were used without further purification. Radioactive samples were counted in a counting unit (Atomlab 100 Dose Calibrator Biodex Medical Systems) and also a gamma counter (Sesa Uniscaller).

2.2. Preparation of MTX-CSNPs

The NPs were prepared by ionotropic gelation of CS with TPP according to a method described previously [23,24]. Briefly, CS was dissolved at a concentration of 0.25% or 0.75% (w/v) in 1% acetic acid solution and the solution of TPP at the concentration of 0.125% was prepared with deionized water. 10 mL TPP aqueous solution was added to 10 mL CS aqueous solution drop by drop and stirred at room temperature for 30 min to obtain blank CSNPs.

To prepare MTX loaded CSNPs; CS was dissolved at a concentration of 0.25% (w/v) and 0.50% (w/v) in 1% acetic acid solution for F1 and F2 respectively. The solution of TPP at the concentration of 0.125% was prepared with deionized water and 1 mg MTX was added to this solution. Then, 10 mL of MTX-TPP solution was added to 10 mL of the CS solution by drop wise under constant stirring. The mixture was stirred at room temperature for 30 min to obtain loaded NPs. Also, spontaneously formed NPs were separated by centrifugation at 4750 rpm for 10 min and supernatant was discarded for the determination of encapsulation efficiency (EE).

2.3. Characterization and stability of MTX-CSNPs

The average particle size, polydispersity index (PdI) and zeta potential of the MTX-CSNPs (F1 and F2) were measured by photon correlation spectroscopy (Nano ZS, Malvern Instruments, UK) at the beginning of the study. Samples were diluted to appropriate concentrations with distilled water before measurement. Also for stability studies F1 and F2 were storaged at stability cabins at 25 ± 2 °C temperature/60 ± 5% humidity and 40 ± 2 °C temperature/75 ± 5% humidity during 6 months. The storage of F1 and F2 will continue for 12 months.

2.4. Fourier transform infrared spectroscopy (FTIR)

For FTIR measurements, MTX-CSNPs (F1 and F2) were separated by centrifugation at 4750 rpm for a period of 10 min at 25 °C. The supernatants were discarded. Formulations were washed three times. Afterward, they were collected and lyophilized to dry powder. Also, to compare, CSNPs in the absence of MTX were prepared with the same method for Blank F1 and Blank F2 as described before [24].

FTIR measurements were carried out on a Perkin–Elmer Spectrum 100 IR spectrometer operated at a resolution of 4 cm^{-1} in the range of 4000–650 cm^{-1}. The sample aliquots were placed on an ATR crystal (ZnSe) and subjected to light within the infrared spectrum. For each sample four scans were collected and averaged to reduce the signal to noise ratio.

2.5. Scanning electron microscopy (SEM) analysis

SEM imaging of the NP formulations was performed with a Philips XL 30S FEG. NP samples were dried at 40 °C, fixed on aluminum plates and coated with a thin gold coating of 10 nm, 1.5 min, 15 mA, 6×10^{-2} mbar under vacuum using K550X Sputter Coater, EMITECH. Then, samples were mounted on to a SEM sample holder and images were taken using an accelerating voltage setting of 4 kV.

2.6. Determination of yield and encapsulation efficiency

MTX-CSNPs (F1 and F2) were separated from aqueous suspension by centrifugation at 4750 rpm for a period of 10 min at 25 °C. The supernatants were discarded. Formulations were washed three times. Afterward, they were collected and lyophilized to dry powder. Yield of MTX-CSNPs was found by weighing out accurately of formulations and calculated using Eq. (1):

$$\%\ \text{Yield} = (\text{Practical weight}/\text{Theoretical weight}) \times 100 \qquad (1)$$

EE of MTX-CSNPs (F1 and F2) were determined by separated NPs from aqueous suspension by centrifugation at 4750 rpm for a period of 10 min at 25 °C. The supernatants were carefully removed and the amount of free MTX in the supernatants was determined by Ultra High Performance Liquid Chromatography (UHPLC) analysis. For this purpose, 1 mL supernatant of MTX-CSNPs were injected into the HPLC system. HPLC method for the quantification of MTX consisted of a Thermo Scientific Accela HPLC system with a reverse phase C18 column (150 mm × 4.6 mm, 3 μm), a mobile phase of ammonium formate solution (pH:2):acetonitrile (80:20 v/v)