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Preparation of epidermal growth factor (EGF) conjugated iron oxide nanoparticles and their internalization into colon cancer cells

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ABSTRACT

Epidermal growth factor (EGF) was conjugated with carboxymethyldextran (CMDx) coated iron oxide magnetic nanoparticles using carbodiimide chemistry to obtain magnetic nanoparticles that target the epidermal growth factor receptor (EGFR). Epidermal growth factor modified magnetic nanoparticles were colloidally stable when suspended in biological buffers such as PBS and cell culture media. Both targeted and non-targeted nanoparticles were incubated with CaCo-2 cancer cells, known to overexpress EGFR. Nanoparticle localization within the cell was visualized by confocal laser scanning microscopy and light microscopy using Prussian blue stain. Results showed that targeted magnetic nanoparticles were rapidly accumulated in both flask-shaped small vesicles and large circular endocytic structures. Internalization patterns suggest that both clathrin-dependent and clathrin-independent receptors mediated endocytosis mechanisms are responsible for nanoparticle internalization.

1. Introduction

Colloidal suspensions of permanently magnetized magnetic nanoparticles in a liquid carrier exhibit a unique combination of fluidity and capability to interact with external magnetic fields, which make them suitable for medical and biological applications. Magnetic particles have been used in medicine for biosensing [1], cell separation [2], and for cancer related applications such as drug delivery [3], imaging [4] and magnetic fluid hyperthermia (MFH) [5]. These applications can be achieved by injecting nanoparticles locally into a tumor or systemically through the blood stream.

It is well-established that when a tumor achieves a size of 1–2 mm, angiogenic processes lead to new abnormal vasculature to feed the tumor [6]. Tumors are highly irrigated by blood vessels with endothelial cells that are not as compact as the rest of the vasculature in the body. Thus, there are gaps in the tumor vasculature, which may allow nanoparticles to permeate from the blood stream to the tumor. As tumors are poorly irrigated by lymphatic vessels, clearance from the tumor is practically non-existent, increasing the retention of nanoparticles [7]. It is expected that nanoparticles injected systemically will locate and be retained by the tumor if a long circulation lifetime can be achieved [6–8]. This is commonly referred to as passive targeting, and although it can be effective in accumulating nanoparticles in tumors, there is significant interest in specific, the so-called active targeting to increase the selectivity and activity of magnetic nanoparticles in the applications mentioned above.

Considerable effort has been invested to obtain biocompatible nanoparticles [9]. There are different strategies to improve the biocompatibility of nanoparticles, such as coating with micelles, liposomes [9], homopolymers such as polyethylene glycol (PEG) [10], dextran [11], and carboxymethyl dextran (CMDx) [12], or block copolymers such as polyethylenoxide (PEO) [13]. These strategies allow nanoparticles to be suspended in water, saline solution, and culture medium, making them suitable for *in vitro* and *in vivo* experiments. However, most nanoparticles exhibit non-specific binding to cancer cells that correlate with low efficiency and low specificity of internalization [9]. In order to increase the specificity of nanoparticles to a target cell [14], different strategies have been used depending on the type of cell and the characteristics of the nanoparticles [9,15]. The most common cell specific strategy is the use of ligands [16], antigens [17], or antibodies [18] that bind specifically to a receptor that is overexpressed in cancer cells but not in normal cells. Epidermal growth factor (EGF) is a 6045 Da mitogenic protein, composed of 53 amino acid residues, involved in proliferation, differentiation, and cell survival [19]. EGF binds specifically to the epidermal growth factor receptor (EGFR), inducing the formation of a dimerized complex, which is internalized by receptor mediated endocytosis into the cell [20].

Two types of receptor mediated endocytosis have been reported for EGFR: clathrin-dependent endocytosis and

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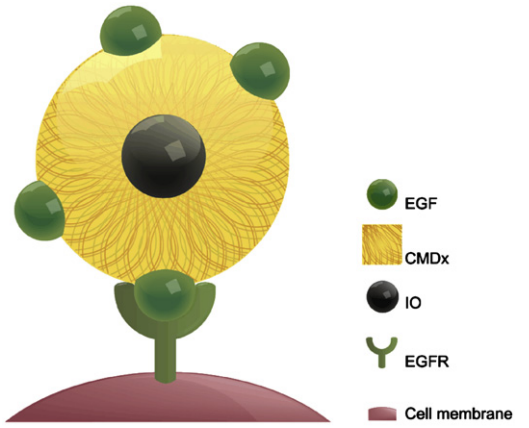

Fig. 1. Iron oxide (IO) nanoparticles modified with carboxymethyl dextran (CMDx) and conjugated with epidermal growth factor (EGF), interacting with epidermal growth factor receptor (EGFR).

clathrin-independent endocytosis. Clathrin-dependent endocytosis occurs when using low concentrations of EGF, ~1.5 ng/mL. When using EGF concentrations higher than 20 ng/mL, both clathrin-dependent and clathrin-independent endocytsosis occur [21]. When EGFR is internalized by clathrin-dependent endocytosis, clathrin coated pits are formed. These are small (100 nm) pits, formed by invagination of the cell membrane [22]. Clathrin-independent endocytosis could lead to caveolae, small (50 nm) flask-shaped, endocytic structures formed from an invagination of the cell membrane rich in caveolin protein [23]. Another clathrin-independent endocytosis mechanism could lead to circular dorsal ruffles (CDR), large concentric structures that could rapidly (5–10 min) internalize large amounts of EGFR. Various studies have been done to elucidate the way in which EGFR is internalized but the mechanisms are not completely understood [20].

Previous reports have shown that EGF could increase the specificity and effectiveness of nanodevices and treatments by increasing the internalization rate in cells that overexpress EGFR [17,24–27]. Studies targeting EGFR have been done using copolymer micelles [24], carbon nanotubes [17], PEGylated adenovirus [25], PEI/DNA complexes [26] and the so-called vault nanoparticles [27] showing an increase in the internalization rate.

In this study, we describe the synthesis of EGF conjugated iron oxide (IO) magnetic nanoparticles, Fig. 1, and their internalization in the EGFR overexpressing cell line Caco-2. IO magnetic nanoparticles were synthesized by the thermodecomposition method, obtaining nanoparticles with a narrow size distribution. In order to make them biocompatible and colloidally stable, we functionalized them with negatively charged carboxymethyldextran attached covalently to the nanoparticles by carbodimiide reaction [28]. We conjugated EGF to carboxymethylated nanoparticles and incubated them with CaCo-2, cells, which overexpress EGFR, to demonstrate improved internalization rate of the targeted nanoparticles.

2. Materials and methods

2.1. Materials

Iron (III) chloride hexahydrate 97%, oleic acid, dextran, carboxymethyl dextran sodium salt, chloroacetic acid 99% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) 98%, acetic acid 99.7%, fluorescein–amine isomer I, Dulbecco's modified eagle's medium (DMEM), phosphate buffered saline (PBS) and Prussian blue staining kit were purchased from Sigma Aldrich. Hexane and anhydrous ethanol were purchased from Fisher Scientific. 3-Aminopropyltriethoxysilane (APS) and sodium oleate were obtained from TCI America. 1-octadecene was obtained from Alfa-aesar. Epidermal growth factor was purchased from GenScript Corporation. Fetal bovine serum (FBS) was obtained from Invitrogen. Fluorescent dye DiD and DAPI were purchased from Molecular Probes. Micro-BCA protein assay was purchased from Piercenet. CaCo-2 cells were purchased from American Type Culture Collection. All materials were used as received.

2.2. Synthesis of APS coated magnetite nanoparticles.

The thermodecomposition method was used to synthesize iron oxide nanoparticles with a narrow size distribution [28,29]. In this methodology, an iron–oleate was first prepared by mixing iron (III) salt and sodium oleate in presence of distilled water, ethanol, and hexane at 70 °C [29]. Afterwards, this iron–oleate solution was washed three times with distilled water and dried in a vacuum oven. To obtain iron oxide magnetite nanoparticles, we decomposed the iron–oleate in presence of oleic acid (OA) using 1-octadecene as the solvent medium [29]. This mixture was heated to 320 °C at a heating rate of 3.5 °C/min. Nanoparticle growth occurred at 320 °C for 1.5 h. Afterwards, the reaction mixture was cooled to room temperature and then iron oxide-OA nanoparticles were washed with anhydrous ethanol (1:3) at 7500 rpm for 15 min and suspended in hexane. Then, iron oxide-OA nanoparticles were modified with APS molecules via ligand exchange using acetic acid as a catalyst to increase the hydrolysis and condensation of APS molecules onto the nanoparticle surface [28,30]. To this end, 6 ml of APS and 50 μL of acetic acid were added to 115 ml of the colloid formed by 35 mL of iron oxide-OA nanoparticles suspended in 80 mL of hexane. This solution was mechanically stirred at room temperature for three days. Afterwards, iron oxide-APS nanoparticles were removed by magnetic decantation and washed three times with hexane and once with ethanol. The modified iron oxide-APS nanoparticles were dried at room temperature.

2.3. Synthesis of carboxymethyl substituted dextran (CMDx).

A carboxymethylation route was employed to incorporate carboxylic groups (–COOH) into dextran chains [31,32]. To this end, 25 ml of an aqueous dextran solution at 20% w/v was cooled in an ice bath at approximately 4 °C, and then 17 ml of sodium hydroxide at 3 M and 7.3 g of monochloro acetic acid were slowly added. This reaction mixture was placed in a water bath at 60 °C and stirred at 100 rpm for 75 min. Afterwards, it was cooled to room temperature, and then neutralized with glacial acetic acid. Finally, the product was precipitated and washed twice with ethanol, dialyzed to a conductivity ≤ 6 μS/cm, concentrated using a Brinkmann RE 121 rotary evaporator and dried at 60 °C. A second carboxymethylation reaction was performed on the dried sample of the modified dextran to increase the incorporation of –COOH groups in the dextran chains [31].

The amount of carboxylic (–COOH) groups incorporated in the synthesized CMDx molecules were determined by acidimetric titration [33]. Briefly, CMDx was washed overnight with a solution of anhydrous methanol and nitric acid 70% v/v (10:1). Then, the acid liquor was removed by vacuum filtration and the solid product was subsequently washed several times with ethanol and dried in a vacuum oven at 60 °C. For the acidimetric titration, a solution at 1% w/v of CMDx in a mixture of distilled water/acetone (1:1) with 10 ml of NaOH 0.012 N was prepared.