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Synthesis, characterization, and cytotoxicity of the plasmid EGFP-p53 loaded on pullulan–spermine magnetic nanoparticles

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ABSTRACT

Magnetic nanoparticles have been used as effective vehicles for the targeted delivery of therapeutic agents that can be controlled in their concentration and distribution to a desired part of the body by using externally driven magnets. This study focuses on the synthesis, characterization, and functionalization of pullulan–spermine (PS) magnetic nanoparticles for medical applications. Magnetite nanopowder was produced by thermal decomposition of goethite (FeOOH) in oleic acid and 1-octadecene; pullulan–spermine was deposited on the magnetite nanoparticles in the form of pullulan–spermine clusters. EGFP-p53 plasmid was loaded on functionalized iron oleate to transfer into cells. Synthesized nanoparticles were characterized by Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), vibrating sample magnetometry (VSM), and transmission electron microscopy (TEM). The encapsulation efficiency and drug loading efficiency of the nanocomplexes were tested. FTIR studies showed the presence of oleic acid and 1-octadecene in the iron oleate nanopowder and verified the interaction between spermine and pullulan. The characteristic bands of PS in the spectrum of the pullulan–spermine-coated iron oleate (PSCFO) confirmed that PS covered the surface of the iron oleate particles. TEM studies showed the average size of the iron oleate nanopowder, the PSCFO, and the plasmid-carrying PSCFO (PSCFO/pEGFP-p53) to be 34 ± 12 nm, 100 ± 50 nm and 172 ± 3 nm, respectively. Magnetic measurements revealed that magnetic saturation of the PSCFO was lower in comparison with the iron oleate nanopowder due to the presence of organic compounds in the former. In cytotoxicity tests performed using U87 cells as glioblastoma cells, a 92% survival rate was observed at 50 μg/μl of the plasmid-carrying PSCFO, with an IC_{50} value of 189 μg/μl.

1. Introduction

Cancer is commonly treated with a combination of surgery, radiation therapy, chemotherapy, and photodynamic therapy (PDT) [1]. While chemotherapy is an effective treatment, the side effects of its toxicity are often severe and devastating when delivered as a full body dose to the patient. Recent developments employing nanotechnology have made it possible to deliver a drug to the targeted tissue across biological barriers, and to release it at a controlled rate that minimizes cell and tissue degradation [2]. In this regard, magnetic nanoparticles have shown great potential in targeted drug delivery for cancer treatment [3]. For example, magnetic nanoparticles coated with activated carbon are able to transport pharmaceuticals to a specific site in the body when facilitated by an external magnetic field. This allows more concentrated doses of drugs to be delivered to relevant cells, and kept on site longer. Chemical and physical vapor deposition and mechanical attrition are used in the preparation of nanoparticles [4] where homogeneity, particle size and size distribution, morphology, and agglomerate size are the most important parameters in product specification [5]. The thermodynamically equilibrated state of the particles is restored by condensation of nuclei of the reaction product, and controlled by the kinetics of the nucleation and growth [6]. Kinetic factors (e.g. reaction rates, transport rates of reactants, and the removal and redistribution of matter) control

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the dynamics of the thermodynamic equilibrium of the system [7]. The reaction and transport rates are affected by the temperature, pH and mixing of the reactants, as well as their respective concentrations. Particle morphology is influenced by factors such as supersaturation, colloidal stability, nucleation and growth rates, recrystallization, and aging times [8]. In particular, supersaturation has a dominant role in determining the morphology of precipitates [9]. To prevent dangerous agglomeration of the particles in the blood stream, magnetic nanoparticles must be tailor-made [10] to avoid agglomeration of particles occurring during drying, handling, and post-processing [11]. In this regard, surfactants can help control particle dispersion during chemical synthesis [12]. Another aspect requiring attention in the synthesis of nanoparticles is the annealing process which causes an increase in crystallinity, that in turn greatly affects the magnetic and electronic properties of particles [13]. Molecular weight, surface charges, amphiphilicity, as well as the structure and shape of particles, also affect the efficiency of gene transfection through polymer-based vectors [14]. While cationic polymers such as polyethyleneimine (PEI), poly L-lysine (PLL), chitosan, and polyamidoamines (PAMAM) are commonly used in gene delivery [15], pullulan–spermine complexing with plasmid DNA has been shown to be a potent carrier system for non-viral gene therapy [16]. Such complexes are known to undergo cellular endocytosis via clathrin-dependent endocytosis [17]. Pullulan is recognized by the asialoglycoprotein receptor (ASGPR) found primarily in the liver, but cells which do not express ASGPR are also able to internalize pullulan–spermine [16,18]. Oncogenes and tumor suppressor genes are the two main types of genes that play a role in cancer [19,20]. Tumor suppressor genes have been found, including TP53 (p53), BRCA1 and BRCA2 (for breast cancer), APC (colorectal tumors) and RB1 (retinoblastoma) [21]. Abnormalities of the TP53 gene have been found in more than half of human cancers [22]. The p53 protein is involved in the pathway to apoptosis and a cell with DNA damage that cannot be repaired continue to grow and divide, and then lead to cancer [23,24]. The outcomes of common cancer therapy for malignant glioblastoma are still very poor with less than 5% of patients surviving five years post diagnosis even with the best current treatment [25]. Bearing all this in mind, we hypothesized that the incorporation of magnetic nanoparticles to pullulan–spermine gene delivery nanocarriers could promote effective cellular uptake via a targeting delivery system. Hence, the main aim of this study was to develop a chemically modified magnetic nanoparticle vector for gene delivery systems to improve their targeting gene therapy. Accordingly, magnetic nanoparticles were prepared and characterized by FTIR, DLS, VSM, and TEM techniques. Their cytotoxicity in the human glioblastoma cell line U87 and their ability to protect genes from physical, chemical, and enzymatic degradation were evaluated. Finally magnetic nanoparticles were experienced against glioblastoma cell line, U87, to show their therapeutic efficacy in vitro and in vivo experiments.

2. Experimental

Goethite (FeOOH), 1-octadecene, spermine, pullulan, carbonyldiimidazole (CDI), Dulbecco's modified eagle's medium F12 (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (100 μg/ml), phosphate-buffered saline (PBS), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium (WST-1 reagent), poly vinyl alcohol (PVA), and dimethyl sulphoxide (DMSO) were purchased from the Sigma Aldrich Company (Mo, USA). Dimethyl formamide (DMF) and oleic acid were obtained from the Merck Company (Darmstadt, Germany). The GeneJET Plasmid Miniprep Kit was obtained from Thermo Scientific while U87 glioblastoma cells were obtained from the Pasteur Institute of Iran.

2.1. Construction of plasmid

The construction of the pEGFP-p53 vector (5.89 kb) coding for the enhanced green fluorescent protein (EGFP), which contained the tumor protein p53 gene that acts as a tumor suppressor, was undertaken based on methods previously described [26]. The full-length 1191 bp tumor protein p53 (Tp53) gene (GenBank AAD28628.1) that encodes 397 amino acids was synthesized using a DNA synthesizer and sub-cloned into the pBR322 vector using the PstI and BamHI sites. The recombinant pBR322-p53 plasmid containing the Tp53 gene was verified by restriction enzyme digestion and sequencing. The Tp53 was then sub-cloned into the pEGFP-N1 vector, and the digested products of the recombinant plasmid were verified by agarose gel electrophoresis. Then pEGFP-p53 was propagated in an *Escherichia coli* strain Top 10 (ATCC® PTA-10989™) and purified by the GeneJET Plasmid Miniprep® Kit according to the manufacturer's protocol. Both the yield and purity of the pEGFP-p53 were evaluated by UV spectroscopy. The absorbance ratio at wavelengths of 260–280 nm for DNA solution was between 1.8 and 2.0 after dilution to between 1:500 and 1:1000 in TE buffer.

2.2. Synthesis of magnetite nanoparticles

The synthesis of magnetite nanoparticles (Fe_3O_4) basically followed the procedure reported by Colvin [27]. Magnetite nanocrystals were synthesized in a three-neck flask equipped with a condenser, magnetic stirrer, thermocouple, and heating mantle. Typically, a mixture of 0.178 g FeOOH fine powder (2.00 mmol), 2.26 g oleic acid (8.00 mmol) and 5.00 g 1-octadecene (20 mmol) was heated with stirring to 320 °C and maintained at this temperature for 1 h. On completion of the reaction, the product was allowed to cool to room temperature and 30 ml ethanol was added. The resulting black precipitate was separated by centrifugation at 6300 g for 20 min. During this time the supernatant turned from turbid black to clear brown as the iron source material dissolved and formed an iron carboxylate salt. The reaction was protected under argon in order to avoid any undesired side-reactions (e.g. oxidation of oleic acid).

2.3. Preparation of pullulan–spermine (PS)

Spermine was introduced to the hydroxyl groups of pullulan by a CDI activation method [28]. Pullulan (25 mg) was dissolved in 2.5 ml DMSO and 435 mg of CDI was then added to reach a molar ratio of 3:1 for CDI to the hydroxyl groups of pullulan. The CDI–pullulan mixture was incubated for 5 min at room temperature. Spermine (2500 mg) was dissolved in 22.5 ml DMSO was added drop-wise to reach a large molar excess of spermine in order to prevent cross linking of pullulan. The mixture was then incubated overnight at 40 °C and purified by dialysis in deionized water.

2.4. Surface functionalization of nanoparticles

Pullulan–spermine-coated iron oleate (PSCFO) was prepared by a solvent diffusion method. Wet magnetite gel (50 mg) and 100 mg of pullulan–spermine were dissolved in separate 5 ml portions of DMF and the two portions were mixed using a vortex mixer. The resulting mixture was then added to 90 ml of PVA (0.5%, w/w) by means of a syringe that was positioned with the needle directly in the medium, with moderate mechanical stirring. The magnetic particles were isolated by ultracentrifugation at 29,000g for 20 min at 4 °C and washed three times with deionized water before freeze-drying for later characterization.