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Folic acid armed Fe_3O_4-HPG nanoparticles as a safe nano vehicle for biomedical theranostics

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ABSTRACT

Targeted nano drug delivery systems can be considered as *in vivo* tiny physicians that can accurately detect the disease location and prescribe drugs at the same time. In some cases, these skilled doctors are equipped with diagnostic agents that can monitor the treatment process. These nano-robots are known as nanotheranostic agents. The aim of this study is to inset folic acid armed polymeric core–shell iron oxide nanoparticles as a new type of nanotheranostic agent. Here, iron oxide nanoparticles were synthesized by polyol method and coated with hyperbranched polyglycerol. Different *in vitro* tests (cell viability and hemocompatibility) were conducted on the nanoparticles to evaluate their biocompatibility. Then they were decorated with folic acid as targeting agents and examined with different *in vitro* test to detect the influence of targeting on cell uptake properties. Results represented that this new type of nano-robot with size around 11 nanometer was biocompatible and had a positive effect on cellular uptake of nanoparticles. Thus, these nanoparticles could be used as potentially useful nanotheranostic systems to defeat cancer cells with high folate expression.

1. Introduction

As a drug carrier, superparamagnetic iron oxide nanoparticles (SPIONs) need to have some critical features like high biocompatibility, good hydrophilicity, high stability in the physiological environment and the surface chemical functionality. Although SPIONs are partially biocompatible, their high superficial area and hydrophobic surfaces may result in aggregation and cause their uptake by the reticuloendothelial system (RES), thus in most cases, they should be engineered with other nanomaterials like proteins, lipids, and polymers [1].

Coating the surface of nanoparticles with biocompatible polymers not only improves their biological properties but also creates a suitable support for drug loading [2]. Hyperbranched polyglycerol (HPG) is a new class of polymer with a biocompatible polyether scaffold, compact and well-defined dendrimer-like architecture and abundant functional end group [3,4] that is used in this study to coat the SPIONs.

Folic acid (FA) could be used for targeting cancers where folate receptors are over expressed. Folate binding protein, a glycosylphosphatidylinositol (GPI) anchored cell surface receptor for folate, has been known to be present in small amounts on the surface of the normal epithelial cells in the kidney, thyroid, lungs and brain while typically they are present with high expression in several human tumors including ovarian and breast cancers which is likely related to the vital role of FA in cancer cell proliferation [5,6]. Thus, attachment of FA on the surface of polymeric nanocarriers could enhanced the bioavailability of drugs in the tumors location and thus increased the therapeutic efficacy of the drugs [7,8].

To the best of our knowledge, there are currently no reports using folic acid for targeting of SPIONs grafted hyperbranched polyglycerol (HPG). To achieve this aim, in this study SPIONs were synthesized by polyol method and then were coated by HPG through anionic ring opening polymerization method. The effect of HPG coating on the biocompatibility enhancement of SPIONs was evaluated in the next step. Then, FA was covalently bonded to the terminal hydroxyl groups of polyglycerol. The physicochemical properties of nanoparticles were examined by FTIR, TEM and CHNS techniques. Then, cell viability (MTT assay) and hemocompatibility tests (coagulation tests (prothrombin time (PT), activated partial thromboplastin time (APTT)), complement activation and red blood cells (RBCs) aggregation) were used to investigate the effect of coating on the biocompatibility of the nanocarrier. Then, the ability of the new carrier for cell targeting was investigated by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) *in vitro* tests and magnetic resonance imaging (MRI).

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2. Experimental section

2.1. Materials

Iron (III) acetylacetonate ($Fe(acac)_3$), dicyclohexylcarbodiimde (DCC), dimethylaminopyridine (DMAP) and ethyl acetate were purchased from Merck. Triethylene glycol (TREG) > 98% was gotten from Noba chem (India). Folic acid, glycidol and anhydrous dimethylsulfoxide (DMSO) were purchased from Sigma–Aldrich.

2.2. Synthesis of SPION–HPGs–FA

Synthesis, functionalization, targeting and characterization procedures are described in detail in the supplementary information.

2.3. Biocompatibility testing

2.3.1. Cell culture

The first step for evaluating the biocompatibility of a material is cell culture studies which can provide an investigation of toxicity of the material in a simplified system by minimizing effects of interfering factors [9]. In this study, MCF-7 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin – streptomycin at 37 °C in 5% CO_2.

2.3.2. Cell viability (MTT assay)

Here, the cell viability was determined by MTT assay which is a widely used test for cell viability assay and is based on the reduction of the yellow tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) to a purple water insoluble formazan in mitochondria of alive cells [10].

2.3.3. Hemocompatibility

Coagulation: Blood clotting, or coagulation, is an extremely high complex process that employs various components to create fibrin clot [11]. Here in, PT and APTT were measured to determine the effect of nanoparticles on blood coagulation time. PT and APTT, as parameters of blood biocompatibility for biomaterials in contact with blood, were measured by means of a coagulation analyzer, using mechanical end point determination (ST4, Diagnostica Stago).

For the PT determination, the extrinsic and common coagulation was activated by Innovin® reagent when incubated with plasma, and then the clotting time was measured. Innovin® is a lyophilized reagent consisting of recombinant human tissue factor, synthetic phospholipids (thromboplastin), calcium ions, a heparin-neutralizing compound, buffers, and stabilizers (bovine serum albumin). For the APTT determination, the intrinsic and common coagulation pathways were activated by adding actin as partial thromboplastin reagent and calcium chloride to plasma, and then the clotting time was measured [12].

Nanoparticles' effect on coagulation behavior was studied by adding plasma to different concentrations of nanoparticles solution in isotonic saline (9:1 dilution) to the final concentrations of 5, 25, 50, 100 and 200 μg ml^{-1}, and mixing at 37 °C for 5 min before applying the coagulation reagents. For control samples, equal volumes of isotonic saline instead of nanoparticle solution were added to the plasma.

Complement activation: The complement activation was determined by monitoring the cleavage of C_3 and C_4 component through polarimetry system. For this reason anti-coagulated plasma from whole blood donations added to nanoparticle solution in isotonic saline (1:1 solution) to the final concentrations of 5, 25, 50, 100 and 200 μg ml^{-1}. As the control sample, plasma was added to saline without any polymer traces inside. The mixtures were then incubated for 2 h at 37 °C before samples were withdrawn. Then, each solution was added to the measurement kit and the concentration of C_3/C_4 was measured. The differences between C_3/C_4 concentrations in samples and control were taken into account as bioincompatibility index [13].

Red blood cells aggregation: Erythrocytes are the biggest group of the cells in the blood. Interaction of erythrocytes with nanoparticles is very important especially in the case of *in vivo* applications, where any interaction of these nanomaterials with the cells could influence their circulation half-life and also could affect the body circulatory system [14].

EDTA-anti-coagulated blood (40 μl) was incubated for 30 min at 37 °C with polymer solutions (10 μl) in 0.15 N NaCl to provide a final concentration of 0.005, 0.05, 0.2 and 1 mg ml^{-1} of polymer in the incubation mixture. After incubation, the red blood cells isolated by centrifugation were resuspended in the plasma solutions and examined by transmitted bright field light microscopy (Nikon Eclipse – TS100) using wet mounted slides. Images were captured with a microscope mounted black and white CCD camera.

2.4. Cellular uptake studies

Intracellular uptake behavior of the targeted and non-targeted Fe_3O_4-HPG was investigated using an ICP-OES, where a known number of cells from each sample were analyzed for iron content, through which the effect of folic acid on the cellular uptake of coated nanoparticles could be investigated.

In order to evaluate the effect of folic acid moiety on the nanoparticles uptake by cells, HeLa cells (as an overexpression folate receptor cell line) at the concentration of 1×10^5 cells per well was chosen. In order to investigate the effect of dual targeting, targeting with folic acid and external magnetic field, cellular uptake of Fe_3O_4-HPG and Fe_3O_4-HPG–FA at a concentration of 0.20 mg ml^{-1} in the absence/presence of an external magnetic field was evaluated. To this end HeLa cell line was cultured in 24 well plates at the concentration of 1×10^5 cells per well for 24 h. Then cells were exposed to the 0.20 mg ml^{-1} of Fe_3O_4-HPG and Fe_3O_4-HPG–FA for 1, 2, and 4 h in the presence of an external magnetic field. Then medium was removed, cells were washed with PBS and 500 μl of hydrochloric acid (37%) was added to each well and incubated at 70 °C for 1 h.

To verify the specificity of the targeting by folic acid and determine the optimal concentration of cellular uptake, nanoparticles were exposed to two cancer cell lines. In this experiment, the MCF-7 and HeLa cells as overexpression folate receptor cancer cells were used. Cells were seeded in 24-well plates at a concentration of 1×10^5 cells per well for each cell line. After 24 h, Fe_3O_4-HPG, and Fe_3O_4-HPG–FA, with concentrations of 5, 10 and 20 μg ml^{-1} were exposed to the cells and plates were incubated for 2 h at 37 °C. Next steps were conducted as previously described.

Samples were analyzed in the volume of 2 ml with ICP-OES (Varian-735-ES, Australia) to quantify the iron content in each sample. With a known number of cells from each sample, the concentration of iron absorption (pg $cell^{-1}$) was quantified.

2.5. In vitro MRI experiment

In vitro magnetic resonance imaging was evaluated in phosphate buffer solution. A clinical MR scanner (Siemens Magnetom Avanto, 1.5 T) was used to measure T2-weighted signal intensities. HeLa cells (5×10^5 cells per well) were seeded into a 6-well culture plate. After 24 h, nanocarriers including Fe_3O_4, Fe_3O_4-HPG, and Fe_3O_4-HPG–FA (with 12 wt% folic acid) with a concentration of 0.20 mg ml^{-1}, were exposed to the cells. Cells with fresh medium were selected as control.

In another experiment, to evaluate the effect of dual targeting on T2-weighted signal, Fe_3O_4-HPG, and Fe_3O_4-HPG–FA (with 12 wt% folic acid) at the concentration of 0.20 mg ml^{-1} were

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