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Multifunctional fluorescent magnetic nanoparticles for lung cancer stem cells research

Xuan Zhou[a,1], Lisha Chen[a,b,1], Anxin Wang[a,b], Yufei Ma[a], Hailu Zhang[c], Yimin Zhu[a,*]

[a] Key Laboratory for Nano-Bio Interface Research, Suzhou Key Laboratory for Nanotheranostics, Division of Nanobiomedicine, Suzhou Institute of Nano-Tech and Nano-Bionics, Chinese Academy of Sciences, Suzhou 215123, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China
[c] Laboratory of Magnetic Resonance Spectroscopy and Imaging, Suzhou Institute of Nano-Tech and Nano-Bionics, Chinese Academy of Sciences, Suzhou 215123, PR China

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ABSTRACT

In this paper, a multifunctional peptide-fluorescent-magnetic nanocomposites (Fe_3O_4@PEI@Cy5.5@PEG@HCBP-1 NPs) was synthesized *via* a layer-by-layer approach for potential application to cancer diagnoses. The multifunctional nanocomposites have great dispersibility and homogeneous particle sizes in aqueous solution. Meanwhile, it has perfect hemocompatibility and satisfying cytocompatibility in a relatively high concentration. Data from *in vitro* cytotoxicity assay indicated that the nanocomposites could recognize the lung cancer stem cells (CSCs) specifically and enrich the HCBP-1 positive CSCs from H460 tumor xenografts effectively. Additionally, the results of *in vivo* live fluorescent imaging and magnetic resonance imaging (MRI) showed that the nanocomposites could identify lung CSCs in tumor xenografts. These results suggested that the nanocomposites could be used as a potential cancer diagnostic agent through modifying diverse fluorescence dyes and targeting ligands on its surface.

1. Introduction

Cancer is one of the world's largest threats to human health. It is the leading cause of death and becoming a growing issue in most countries [1]. Based on the GLOBOCAN 2012 estimates from the International Agency for Research on Cancer (IARC), there are about 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people suffering potential cancer worldwide in 2012 [2]. Cancer is becoming a severe problem in the United States as well as many other countries and about one fourth deaths in the United States are due to cancer [3,4]. Early detection, accurate diagnosis and effective therapy are the important strategies for cancer therapeutics. Many researchers have focused on cancer treatment or diagnosis by targeting drugs and gene delivery [5,6]. The relevant delivery materials refer to nanoparticles [7], hydrogel, liposome [8] and magnetic nanoparticles. Among them, detection agents attract more and more attentions since early detections provide the information of cancer progression directly.

Over the last decade, there are hypotheses that some cancers are derived from a small particular subset of cancer cells which is so-called cancer stem cells (CSCs) or cancer stem-like cells (CSLCs) [9–11]. The CSCs have proliferation capacities, self-renewal ability, propagation, tumorigenicity and differentiation *in vivo* [12–15]. Moreover, it may play a key role in tumorigenesis, metastasis and cancer relapse [16]. At present, identification and isolation of CSCs are highly dependent on markers or marker combinations specifically expressed on the surface of CSCs [17], including CD133 [18], CD34 [11], CD44 [19], CD90 [20]. In our previous work [21], a novel and effective targeting peptide named HCBP-1 has been isolated by bacterial surface display method, which could definitely bind to lung cancer stem cells.

Magnetic iron oxide nanoparticles (Fe_3O_4 NPs, MNPs) have drawn many attentions due to its outstanding superparamagnetic properties, attractive physicochemical characteristics and multifunctional modifiable surface [22–24]. It has excellent potential for physiological applications such as targeted drug and gene delivery[6,25,26], cell and protein separation [8,27], magnetic resonance (MR) imaging [5,28] and cancer therapy (hyperthermia) [7,29]. The MNPs could be flexibly designed and synthesized as

* Corresponding author at: Division of Nanobiomedicine, Suzhou Institute of Nano-Tech and Nano-Bionics, Chinese Academy of Sciences (CAS), 398# Ruoshui Road, Suzhou Industrial Park, Suzhou, 215123, China. Fax: +86 512 62603079.
E-mail address: ymzhu2008@sinano.ac.cn (Y. Zhu).
[1] Zhou and Chen contributed equally to this manuscript as co-first authors.

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multifunctional nanoparticles *via* modifying ligands on the surface according to different application purposes [30,31]. Meanwhile, there is report that MNPs could be used in CSCs identification *via* the specific targeting ligands on the surface [32].

In this paper, nanocomposites of fluorescent magnetic nanoparticles modified with specific targeting peptides (HCBP-1) were synthesized through layer-by-layer assembly technique. Thus, the nanocomposites possessed multifunctional properties such as targeting to lung CSCs, superparamagnetism and fluorescence. The physicochemical characteristics of these nanocomposites were investigated. Its bioactivities were examined including the haemocompatibility, cytotoxicity and targeting properties *in vivo*. All the data highlighted that the nanocomposites could be effective agent for tumor diagnosis and therapeutics.

2. Materials and methods

2.1. Materials

$FeCl_2{\cdot}4H_2O$, dimethyl sulfoxide (DMSO), Polyethyleneimine (PEI) (branched, Mn = 10,000), *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS) and Poly(ethylene, glycol) bis(carboxymethyl) ether (Mn = 600) (PEG for short in whole paper unless otherwise specified) were purchased from Sigma–Aldrich (St. Louis, MO USA.). Cy5.5 Dye mono-reactive NHS Esters (Cy5.5 NHS ester, M_W = 791) was supplied by Fanbo Biochemicals (Beijing, China). Human lung cancer cell lines (H460), human embryonic lung fibroblast cell line (HLF) and human mesenchymal stem cell (MSC) were provided by Shanghai type culture collection, CAS (Shanghai, China). RPMI-1640 medium, DMEM medium, DMEM-12 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Hyclone (Thermo Fisher. USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA.). All the chemical agents were of analytical grade. The 18.2 MΩ water used in all experiments was obtained from a Purelab Ultra Genetic instrument (ELGA, U.K).

Specific targeting peptides were synthesized by Shanghai Biotech bioscience and technology Co., Ltd. (Shanghai, China) referring to our previous reports [21]. (HCBP-1: GGLGCF-PEGEMACWWSGGSGK, M_W = 2114; control peptide: GSSGCSSGSS-GSCSGSSGSSK, M_W = 1773).

Healthy Female nude mouse (16 ± 2 g) in this study was supplied by Shanghai Laboratory Animal Center (SLAC) (Shanghai, China). The animal experiment protocol was approved by Suzhou Medical Laboratorial Animal Administration Committee. All animal studies were performed in compliance with the National institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23) revised in 1996.

2.2. Synthesis of fluorescent magnetic nanoparticles (Fe_3O_4@PEI@Cy5.5@PEG NPs, PCPMNPs)

Fe_3O_4@PEI NPs (PMNPs) were synthesized according to Li & Cai' literature with minor modification [33,34]. Briefly, $FeCl_2{\cdot}4H_2O$ (1.2 g) was dissolved in 7.5 mL water. Ammonium hydroxide (6.5 mL) was added drop-wise into the solution with vigorous stirring for 10 min. In addition, PEI solution (5 mL, 0.2 g/mL) was mixed with stirring. Next, the mixture was transferred into a 50 mL Teflon vessel and put into a sealed reactor at 140 °C for 3 h. After the mixture cooled down to room temperature, the black precipitate was separated and rinsed for 5 times in water. Finally, the product (PMNPs) was collected and ultrasonically dispersed in water.

The preparation of Fe_3O_4@PEI@Cy5.5@PEG NPs (PCPMNPs) was based on synthesis mentioned above. The PMNPs (10 mg) were separated by a magnet followed by ultrasonically rinsed for 2 times with DMSO, and finally dispersed in 0.5 mL DMSO. Cy5.5 NHS ester (1.1 mg) was fully dissolved in 0.5 mL DMSO and then mixed into the PMNPs solution with vigorous vibration in dark at room temperature for 12 h. Subsequently, the reaction product (Fe_3O_4@PEI@Cy5.5 NPs, CPMNPs) was collected, rinsed and dispersed in 1 mL DMSO. HOOC-$(PEG)_n$-COOH (9 mg), EDC (6.9 mg) and NHS (4.1 mg) were dissolved successively in 2 mL DMSO and stirred for 12 h to activate the carboxyl groups of PEG. Additionally, the DMSO solution of CPMNPs (1 mL) was added drop-wise into the activated PEG in DMSO with vigorous vibration in dark at room temperature for another 48 h. Finally, the product PCPMNPs was collected by a magnet and then ultrasonically rinsed 2 times with DMSO and dispersed in 1 mL DMSO.

2.3. Modification of HCBP-1 on the suface of fluorescent magnetic nanoparticles (Fe_3O_4@PEI@Cy5.5@PEG@HCBP-1 NPs, HPCPMNPs)

HPCPMNPs were synthesized by the EDC crosslinking method. HCBP-1 peptide (3.5 mg) was dissolved in 1 mL DMSO and then added drop-wise into 1 mL DMSO solution of PCPMNPs with vigorous vibration in a dark environment at room temperature for 48 h reaction. After reaction, the mixture was collected by a magnet and carried out 3 cycles of rinse-dispersion–separation in water. Eventually, the final products HPCPMNPs were dispersed in 1 mL water. In the paper, the parts of sample were taken out for freeze–drying and weighting accurately. After that, the freeze–dried samples were used for testing physicochemical properties and the rest of sample solutions were used for testing biological characteristics. The control peptides were used for comparison under an identical synthetic process (Fe_3O_4@PEI@Cy5.5@PEG@control peptide NPs, CPCPMNPs). In order to avoid a tedious and long presentation about the series of nanoparticles production in the paper, all nanoparticles were abbreviated at appropriate positions as mention above.

2.4. Characterization

The size and morphology of samples were assessed by transmission electron microscopy (TEM) (Tecnai G2 F20 S-TWIN, FEI, USA). The size distribution and zeta potential were measured with dynamic light scattering (DLS) method by Zetasizer Nano ZS90 (Malvern Instruments, UK). The FTIR spectra of samples were recorded by Nicolet FTIR 6700 spectrophotometer (Thermo, USA.). The weight loss of samples was evaluated by thermo gravimetric analysis (TGA) method using an EXSTAR6000 TG/DTA (SII Nano-Technology Inc. Japan). The crystalline structure of samples was measured with X-ray diffraction (XRD) method using a D8 Advance X-ray diffractometer (Bruker Cop. German). The fluorescence of nanocomposites was recorded by LuxScan 10 K-A Microarray Scanner (CapitalBio, China).

2.5. Cell culture

Human lung cancer cell line (H460), Human lung fibroblast (HLF) cells and mesenchymal stem cells (MSCs) were cultured in RPMI-1640 medium, DMEM medium and DMEM-F12 medium, respectively (Hyclone). The culture medium was supplemented with FBS (10%, v/v), penicillin (100 U/mL) and streptomycin (100 U/mL). Cells were incubated at condition of 37 °C, 5% CO_2, and 95% relative humidity (RH).

Specially, in order to obtain tumor sphere, lung CSCs enriched by sphere-forming assay were prepared following the processes described previously [21]. Briefly, H460 cells were seeded in a culture plates (1×10^4 cells/mL) in the serum-free DMEM-F12 medium, which was supplemented with epidermal growth factor (EGF, 20 ng/mL), basic fibroblast growth factor (bFGF, 20 ng/mL)