Preparation and properties of magnetic-fluorescent endoglin aptamer nanoprobe

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Abstract. In this study, a kind of magnetic-fluorescent endoglin aptamer nanoprobe, Cy5.5-End-Fe3O4/KCTS, was synthesized with magnetic functions of Fe3O4/KCTS nanoparticles and the fluorescence ability of Cy5.5 through SMCC cross-linking with endoglin aptamer labeled with Cy5.5. The microstructure, fluorescence and magnetic properties of the samples were investigated and discussed by Fourier transform-infrared (FT-IR) spectra, X-ray diffraction (XRD), transmission electron microscopy (TEM), photoluminescence spectra, dynamic light scattering (DLS) and Zeta potential. The magnetic-fluorescent aptamer nanoprobe has an average particle size of about 240 nm, Zeta potential of -8.9 mV, excellent fluorescence and magnetic properties. This study provides a new MRI/fluorescent bimodal molecular probe for early diagnosis of hepatocellular carcinoma.

1. Introduction
Hepatocellular carcinoma (HCC) is a common and highly malignant tumor, has been the third cause of cancer death and the leading cause of mortality due to its difficult to diagnosis at the onset [1]. Therefore, early diagnosis and timely treatment is a key for improving the prognosis and cure rate of patients. Magnetic resonance imaging (MRI) is non-invasive early diagnosis of HCC and widely used for imaging liver lesions, valuable in tumor molecular imaging for its soft tissue resolution, unlimited image depth, multi-parameter imaging, and lack of radioactivity [2, 3]. Magnetic nanoparticles (Fe3O4 NPs) are common MRI contrast agents that significantly reduce T2 relaxation times, thereby darkening the T2-weighted image signal [4-6].

To date, increasing attention has been paid to the fabrication of bifunctional nanoprobe consisting of discrete functions [7-9]. More importantly, a nanoprobe integrating both fluorescent and magnetic properties can be used in cell separation, biological labeling and MRI/fluorescent bimodal molecular imaging, because they possess both magnetic and fluorescent properties that can be traced and visualized [8, 10].

Endoglin (also known as CD105), an auxiliary receptor component of the transforming growth factor beta signalling pathway, is expressed mainly by endothelial cells and has been found to be involved in angiogenesis and vascular remodeling [11, 12]. The potential application of Endoglin in the diagnosis and treatment of HCC has become important [13]. Aptamer is a short single-stranded deoxyribonucleic acid (ssDNA) or ribonucleic acid (RNA) molecules screened by systematic evolution of ligands by exponential enrichment (SELEX) and hold promise for applications in disease diagnosis and targeted therapy fields as a potential candidate for biomolecular recognition [14, 15].

In previous studies, we screened for an aptamer that specifically binds to endoglin molecules on murine neovascular endothelial cells. On the basis, a specific MRI/fluorescent imaging endoglin
aptamer nanoprobe (Cy5.5-End-Fe₃O₄/KCTS) was constructed using endoglin aptamers as recognition molecules in this paper. The physical and chemical properties of Cy5.5-End-Fe₃O₄/KCTS were verified. The fluorescence-encoded magnetic nanoparticles, with the magnetic functions of Fe₃O₄/KCTS nanoparticles and fluorescence ability of Cy5.5, can simultaneously realize two functions of magnetic resonance imaging and fluorescence labeling. We believe that such a competitive multimodality probe has the potential to challenge some met limitations in biomedical area.

2. Materials and methods

2.1 Chemicals and reagents

The endoglin aptamer (End), labeled with Cy5.5 at the 5' end and sulfhydryl group modification at the 3' end, was synthesized by Shanghai Bioengineering Company (Shanghai, China). The sequence (5' to 3') of End was CCC CCG ATG CTT TCG CCT TCG CCT TCG TTG TTC GCT TCG TCC CTG GTT CCT TTC TTG; Chitosan (MW 4.9×105, degree of deacetylation 95%) was procured from Dalian Xindie Chitin Co. (Dalian, China). carbodiimide, alpha-ketoglutaric acid was purchased from Qianshan Science and Technology Development Company (Zhuhai, China). NaN₃, FeSO₄ and NH₃·H₂O solution were procured from Tianjin No. 3 Chemical Plant (Tianjin, China). Sodium borohydride (NaBH₄), sulfo-SMCC was supplied by Fluka Co. All other chemicals are analytic grade reagents and used without further purification.

2.2 Preparation of Cy5.5-End-Fe₃O₄/KCTS nanoprobe

The magnetic Fe₃O₄/KCTS nanoparticles were prepared according to our previous reported method with Fe₃O₄ nanoparticles as the magnetic core and chitosan alpha-ketoglutaric acid (KCTS) as the basic skeleton through carbodiimide activation [16]. The Cy5.5-End-Fe₃O₄/KCTS nanoprobe was prepared by sulfo-SMCC cross-linking with endoglin aptamer labeled with Cy5.5 as the target molecule. Firstly, Fe₃O₄/KCTS NPs was dissolved in HEPES buffer (pH 7.2) to form 1.0 mg/mL Fe₃O₄/KCTS suspends, and 100 μL sulfo-SMCC (5.0 mg/ml) was added and incubated at 25 °C for 30 min. After magnetic separation, 10 μL endoglin aptamer labeled with Cy5.5 (10 μmol/L) were added and mixed, and incubated at 25 °C for 2 h. Then, 100 μL of 1% bovine serum albumin was added for 30 min. Lastly, the Cy5.5-End-Fe₃O₄/KCTS nanoprobe was obtained by magnetic separation after washed 2-3 times with HEPES buffers. The prepared nanoprobe was resuspended with 1 mL of 0.01% NaN₃ and 1% bovine serum albumin buffer and stored at 4 °C for further use. The conjugation rate is calculated according to the following equation.

Conjugation rate = \frac{I_i}{I_0} \times 100\%

Where I₀ was the fluorescence intensity of Cy5.5-End and Iᵢ was the fluorescence intensity of Cy5.5-End-Fe₃O₄/KCTS nanoprobe.

2.3 Characterization of the Cy5.5-End-Fe₃O₄/KCTS nanoprobe

The morphology and structure of the Cy5.5-End-Fe₃O₄/KCTS nanoprobe were characterized by Fourier transform infrared spectroscopy (FT-IR, Bruker Tensor 27, Germany), X-Ray diffraction (XRD, D8 Advance, Germany), Transmission electron microscopy (TEM, JEM-2100F, Hitachi, Japan), Zeta potential measurement (Zetasizer Nano Series Nano-ZS, Malvern Instruments, United Kingdom) Fluorescence spectrum measurement (F-4600 fluorescent spectrophotometer, Hitachi High Technologies Corporation, Japan).

3. Result and discussion

3.1 The formation process of the Cy5.5-End-Fe₃O₄/KCTS nanoprobe

Fig.1 was the schematic representation of the formation process of the Cy5.5-End-Fe₃O₄/KCTS nanoprobe. KCTS is a chemical modification version of chitosan and has a better water solubility.
Herein, we introduced KCTS as a wrapping agent to prepare biocompatible and nontoxic Fe$_3$O$_4$/KCTS magnetic nanoparticle through carbodiimide activation. The endoglin aptamer (End), labeled with Cy5.5 at the 5’ end and sulfydryl group modification at the 3’ end, could react with the amine groups containing in the Fe$_3$O$_4$/KCTS by SMCC cross-linking method through amine and sulfydryl group chemistry. The Cy5.5-End-Fe$_3$O$_4$/KCTS nanoprobe, with the fluorescence ability of Cy5.5 and magnetic functions of Fe$_3$O$_4$/KCTS nanoparticles, can simultaneously realize two functions of magnetic resonance imaging and fluorescence labeling.

Fig. 1 Schematic representation of the formation process of Cy5.5-End-Fe$_3$O$_4$/KCTS nanoprobe

3.2 Optimization of experimental conditions

The properties of the Cy5.5-End-Fe$_3$O$_4$/KCTS nanoprobe were influenced by various formulation and process variables like the concentration of aptamer, concentration of cross-linking agent, incubation time, temperature and so on. Fig. 2 presented the result of factor effects on the conjugation rate. It could be seen from Fig. 2A that different aptamer concentration had important effects on the conjugation rate. When aptamer concentration was 0.2μmol/L, the conjugation rate was the highest. Moreover, the conjugation rate increased with the increase of the SMCC concentration and reached the highest value (79.6%) when the SMCC concentration was 1.25 mg/mL (Fig. 2B). In Fig. 2C, it could be found that conjugation rate increased markedly with the increase of incubation time from 0.5 h to 2 h. Over 2 h, conjugation rate decreased sharply. Fig. 2D indicated that conjugation rate increased with the increase of incubation temperature, and reached the peak at 25 °C, and then dropped from 25 to 60 °C. Therefore, the maximum conjugation rate of 84.8% was achieved at 25 °C when the endoglin aptamer concentration was 0.2 μmol/L, the SMCC concentration was 1.25 mg/mL and an incubation time of 2 h was employed.

Fig. 2 (A) The effect of endoglin aptamer concentration on the conjugation rate. (B) The effect of SMCC concentration on the conjugation rate. (C) The effect of incubation time on the conjugation rate. (D) The effect of incubation temperature on the conjugation rate
3.3 Characterization of the Cy5.5-End-Fe₃O₄/KCTS nanoprobe
Fig. 3A illustrated FT-IR spectra of Fe₃O₄ (a), Fe₃O₄/KCTS NPs (b) and Cy5.5-End-Fe₃O₄/KCTS (c). The peak around 3431 cm⁻¹ related to the -OH group and the peak at 588 cm⁻¹ or 586 cm⁻¹ related to Fe-O group. The FT-IR spectrum (Fig. 3A, curve b) of Fe₃O₄/KCTS NPs showed the peak at 3431 cm⁻¹ related to the O-H and N-H stretching vibrations, and 2934 cm⁻¹ related to C-H stretching vibrations. The characteristic absorption bands appeared at 1629 cm⁻¹ (C=O carbonyl stretching vibration), 1458 cm⁻¹ (C-H bending vibration), 1068 cm⁻¹ (C-O stretching vibration). The FT-IR spectrum of Cy5.5-End-Fe₃O₄/KCTS probe (Fig. 3A, curve c) kept the characteristic peaks of Fe₃O₄/KCTS NPs and included the unique peak of the Fe₃O₄ NPs, obviously indicating the successful preparation of the Cy5.5-End-Fe₃O₄/KCTS probe.

The XRD pattern of Fe₃O₄ (a), Fe₃O₄/KCTS NPs (b) and Cy5.5-End-Fe₃O₄/KCTS (c) was shown in Fig. 3B. In the three samples, we observed six characteristic peaks (2θ=30.1°, 35.5°, 43.1°, 53.4°, 57.0° and 62.6°) which marked for Fe₃O₄ by their indices ((220), (311), (400), (422), (511) and (440)) for Fe₃O₄ NPs. These peaks were consistent with the database in JCPDS file (PDF No.65-3107) and revealed that the resultant nanoparticles were Fe₃O₄ with a spinel structure.

TEM images showed that the Cy5.5-End-Fe₃O₄/KCTS nanoprobe nearly spherical in shape and the average diameter was 35 nm (Fig. 3C). Size distribution was determined by DLS (Fig. 3D) in aqueous solution, the size of the Cy5.5-End-Fe₃O₄/KCTS nanoprobe was in the range from 190 to 295 nm and the mean diameter was 240 nm, which was bigger than determined by TEM image, presumably arising from the dry state of the TEM measurement. The Zeta potential of the Cy5.5-End-Fe₃O₄/KCTS probe was approximately -8.93 mV, while the Fe₃O₄/KCTS were approximately +17.01 mV (Fig. 3E).

Fig. 3F showed the representative fluorescence emission spectra of Fe₃O₄/KCTS NPs and Cy5.5-End-Fe₃O₄/KCTS respectively. It showed that the emission peaks of both samples were around 694 nm. Suggesting the Cy5.5-End-Fe₃O₄/KCTS has the same fluorescence effect of Cy5.5.

![Fig. 3 Characterization of Cy5.5-End-Fe₃O₄/KCTS nanoprobe](image)

Fig. 3 Characterization of Cy5.5-End-Fe₃O₄/KCTS nanoprobe ((A) FT-IR spectra; (B) XRD pattern; (C) TEM images; (D) Particle size distribution; (E) Zeta potential; (F) fluorescence emission spectra)

4. Conclusions
In summary, magnetic-fluorescent endoglin aptamer nanoprobe, Cy5.5-End-Fe₃O₄/KCTS, was successfully prepared with the fluorescence ability of Cy5.5 and magnetic functions of Fe₃O₄/KCTS nanoparticles. The maximum Cy5.5-End aptamer’s conjugation rate achieved 84.8% at 25 °C when the aptamer concentration was 0.2 μmol/L, the SMCC concentration was 1.25 mg/mL and incubation time was 2 h. The microstructure, fluorescence and magnetic properties of the samples were characterized by FTIR, XRD, TEM, DLS, Zeta potential and fluorescence. The magnetic-fluorescent aptamer nanoprobe has an average particle size of about 240 nm, Zeta potential of -8.9 mV, excellent
fluorescence and good magnetic properties. In the future, the Cy5.5-End-Fe3O4/KCTS would be applied in MRI/fluorescent bimodal molecular imaging for accurate molecular diagnostics and therapeutics for HCC.

**Acknowledgments**
This work was supported by the National Nature Science Foundation of China (Nos. 81372362, 81460451, 81760534 and 81430055), the Innovation Project of GUET Graduate Education (No. 2017YJCX100), Guangxi key industry science and technology innovation project (No.1598005-3) and the National Science Foundation of Guangxi province of China (Nos. 2016GXNSFAA380011 and 2016GXNSFAA380080)

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