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Studies on the Biocompatibility and Targeting Properties of Magnetic Nanomaterials

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ABSTRACT
This study aims to explore the biocompatibility and targeting of folate modified magnetic iron oxide nanoparticles, and provide a theoretical basis for the precise diagnosis and treatment of tumors. In this experiment, Fe_3O_4 nanoparticles were prepared by chemical synthesis and folic acid molecules were modified onto their surface to form FA- Fe_3O_4 MNPs. Fourier transform infrared (FTIR) spectroscopy was used to verify whether folic acid was successfully coupled to the Fe_3O_4 nanoparticles; and a CCK-8 assay was used to assess the effect of FA-Fe_3O_4 MNPs on the cytotoxicity of HK2 cells and HeLa cells. Meanwhile, the uptake of FITC-labelled FA-Fe_3O_4 MNPs by both cells was observed. FTIR characterisation showed that folic acid was successfully modified on the surface of Fe_3O_4 nanoparticles. In CCK-8 experiments, 12.5 and 25 μg/mL of FA-Fe_3O_4 MNPs were virtually non-toxic to HK2 cells and HeLa cells, and cell viability was maintained at a high level, whereas cell viability decreased at higher concentrations. Cellular uptake assays showed that HeLa cells showed significantly higher uptake of FITC-labelled FA-Fe_3O_4 MNPs than HK2 cells. FA-Fe_3O_4 MNPs have good biocompatibility and targeting properties, and can effectively target tumour cells with high folate receptor α expression.

KEYWORDS
Folic acid; Magnetic nanomaterials; Biocompatibility

1 Introduction

Cancer remains a significant public health challenge worldwide, severely endangering human health and creating substantial healthcare and economic burdens. Conventional approaches to treating malignancies including surgical intervention, radiation therapy, and chemical agents remain prevalent yet exhibit substantial drawbacks. While these modalities are extensively employed in clinical practice, they demonstrate considerable constraints that impact treatment efficacy. The standard therapeutic triad of operative procedures, ionizing radiation, and cytotoxic drugs continues to dominate oncology despite presenting multiple challenges [1]. The non-specific distribution of chemotherapeutic drugs leads to severe side effects, while drug resistance in cancer cells further undermines the therapeutic efficacy [2]. In recent years, magnetic nanomaterials have shown great potential in cancer diagnosis and treatment due to their unique physical and chemical properties [3]. Magnetic iron oxide nanoparticles (Fe_3O_4 MNPs) have become a focus of research because of their good biocompatibility, superparamagnetism, and responsiveness to external magnetic fields. These materials can not only achieve highly sensitive tumor detection through magnetic resonance imaging (MRI) but also generate heat for magnetic hyperthermia in alternating magnetic fields or enable precise drug delivery through magnetic targeting technology.

Magnetic iron oxide nanoparticles (Fe_3O_4 MNPs) are magnetic nanomaterials with unique magnetic properties, possessing a crystal structure of magnetite and exhibiting good superparamagnetism and biocompatibility [4]. The size of Fe_3O_4 MNPs typically ranges between 10 and 100 nanometers. This size range allows them to utilize the enhanced permeability and retention (EPR) effect to achieve passive targeting in tumor tissues [5]. The magnetic properties of Fe_3O_4 MNPs enable them to respond to external magnetic fields, thus facilitating magnetic targeting and magnetic hyperthermia [6]. Fe_3O_4 MNPs demonstrate excellent biocompatibility due to their low toxicity and good biodegradability. For example, ferumoxytol, an Fe_3O_4-based magnetic nanoparticle (MNP), has been clinically approved for iron deficiency anemia treatment and demonstrates promising potential in cancer detection and therapy [7]. The surface of Fe_3O_4 MNPs can be chemically modified to introduce various functional groups, such as polyethylene glycol (PEG) and folic acid (FA), to enhance their biocompatibility and targeting properties.

Functioning as a crucial B-vitamin, folic acid (FA) significantly contributes to cellular multiplication, genetic material production, and mitotic processes. The folate receptor (FR) system comprises four distinct variants: FRα, FRβ, FRδ, and FRγ. Notably, both FRα and FRβ exist as surface-bound molecules tethered to cellular membranes through glycosylphosphatidylinositol (GPI) anchors[8-9]. Studies have shown that folate receptor α is significantly upregulated in various cancers such as ovarian, lung, colon, and breast cancer, while its expression level is relatively low in normal tissues [10-11]. This difference makes FRα an ideal target for tumor-targeted therapy. FRα transports folic acid into cells via endocytosis. When folic acid binds to FRα, it induces a structural change, leading to membrane invagination and endosome formation, ultimately releasing folic acid into the cytoplasm [8]. Folic acid-modified magnetic nanoparticles (FA-Fe_3O_4 MNPs) can significantly improve the enrichment efficiency of drugs in tumor tissues through the specific binding of

folic acid to FRα, while reducing toxicity to normal tissues, thereby achieving precise targeting of tumor cells [12]. The high affinity, non-immunogenicity, and stability of folic acid make it an ideal targeting ligand, widely used in magnetic resonance imaging and magnetic targeted drug delivery systems [13]. Novel therapeutic approaches such as antibody-drug conjugates (ADCs) and CAR-T cell therapy have already demonstrated significant anti-tumor effects.

The objective of this study is to investigate the biocompatibility and targeting capability of FA-Fe_3O_4 MNPs, which will contribute to the optimization of nanomaterial design and provide a theoretical foundation for precise tumor diagnosis and therapy.

2 Materials and methods

2.1 Experimental materials

2.1.1 Experimental reagents

Table 1 Main experimental reagents

Name	Manufacturer
$FeCl_3 \cdot 6H_2O$	Sinopharm Chemical Reagent Co., Ltd.
Sodium Citrate	Sinopharm Chemical Reagent Co., Ltd.
Polyacrylamide	Sinopharm Chemical Reagent Co., Ltd.
Urea	Sinopharm Chemical Reagent Co., Ltd.
Polyetherimide	PolyK Technologies, USA
N-Methylpyrrolidone	Sinopharm Chemical Reagent Co., Ltd.
Folic Acid	Aladdin Reagent (Shanghai) Co., Ltd.
EDC	Hangzhou Xinqiao Biotechnology Co., Ltd.
NHS	Hangzhou Xinqiao Biotechnology Co., Ltd.
DMSO	Aladdin Reagent (Shanghai) Co., Ltd.
FITC	Aladdin Reagent (Shanghai) Co., Ltd.
Fetal Bovine Serum	Gibco
Penicillin-Streptomycin	Beyotime
DMEM Medium	Gibco
CCK-8 Detection Kit	Solarbio
DAPI	Beyotime

2.1.2 Experimental instruments

Table 2 Main experimental instruments

Name	Manufacturer
Fourier Transform Infrared Spectrometer	Varian
Magnetic Stirrer	Hangzhou Ruicheng Instrument Co., Ltd.
Oven	Shanghai Jinghong Experimental Equipment Co., Ltd.
Microplate Reader	BIO-TEK
Fluorescence Microscope	Zeiss

2.2 Experimental methods

2.2.1 Synthesis of Fe_3O_4 Nanoparticles

Dissolve 1.084 g of $FeCl_3 \cdot 6H_2O$ completely in 80 mL of deionized water to form an iron salt solution. Subsequently, a mixture of 2.352 g of sodium citrate, 0.8 g of polyacrylamide, and 0.72 g of urea was added to the iron salt solution, and the mixture was continuously stirred for 30 minutes to ensure that all components were fully dissolved and mixed. The entire mixture was transferred into a reaction kettle, which was then sealed and heated at 200°C for 24 hours to promote the formation of Fe_3O_4 nanoparticles. After the reaction kettle had naturally cooled to room temperature, the product was washed sequentially with deionized water and alcohol, each time using centrifugation at 3000 rpm for 5 minutes (repeated 3 times) to remove unreacted chemicals and impurities. Finally, the sample was dried in an oven at 60°C for 12 hours to obtain the black Fe_3O_4 nanoparticle material.

2.2.2 Folic Acid-Linked Fe_3O_4 Nanoparticles

The 0.2 g of Fe_3O_4 nanoparticle powder synthesized above was dissolved in ultrapure water and set aside. 0.3 g of polyetherimide (PEI) was dissolved in N-methylpyrrolidone (NMP) [14]. With continuous magnetic agitation, the PEI solution was gradually introduced into the Fe_3O_4 suspension. This combined system underwent sustained magnetic stirring at 80° C over a 12-hour period to guarantee thorough PEI coating on the Fe_3O_4 nanoparticle surfaces. Following this, the suspension was filtered using a 0.22 μm membrane filter. Subsequent purification involved alternating washes with ddH_2O and ethanol (three cycles each) to eliminate residual PEI. The resulting material was then subjected to oven drying at 60°

C for a full day, yielding the final black PEI-Fe_3O_4 composite powder.

0.2 g of PEI-Fe_3O_4 powder and 0.2 g of folic acid were dissolved thoroughly in ultrapure water and set aside. A mixture of 0.5 g EDC and 1.5 g NHS was sonicated in a water bath for 45 minutes, then slowly added to the mixed solution of PEI-Fe_3O_4 and folic acid, with 3-5 mL of DMSO being added continuously as a cosolvent during the process. Under room temperature and light-protected conditions , the mixture was stirred magnetically continuously for 24 hours. The product was then dialyzed using a dialysis membrane, and the resulting FA-Fe_3O_4 nanoparticle solution was obtained after filtration through a 0.22 μm filter membrane. The solution was stored at 4°C for subsequent use.

2.2.3 Fourier-transform infrared spectroscopy detection

To verify the conjugation of folic acid on the surface of FA-Fe_3O_4 nanoparticles, Fourier-transform infrared spectroscopy (FTIR) was employed for characterization.

The Fe_3O_4 nanoparticles and FA-Fe_3O_4 nanoparticles were ground into very fine powders. Each powder was then mixed with an appropriate amount of potassium bromide (KBr) solid and ground together. Transparent KBr pellets were subsequently pressed and used as test samples. The resolution was set to 4 cm^{-1}, and 32 scans were accumulated. Background interference from the instrument needed to be subtracted before testing.

2.2.4 Cell culture

Human cervical cancer cells (HeLa cells) and human renal cortical proximal tubule epithelial cells (HK2 cells) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2. The cells were passaged every 2-3 days, and cells in the logarithmic growth phase were used for experiments.

2.2.5 CCK-8 assay for cytotoxicity

HeLa cells and HK2 cells in the logarithmic growth phase were trypsinized and centrifuged to prepare a single-cell suspension at a concentration of $1x10^6$ cells/mL. 100 μL of the suspension was seeded into each well of a 96-well plate.

After cell adherence, the original culture medium was removed. Different concentrations (12.5, 25, 50, 100, 200 μg/mL) of FA-Fe_3O_4 nanoparticles were mixed with the cell culture medium, and 100 μL of this mixture was added to each well.

After 24 hours of incubation, the medium containing nanoparticles was removed. Each well was then added with a mixture of 10 μL CCK-8 solution and 90 μL cell culture medium. The cells were further incubated in the incubator at 37°C for 2 hours.

The absorbance (OD value) at 450 nm was measured using a microplate reader to calculate the cell viability of the experimental groups.

$$\text{Viability (\%)} = (OD_{\text{experimental group}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}}) \times 100\%$$

2.2.6 Cell uptake experiment

The prepared FA-Fe_3O_4 nanoparticles were mixed with 0.6 mL of FITC (fluorescein isothiocyanate) and stirred in borate buffer (pH 8.5) in the dark for 12 hours. The resulting mixture was dialyzed to remove free FITC and other residual substances. The dialyzed nanoparticles were centrifuged and resuspended to obtain the FITC-labeled FA-Fe_3O_4 nanoparticles.

A newly prepared nutrient solution was employed to create a cellular mixture with a concentration of 1×10^5 cells per milliliter. One milliliter of this cellular mixture was dispensed into individual compartments of a six-well culture dish, followed by a 24-hour incubation period under controlled conditions. After removing the nutrient solution, each well received one milliliter of medium infused with fluorescently-tagged FA-Fe_3O_4 nanostructures. The cellular samples underwent six hours of exposure in a temperature-regulated chamber maintained at 37° C with 5% carbon dioxide. Following medium removal, the cells underwent triple rinsing with phosphate-buffered saline, underwent fixation using 4% paraformaldehyde for fifteen minutes, and were subsequently treated with DAPI nuclear stain for ten minutes. Cellular internalization of the nanostructures was visualized through fluorescence microscopy.

3 Experimental results

3.1 FT-IR characterization results

The chemical structures of Fe_3O_4 nanomaterials and FA-Fe_3O_4 nanomaterials were analyzed using Fourier-transform infrared spectroscopy (Figure 1). The unmodified Fe_3O_4 nanospheres exhibited a typical Fe-O bond stretching vibration peak at 589 cm^{-1}, indicating the formation of the magnetite lattice structure. After folic acid modification, the FTIR spectrum of FA-Fe_3O_4 MNPs showed a significant C=O stretching vibration peak at 1646 cm^{-1} (attributed to the glutamic acid carboxyl group of folic acid), a C-N bond vibration peak at 1384 cm^{-1} (benzoic acid moiety in the folic acid molecule), and a C-O bond vibration peak at 1194 cm^{-1} (hydroxyl functional group of folic acid).

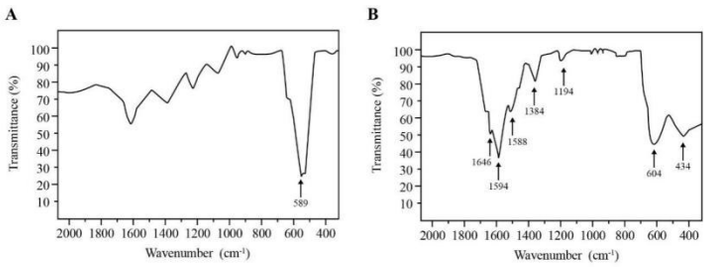

Figure 1 FT-IR spectra of Fe_3O_4 nanomaterials (A) and FA-Fe_3O_4 nanomaterials (B)

3.2 Cytotoxicity experiment

To evaluate the biocompatibility of FA-Fe_3O_4 MNPs, cytotoxicity tests were conducted on HK2 cells and HeLa cells using the CCK-8 reagent. After co-culturing the two cell types with FA-Fe_3O_4 nanoparticles at different concentrations (12.5, 25, 50, 100, 200 μg/mL) for 24 hours, cell viability was measured to assess their toxicity (Figure 2). The experimental results showed that at lower concentrations (12.5 μg/mL and 25 μg/mL), the viability of both cell types remained at a high level. For HK2 cells, the viability was 99.2±0.9% and 95±1.2%, respectively. For HeLa cells, it was 98.5±1.2% and 94.7±0.9%, respectively. However, under treatment with 100 μg/mL and 200 μg/mL of FA-Fe_3O_4 nanoparticles, the cell viability gradually decreased. The results indicate that FA-Fe_3O_4 nanoparticles have minimal toxic effects on both cell types at lower concentrations.

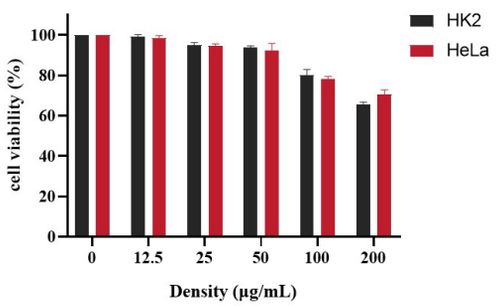

Figure 2 CCK-8 experimental results of FA-Fe_3O_4 MNPs at different concentrations on MCF-7 and MCF-10A cells after 24 hours.

3.3 Cell uptake experiment

Folate Receptor alpha (FRα) is a transmembrane receptor highly expressed in many tumor cells and widely present in solid tumors such as ovarian cancer and fallopian tube cancer. FRα is typically expressed at high levels in certain cancer cells. A cell uptake experiment was conducted using FITC-labeled FA-Fe_3O_4 MNPs (Figure 3). The results showed that HK2 cells (low FRα expression) displayed only weak fluorescence (Figure 3A), whereas HeLa cells (high FRα expression) exhibited strong green fluorescence signals in the cytoplasm after 6 hours of incubation, with a significant number of nanoparticles present (Figure 3B). This indicates that folic acid modification significantly enhances the accumulation ability of the nanoparticles in tumor cells with high folate receptor expression. DAPI nuclear staining revealed that the nanoparticles were primarily distributed in the cytoplasm, with no significant nuclear uptake observed.

4 Discussion

Magnetic nanomaterials have garnered significant attention in the biomedical field due to their unique physical and chemical properties. This study focused on folic acid-modified magnetic iron oxide nanoparticles to investigate their biocompatibility and targeting properties. The experimental results demonstrated that FA-Fe_3O_4 MNPs exhibit good biocompatibility at various concentrations for both tumor and normal cells, while showing significant targeting ability in tumor cells with high folate receptor expression. These findings provide important references for the further development of precision diagnosis and treatment tools based on magnetic nanomaterials.

From the perspective of biocompatibility, this study used the CCK-8 method to evaluate the toxicity of FA-Fe_3O_4 MNPs at different concentrations on HeLa cells (tumor cells) and HK2 cells (normal cells). Within the concentration range of 12.5 and 25 μg/mL, FA-Fe_3O_4 MNPs exhibited low cytotoxicity towards both cell types. The results indicate that folic acid modification did not significantly affect the biocompatibility of the nanoparticles. Folic acid modification may improve the surface properties of the nanoparticles, thereby reducing non-specific adsorption and toxicity towards normal cells.

FA-Fe_3O_4 MNPs significantly enhanced their accumulation in tumor cells with high folate receptor expression. The cell uptake experiment using FITC-labeled nanoparticles showed that HeLa cells exhibited strong green fluorescence signals in the cytoplasm after 6 hours of incubation, whereas HK2 cells only displayed weak fluorescence. This differential uptake

suggests that folic acid modification significantly increases the uptake efficiency of the nanoparticles in tumor cells through specific binding to Folate Receptor alpha (FRα). Since FRα is highly expressed in various tumor cells but expressed at low levels in normal tissues, this characteristic allows FA-Fe_3O_4 MNPs to achieve precise targeting of tumor cells, thereby reducing toxicity to normal tissues. Further analysis of the mechanism of folic acid modification reveals that FRα transports folic acid into the cell via endocytosis. Upon binding to FRα, folic acid induces a conformational change, leading to membrane invagination and endosome formation, ultimately releasing folic acid into the cytoplasm [15]. FA-Fe_3O_4 MNPs utilize this mechanism to achieve efficient uptake of the nanoparticles through specific binding between folic acid and FRα. This targeting mechanism not only improves the accumulation efficiency of nanoparticles in tumor tissues but also provides theoretical support for magnetic resonance imaging and magnetic targeted drug delivery systems.

However, the targeting specificity of folic acid modification is not absolute. Although FRα is highly expressed in tumor cells, it may also be present at low levels in certain normal tissues, potentially leading to some degree of non-specific uptake. Additionally, factors such as the size, surface charge, and modification density of the nanoparticles can also influence their targeting efficiency. Therefore, in future research, the targeting efficiency and biocompatibility can be further improved by optimizing the surface modification and physicochemical properties of the nanoparticles.

This study verified the biocompatibility and targeting properties of FA-Fe_3O_4 MNPs through CCK-8 and cell uptake experiments, but in vivo experiments were not conducted. In vivo experiments can provide a more comprehensive evaluation of the biodistribution and hepatic metabolism of the nanoparticles, which is crucial for their clinical application. Future research can further conduct animal experiments to observe the in vivo targeting effect and biocompatibility of FA-Fe_3O_4 MNPs, providing more robust evidence for clinical translation. The superparamagnetism and responsiveness to external magnetic fields of FA-Fe_3O_4 MNPs offer possibilities for their application in magnetic hyperthermia. Future research can combine alternating magnetic fields to explore the synergistic therapeutic effects of FA-Fe_3O_4 MNPs in magnetic hyperthermia, further expanding their potential applications in cancer treatment.

5 Conclusion

(1) FTIR characterization results show that folic acid was successfully modified onto the surface of Fe_3O_4 nanoparticles.

(2) FA-Fe_3O_4 MNPs exhibited low cytotoxicity towards MCF-7 and MCF-10A cells within the concentration range of 12.5–25 μg/mL, with cell viability remaining above 90%.

(3) The cell uptake experiment using FITC-labeled nanoparticles showed that HeLa cells exhibited strong green fluorescence signals in the cytoplasm after 6 hours of incubation, whereas HK2 cells only displayed weak fluorescence.

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