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Acridine orange coated magnetic nanoparticles for nucleus labeling and DNA adsorption

Chi-Hsien Liu*, Soubhagya Laxmi Sahoo, Min-Han Tsao

Graduate Institute of Biochemical and Biomedical Engineering, Chang Gung University, Taiwan, ROC

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ABSTRACT

The magnetic–fluorescent nanoparticles are the integration of fluorophores and magnetic nanoparticles (MNP), which are superior to traditional single-modal nanoparticles. Here, we develop magnetic nanoparticles functionalized by acridine orange (ACO) for labeling nucleus and separating DNA. The ACO, a cell-permeant nucleic acid binding dye, is conjugated with amine on magnetic nanoparticles by glutaraldehyde-mediated coupling and characterized by TEM and FT-IR. Fluorescence spectroscopy, INCell analyzer, and confocal microscopy analyses confirmed the fluorescent property of ACO modified MNP. Furthermore, the modified magnetic nanoparticles showed strong intracellular fluorescence when incubated with 293T cells for a short period of time. The adsorption capacities measured at various concentrations showed enhanced adsorption capacities for double or single stranded DNA when compared to amine MNP conjugated with glutaraldehyde. The cell viability tests of the composite nanoparticles on 293T cells showed low cytotoxicity indicating the safeness of the nanoparticles. The modified magnetic nanoparticles pave a versatile platform for biological applications such as cell labeling and DNA adsorption.

1. Introduction

Magnetic nanoparticles (MNP) are being of great interest due to their unique physical properties and ability to function at the cellular and molecular level of biological interactions. However, the bare nanoparticles have limited use in biomedical applications due to their restricted functions and aggregation of the nanoparticles [1]. To overcome this problem, surface modification with suitable ligands by crosslinkers is required to functionalize these bare nanoparticles. The linker is important to keep appropriate space between the magnetic nanoparticles and ligands for avoidance of steric hindrance and fluorescent quenching [2]. Recently, functionalized MNP have been used for magnetic resonance imaging [3], enzyme isolation [4,5], drug delivery [6], gene therapy [7], and fluorescence imaging [8–10]. Thus, the development of multifunctional nanoparticles that simultaneously fulfill different functionalities is promising. The magnetic–fluorescent nanoparticles can be served as an all-in-one diagnostic tool, which could be used in the diagnostic images based on magnetic resonance imaging [11]. The fluorescent part emitting at an appropriate wavelength can provide the visual imaging by fluorescence microscope. These nanoparticles also allow us to perform optical tracking of biological processes as well as magnetic manipulation [12].

Different fluorophores such as fluorescein, rhodamine, and cyanine are combined with MNP for fluorescent labeling applications [3,13,14]. These MNP serving as dual function contrast agents can provide more realistic imaging of live and intact organisms in vivo and in vitro by using fluorescence microscopy and MRI techniques. A few researchers are investigating the dye-doped nanoparticles for tumor cell labeling. For example, amino acids have been used as a spacer to conjugate fluorescein and a magnetic nanoparticle in a two-step protocol [9]. The developed fluorescent nanoparticles maintain the excitation and emission properties of fluorescein which can be used in wide-ranging applications including laser induced fluorescence detection techniques and flow cytometry. Hoffmann et al., have developed a fibronectin-nanoparticle conjugate for multiple detection imaging assays [15]. The core shell composed of Gd(OH)CO3·SiO2 nanoparticles embedded with rhodamine could be used as MRI and optical imaging in biology [3]. Tallury and co-workers design magnetic chitosan nanoparticles for fluorescent imaging and stimulus-responsive drug release [16]. However, these researches have provided important information only on the development of novel nanoparticles with cell labeling function, but are limited in the detailed analysis of endocytotic mechanism. The characteristics of nanoparticles such as size, shape, and surface properties are important for the cellular internalization and intracellular trafficking of nanoparticles. For example, the size effect on the endocytosis pathway and subsequent intracellular

* Corresponding author at: Chang Gung University, Graduate Institute of Biochemical and Biomedical Engineering, Tao-Yuan, Taiwan.
Tel.: +886 32118800x3146; fax: +886 32118800x3140.
E-mail address: chl@mail.cgu.edu.tw (C.-H. Liu).

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fate has been investigated by using fluorescent latex beads of defined sizes (50–1000 nm) in non-phagocytic B16 cells [17]. Internalization of nanoparticles with a diameter <200 nm involves clathrin-mediated pathway. With increasing size, a shift to a mechanism that relied on caveolae-mediated internalization becomes the dominant pathway of entry. In B16 cell line the nanoparticles internalized through clathrin-mediated endocytosis are destined for a lysosomal compartment, whereas those through a caveolin-mediated process are not. The shape also influences the cellular internalization of nanoparticles in different cell lines. The particles with an aspect ratio of 3 were internalized four times more rapidly than those with an aspect ratio of 1 in non-phagocytic HeLa cells [18].

Acridine orange (ACO) is a fluorescent probe commonly used to label nucleus in both living and dead microorganisms. ACO is a cell-permeable probe that interacts with DNA and RNA by intercalation or electrostatic attractions [19]. ACO also enters acidic compartments such as lysosomes and becomes protonated and sequestered. In these low pH conditions, the dye will emit orange light when excited by blue light. Therefore, the acidified apoptotic cells can be identified by ACO using fluorescence microscopy. ACO can be derivatized at several positions while still keeping its ability to absorb DNA [8,20]. Although many works have been reported on different kinds of fluorophores, according to our knowledge ACO modified MNP have not been reported so far. Hence, our aim was to design and characterize novel magnetic nanoparticles for imaging and separation. In addition, the potential implications of ACO modified MNP as the fluorescent marker for cell imaging and as the adsorbent for DNA are investigated.

2. Materials and methods

2.1. Materials

Acridine orange (ACO), glutaraldehyde, KBr, hydroxylamine, o-phenanthroline are purchased from Sigma–Aldrich (St. Louis, MO, USA). Paramagnetic iron oxide nanoparticles (USPIO101, NH_2-surface modified) are purchased from Taiwan Advance Nanotech (Taoyuan, Taiwan). The amine modified MNP has the 6.2 ± 2.0 nm diameter with the 7 emu/g saturated magnetization. Plasmid EGFP-C3 (size 4.7 kb) is obtained from Takara Bio (Shiga, Japan). The plasmid was amplified in *Escherichia coli* DH-5 alpha and purified using a purification kit (GeneMark, Taipei, Taiwan). Single strand DNAs from salmon milt are obtained from Maruha-Nichiro Foods Inc. (Tokyo, Japan). The size of the salmon milt DNA ranges from 300 to 500 bp. The purity of pDNA and ssDNA is higher than 99%. All reagents are used without further purification.

2.2. Preparation of dye coated magnetic nanoparticles

One gram of amine magnetic nanoparticles (AM-MNP) were mixed with 1 ml of a solution containing glutaraldehyde (10%) and reacted at a constant vortex rate (of magnitude 4) for 1 h. The glutaraldehyde modified nanoparticles (GL-MNP) were obtained and washed three times with deionized water using a magnetic separation system (Millipore). The ACO dye (0.17%) was added to the nanoparticles with vortex for 2 h to further crosslink the particles. The un-reacted ACO was removed by three washes with water. The iron content of MNP was quantified via modified o-phenanthroline method as described previously [21]. All of ferric iron in the MNP shall be reduced from Fe^{3+} to Fe^{2+} by the use of an excess of hydroxylamine. The ferrous iron reacts with o-phenanthroline to form orange complexes. The absorbance is measured by Epoch Spectrophotometer (Biotek, Winooski, VT) at a wavelength of 510 nm. The density of ACO on the MNP surface was estimated by the mass balance of initial and final ACO concentrations in the reaction solution.

2.3. Cell culture and MNP uptake analysis

The HEK-293T cell line is obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and is maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 10% serum. For MNP uptake experiments, cells were seeded in 48 well plates at a density of 6×10^4 cells/well and allowed to grow for 24 h. DMEM medium (0.2 mL) containing 20 μg of MNP solution was added to the 48-well plate at 37 °C for various incubation intervals and the cells were washed with 0.2 mL PBS to remove the remaining particles. At the end of the incubation, cells were fixed by 0.15 mL of 4% formaldehyde and analyzed by INCell Analyzer 1000 (GE Healthcare, Piscataway, NJ). Cells in 48 well plates were analyzed for fluorescence intensity and ACO positive percentage by the INCell Analyzer. The fluorescence at 455 nm in the nucleus stained with Hoechst 33342 was recorded after excitation at 350 nm. The fluorescence of ACO at 525 nm was quantitatively analyzed after excitation at 480 nm. To analyze the fluorescent images, the INCell Investigator was used to identify the viable cells (blue fluorescence) and ACO-positive cells (green fluorescence). The percentage of fluorescence positive cells is defined as follows:

$$\left(\frac{\text{the number of cells exhibiting both blue and green fluorescence}}{\text{the number of cells exhibiting blue fluorescence}}\right) \times 100\%$$

The cell viability is defined as follows:

$$\left(\frac{\text{the number of nuclei in treated cells}}{\text{the number of nuclei in controlled cells}}\right) \times 100\%.$$

2.4. Adsorption of pDNA or ssDNA by magnetic nanoparticles

The amount of 10 μg MNP was added to the DNA solution (10 μl) at various DNA concentrations containing 0.5 M NaCl in an eppendrof tube and incubated for 30 min. The MNP were removed magnetically from the DNA solution by a magnet. The amounts of DNA adsorbed on the magnetic nanoadsorbents were estimated from the mass balance of DNA in solution. The concentration of nucleic acids was calculated by the NanoDrop spectrophotometer (Thermo, Wilmington, DE). For all experiments, four separate experiments were performed under each condition. The standard deviation of the measurement was calculated using the STDEV function of Microsoft Excel (Microsoft, Redmond, WA, USA).

2.5. Adsorption isotherms

The adsorption data were fitted by the two isotherm equations including Langmuir (Eq. (1)) and Freundlich (Eq. (2)) models employing the iterative fitting method of Sigmaplot (Systat software, San Jose, CA).

$$q = \frac{q_m[C_e]}{([C_e] + K_L)} \quad (1)$$

$$q = K_F C_e^n \quad (2)$$

where q is the equilibrium adsorption capacity (μg/mg), $C_e$is the equilibrium DNA concentration in solution μg/mL), q_m is the maximum capacity (μg/mg), K_L is the Langmuir adsorption constant (μg/mg), K_F is the Freundlich adsorption constant and n is the Freundlich coefficient. The intercept K_F can be obtained from the plot of log q versus log C_e, where K_F is a measure of the adsorption capacity and the slope n in the plot is dependent on the adsorption homogeneity [22].