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Enzyme-functionalized gold-coated magnetite nanoparticles as novel hybrid nanomaterials: Synthesis, purification and control of enzyme function by low-frequency magnetic field

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ABSTRACT

The possibility of remotely inducing a defined effect on NPs by means of electromagnetic radiation appears attractive. From a practical point of view, this effect opens horizons for remote control of drug release systems, as well as modulation of biochemical functions in cells. Gold-coated magnetite nanoparticles are perfect candidates for such application. Herein, we have successfully synthesized core–shell NPs having magnetite cores and gold shells modified with various sulphur containing ligands and developed a new, simple and robust procedure for the purification of the resulting nanoparticles. The carboxylic groups displayed at the surface of the NPs were utilized for NP conjugation with a model enzyme (ChT). In the present study, we report the effect of the low-frequency AC magnetic field on the catalytic activity of the immobilized ChT. We show that the enzyme activity decreases upon exposure of the NPs to the field.

1. Introduction

The integration of nanotechnology into biology and medicine is expected to produce major advances in molecular diagnostics, therapeutics, molecular biology and bioengineering. Success in the synthesis of nanoparticles (NPs) has led to the development of functional NPs able to react to an applied external stimulus. These NPs can be covalently linked to biological molecules such as peptides, proteins and nucleic acids. Due to their size-dependent properties and dimensional similarities to biomacromolecules, NPs offer exciting new opportunities for many biotechnological applications. In this frame, the possibility of remotely inducing a defined effect on NPs by means of electromagnetic radiation appears particularly attractive. For example, a gold nanocrystal has been covalently attached to a double-helical DNA; upon exposition to an oscillating radio frequency (RF) magnetic field, the DNA double helix opened up and closed back, i.e., denaturated and renaturated [1]. In a similar way, it has been reported that it is possible to switch on and off an enzyme in vitro by attaching to it a gold nanocrystal and modulating an applied RF field [2]. Recently, we have demonstrated that super low-frequency non-heating magnetic field can alter the kinetics of chemical reactions catalyzed by the enzymes α-chymotrypsin (ChT) and β-galactosidase (β-GaL) immobilized on nanoscale magnetic NP aggregates and core–shell magnetic NPs [3]. The observation is unprecedented and suggests the significance of magneto-mechanochemical effects induced by realignment of MNP magnetic moments in an alternating current (AC) magnetic field rather than traditional heating. Such low frequency and amplitude fields are safe and are not expected to cause any damage to biological tissues [4]. Thus, from a practical point of view, this investigation opens horizons for remote control of drug release systems, as well as modulation of biochemical functions in cells. However, to fully benefit from these potential opportunities there is a need to advance the NP surface chemistry approaches allowing

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functionalization of magnetite NPs with various biological molecules. It is well known that gold NPs offer excellent opportunities for surface chemical modification and facile attachment of broad range of small molecules as well as synthetic biological macromolecules.

In the present report, we demonstrate a facile and simple aqueous-based method to fabricate Fe_3O_4/Au bifunctional hybrid materials based on reduction of $AuCl_4^-$ on the surface of magnetite NPs [5]. Herein, we first develop a procedure for the purification of synthesized gold-coated magnetic NPs using flash chromatography on a Sepharose gel. Resulting NPs were consistently modified by thiolated carboxylic acids with different chain lengths (to form covalent bond Au–S) and ChT using the carbodiimide method. To investigate the influence of the spacer nature on the kinetics of chemical reaction catalyzed by the ChT, the resulting hybrid nanomaterials were investigated under the influence of non-heating magnetic fields.

2. Materials and methods

2.1. Materials

Iron chloride (II) tetrahydrate ($FeCl_2{\cdot}4H_2O$, 98%), hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4{\cdot}3H_2O$, 98%), iron (III) chloride ($FeCl_3$, anhydrous, 97%), ammonium solution ($NH_3{\cdot}H_2O$, 29%), nitric acid (HNO_3, 69%), hydrochloric acid (HCl, 37%) and 11-mercaptoundecanoic acid (11-MUA, 98%) were obtained from Sigma–Aldrich. Perchloric acid ($HClO_4$, 70%) and L-cysteine (99%) were purchased from Acros Organics, sodium citrate from Reachem (Russia), HS-PEG-COOH (5 kDa) and HS-PEG-OMe (5 kDa) from Nanocs Inc. All water used in experiments was deionized (18.2 MΩ cm^{-1}, Millipore Milli-Q Academic System). All vessels were washed with hot solution of aqua regia and then rinsed with DI water before conducting syntheses.

2.2. Synthesis of Fe_3O_4 NPs

Fe_3O_4 NPs were prepared by co-precipitation of Fe(II) and Fe(III) salts according to the procedure [5].

2.3. Synthesis of gold-coated Fe_3O_4 NPs

Gold-coated magnetite NPs were synthesized by a modified protocol [5,6]. Briefly, 120 ml of $HAuCl_4$ water solution (35 mg of $HAuCl_4{\cdot}3H_2O$) under vigorous stirring was heated under reflux to boiling-state, and 5 ml of freshly prepared Fe_3O_4 NP dispersion was quickly added to $HAuCl_4$ solution. After 10 min, 5 ml of sodium citrate (80 mM) was rapidly added to the reaction mixture. This mixture was boiled under reflux with vigorous stirring for 5 min; the heating was then turned off, and the mixture was cooled to room temperature (r.t.).

2.4. Purification and modification of Fe_3O_4@Au core–shell NPs by organic ligands

The 0.15 g of Sephadex G-100 was mixed with an excess of water and kept overnight at 5 °C and then placed into the empty 12 g chromatography column cartridge (Interchim PF-50SIHP) with a downside filter. All chromatography procedures were made under gravity flow conditions. Sephadex was washed with 50 ml of citrate buffer solution (pH 5.0), and 15 ml of as-prepared Fe_3O_4@Au core–shell freshly sonicated (30 min) NP dispersion was added to the column. After all of the dispersion entered into the Sephadex, the column was washed with 20 ml of citrate buffer to remove the uncovered Fe_3O_4 NPs. Then, the Sephadex gel with purified Fe_3O_4@Au NPs was suspended in 50 ml of an aqueous ligand solution (0.103 μmol of ligand) and stirred overnight. After decantation of the NP solution from Sephadex and dialysis in DI water (3 times in 1 L), the NP solution was purified from any Sephadex traces using 0.22 μm hydrophilic PES filters.

2.5. Immobilization of ChT on Fe_3O_4@Au core–shell NPs

The 0.75 ml of as-purified NP solution (freshly sonicated for 20 min) was mixed with 0.25 ml of citrate buffer (20 mM, pH 5.9). Then, the mixture was supplemented first by 0.7 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.2 mg of *N*-hydroxysulphosuccinimide (Sulpho-NHS) (both used as 10 mg/ml aqueous stock solutions) and second by 600 μl of 10 mg/ml ChT aqueous solution. The final solution was shaken for 2 h at r.t. The last step was the removal of any non-bound enzyme by multiple centrifugal filtrations (1800 × *g*, rotary 100 kDa filters).

2.6. Enzyme activity study

Activity of free or conjugated ChT was determined by UV–vis spectrometry by measuring the rate of enzymatic hydrolysis of a specific substrate, *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTNA). Briefly, 1–2 μl of BTNA solution (17 mg/ml) in a dioxane–acetonitrile mixture (1:1 v:v) was added to 1 ml of 20 mM Tris–HCl buffer, pH 8.2 and then mixed with 2 μl of the free or immobilized enzyme solutions. Formation of the product (*p*-nitroaniline) was recorded at 380 nm over time. The immobilized enzyme activity was calculated and compared with a standard curve of free enzyme activity. The enzymatic hydrolysis measurements followed Michaelis–Menten kinetics, showing a linear dependence on enzyme concentration and hyperbolic dependence on substrate concentration. Substrate concentrations were adjusted to maintain constant hydrolysis rates for time periods required for AC field exposures (approx. 1 h).

2.7. Immobilized enzyme quantity

Total immobilized ChT quantity was determined using a Micro BCA protein assay kit. 150 μl of reagents were added to 150 μl samples, which contained ChT (the measurements were conducted in microplates). The mixture was intensively stirred in the shaker for 1 min and then thermostated at 37 °C for 2 h. After that, the microplate was cooled to r.t. and solution absorbance at 562 nm was measured. The quantity of immobilized enzyme was determined from the linear (in the range of 2–40 μg of enzyme/ml) calibration graph for standard samples. The studied samples were several times diluted.

2.8. Number of free amino groups

Free amino groups on the surface of NPs were determined using 2,4,6-trinitrobenzenesulphonic acid (TNBSA) as described in [7]. 25 μl of 0.1 M sodium tetraborate $Na_2B_4O_7$ in 0.1 M sodium hydroxide NaOH and then 1 μl of 1 M TNBSA solution were added to 25 μl of studied/standard samples (in microplates). The mixture was incubated at 23 °C for exactly 5 min, and then 100 μl of 0.1 M sodium sulphite Na_2SO_3 solution and 0.1 M sodium dihydrogen phosphate NaH_2PO_4 mixture (1:65 v:v) were added to it. After that, the absorbance at 420 nm was measured. The quantity of free amino groups was determined from the linear calibration graph for standard samples.