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Regular Article

Aggregation-resistant nanozyme containing accessible magnetite nanoparticles immobilized in monodisperse-porous silica microspheres for colorimetric assay of human genomic DNA

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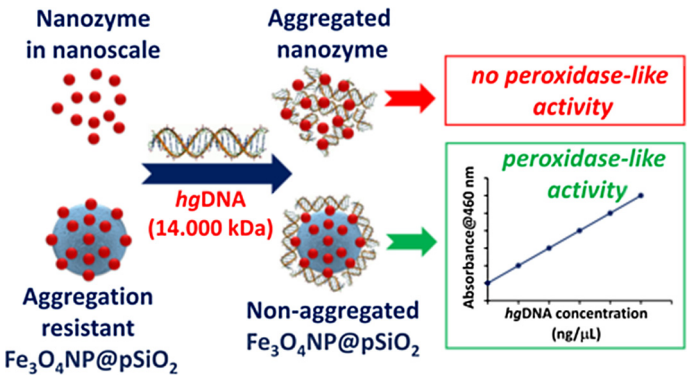
GRAPHICAL ABSTRACT



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ABSTRACT

Bridging-induced aggregation of individual nanozyme particles by long-chain biomacromolecules causes loss of peroxidase mimetic activity for various nanozymes. When a common nanozyme (Fe_3O_4 nanoparticles) was used for colorimetric assay of a long-chain biomacromolecule, human genomic DNA (*hg*DNA, 14.000 kDa, containing 22 kilobases), peroxidase-like activity was absent owing to the irreversible aggregation of Fe_3O_4 nanoparticles in the presence of *hg*DNA. We synthesized an aggregation-resistant nanozyme containing Fe_3O_4 nanoparticles immobilized in 5.1 μm monodisperse-porous silica microspheres. Fe_3O_4 nanoparticles were immobilized in a form accessible to the substrate and large biomolecules in the solution within the monodispersed silica microspheres including both mesopores and macropores. An appreciable and stable spectrophotometric response was obtained, originating from the satisfactorily high peroxidase-like activity of the synthesized nanozyme. No aggregation was observed in the aqueous dispersion of the nanozyme in the presence of *hg*DNA. Large particle size, low particle number density, high surface area, and the presence of macropores were evaluated as factors contributing to the adsorption of *hg*DNA chains onto the synthesized nanozyme without interparticle bridge formation between the individual microspheres causing aggregation. Here, for the first time a colorimetric assay was developed based on the enhancement of peroxidase-mimetic activity of non-aggregated porous silica microspheres to determine *hg*DNA concentration up to 300 ng/μL. *hg*DNA could be also isolated with 47% yield and an equilibrium *hg*DNA adsorption of 19.000 ng/mg, using the same nanozyme. Hence, a material acting as a

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nanozyme for colorimetric determination of *hg*DNA was also evaluated as a magnetic, solid phase extraction sorbent for the first time.

1. Introduction

The intrinsic peroxidase-like activity was first observed for Fe_3O_4 nanoparticles in 2007 [1]. Gao *et al.* reported that Fe_3O_4 nanoparticles possessed an intrinsic enzyme mimetic activity similar to that found in natural peroxidases, which were widely used to oxidize organic substrates as detection tools [1]. Noble metal or transition metal oxide-based nanomaterials exhibiting intrinsic peroxidase-like activity were also evaluated for colorimetric determination of various biomolecules such as glucose, H_2O_2 and nucleic acids [2,3].

DNA is an important agent that has been effectively used for controlling the peroxidase-like activities of various nanomaterials [4–17]. Colorimetric sensing of single nucleotide polymorphisms was performed using the peroxidase-like activities of single-walled carbon nanotubes and hemin–graphene hybrid nanosheets [4,5]. Self-assembled, functionalized graphene was used for DNA detection in the presence of Hg (II) [6]. DNA-mediated inhibition of peroxidase-like activity on platinum nanoparticles was evaluated for sensing of nucleic acids [7]. Recently, fluorescent DNA probing was achieved using nanoscale MnO_2 [8]. DNA assays based on composite nanozymes were developed by immobilization of AuNPs onto different supports such as metal organic frameworks or graphene [9–11]. Composite nanostructures, including magnetic porous nanocomposites, Ag/Pt bimetallic nanoclusters, and mesoporous silica loaded with platinum nanoparticles, were also successfully used for nucleic acid assays that were developed based on their peroxidase-like activities [12–14]. Fe_3O_4-based nanomaterials such as underivatized Fe_3O_4 nanoparticles, three-dimensional graphene/Fe_3O_4–AuNPs, and Fe_3O_4-AuNPs anchored two-dimensional metal–organic framework nanosheets were recently evaluated for sensing of single stranded DNA (*ss*DNA) oligomers and their double stranded forms [15,16]. In all DNA colorimetric assays developed based on peroxidase mimicking activity of nanozymes designed either in the form of underivatized nanostructures or composite nanostructures, DNA oligomers possessing short chain lengths (i.e. mostly oligonucleotides containing up to 30 bases) have been selected to demonstrate the colorimetric DNA detection performance of selected artificial enzymes [4–17]. An appreciable enhancement in the peroxidase-like activity by the adsorption of short chain DNA oligomers was observed using different composite nanomaterials including Fe_3O_4 nanoparticles, graphene supported bimetallic nanocomposites, and Fe_3O_4-AuNPs anchored two-dimensional metal-organic framework nanosheets [15–17].

The inhibition of peroxidase-like activity by the target molecule provides another tool for the colorimetric detection of various biomolecules. Colorimetric assays for target molecules capable of triggering nanozyme aggregation were developed based on the decrease of peroxidase-like activity in relation to target molecule concentration when the aggregation took place under controlled conditions [18–21]. PtNP-catalyzed oxidation of TMB was efficiently suppressed by heparin-triggered aggregation of protamine-coated PtNPs [18]. In the presence of polyphenols, the peroxidase-like activity of protein-conjugated gold nanoclusters was restrained, and this was due to polyphenol-induced aggregation [19]. Cysteine also induced the aggregation of AuNPs, resulting in the reduction of their peroxidase-like activity [20]. Peroxidase-mimicking activity of PdNPs was also significantly inhibited by the aggregation induced by binding of Ag+ ions onto the nanoparticles [21].

In contrast, various large-size biomolecules, such as DNA and proteins, have been demonstrated to be effective for suppressing the catalytic activity of peroxidase-like nanomaterials [22]. Our preliminary studies also demonstrated that the irreversible, extensive aggregation of nanomaterials with peroxidase-like activity was particularly observed in the presence of long-chain, large biomolecules such as *hg*DNA. The complete disappearance of oxidase/peroxidase-mimetic activity due to an irreversible, extensive aggregation induced by clumping of nanozyme particles by the long-chain target biomolecules (i.e. agglutination) is a drawback preventing the colorimetric determination of long-chain, large biomolecules through the use of nanozymes designed in the form of functional nanoparticles. The high number density of currently available, low-sized nanozyme particles and the limited external surface area of nanozyme are likely responsible for irreversible clumping of nanozyme particles induced by the long-chain biomolecules. The formation of large nanozyme-biomolecule aggregates possessing very limited surface area and the blockage of the enzyme-mimetic active sites by the adsorbed biomolecule chains are likely to be largely responsible for reducing or destroying the peroxidase-mimetic activity in the presence of a large-size, target biomolecule.

In this work, a composite structure containing Fe_3O_4 nanoparticles immobilized in the accessible form within monodisperse-porous silica microspheres exhibiting bimodal pore-size distribution (Fe_3O_4NP@pSiO_2) was proposed as a new type of nanozyme that is resistant against aggregation and suitable for the colorimetric determination of large-size biomolecules. Here, we report the peroxidase-mimetic activity behavior of the proposed nanozyme in the absence and presence of selected long-chain biomolecule, *hg*DNA and the first colorimetric assay of *hg*DNA based on the proposed nanozyme that could be also utilized for similar large-sized biomolecules.

2. Experimental section

2.1. Materials

Tetraethyl orthosilicate (TEOS), ammonium hydroxide (containing 25%–28% w/w NH_3), 2-propanol (IPA), tetrabutylammonium iodide (TBAI), Tris-HCl, and EDTA were obtained from Aldrich Chem. Co., U.S.A. Human genomic DNA (*hg*DNA, Deoxyribonucleic acid sodium salt from human placenta, D7011, Supplier's information: Molecular weight: 14.000 kDa, 22 kilobases, prepared from human placental tissue, DNA from human placenta is 42.0 mol% G-C and 58.0 mol% A-T) was obtained from Sigma-Aldrich Co., U.S.A. Guanidium hydrochloride (Gu-HCl), o-phenylenediamine (OPDA), HCl solution (37% w/w), and H_2O_2 solution (50% w/w) were supplied by Sigma-Aldrich Chem. Co. Glucose, glutathione, alanine, histidine, albumin from bovine serum, RNA from torula yeast type VI, NaCl and KCl were also obtained from Sigma-Aldrich Chem. Co.

2.2. Synthesis of Fe_3O_4NP@pSiO_2 microspheres

"Fe_3O_4NP@pSiO_2 microspheres" were synthesized by using a modification of the "*staged shape template hydrolysis-condensation*

method" that was previously used for the synthesis of starting material for Ti(IV) attached-magnetic SiO_2 microspheres as an immobilized metal affinity chromatography sorbent for phosphopeptide purification [23]. The schematic representation of the synthesis protocol is given in Fig. S1 of the Supporting Information. In the present study, the synthesis protocol was modified to increase the magnetite content and by enhancing the porous properties of microspheres for obtaining a nanozyme with appropriate peroxidase-like activity. The characterization methods used for the synthesized Fe_3O_4NP@pSiO_2 microspheres are also given in Section 2 of the Supporting Information.

2.3. Determination of peroxidase-mimetic activity of Fe_3O_4NP@pSiO_2 microspheres

The peroxidase-like activity of Fe_3O_4NP@pSiO_2 microspheres was monitored against time in a reaction medium that included o-phenylenediamine (OPDA) and H_2O_2, by the formation of 2,3-diaminophenazine (DAP). Typically, Fe_3O_4NP@pSiO_2 microspheres (10 mg) were dispersed in 1× Tris-EDTA (TE) buffer (100 mM, 4.0 mL, pH 5.0 or 6.0) containing OPDA at concentrations ranging between 10 μM and 400 μM. The zero reaction time was defined when H_2O_2 solution (2 μL, 50% w/w) was added into the reaction medium. The enzyme-mimicking reaction proceeded for a given time in the dark at room temperature, by rotating the reaction tube at 100 rpm. In these runs, the reaction period varied between 5 min and 180 min, and a separate reaction medium was used for each reaction period. At the end of each period, Fe_3O_4NP@pSiO_2 microspheres were separated from the reaction medium using an external magnet. After removal of microspheres, the absorbance of the reaction medium was measured by a UV–Vis spectrophotometer (Hitachi, Model 230, Japan) at 416 nm. This allowed the data for the variation of absorbance at a given time to be collected for different initial OPDA concentrations. The initial OPDA consumption rate ($-r_{OPDA}$) for a given initial OPDA concentration (C_o) was calculated according to the method provided in Section 3 of the Supporting Information.

2.4. Interaction of Fe_3O_4NP@pSiO_2 microspheres with hgDNA

The experimental procedures followed for determination of adsorption/desorption behavior of *hg*DNA in the presence of Fe_3O_4-NP@pSiO_2 microspheres are given in Section 4 of the Supporting Information.

2.5. Enhancement of peroxidase-like activity in the presence of hgDNA

Typically, Fe_3O_4NP@pSiO_2 microspheres (10 mg) were dispersed into 100 μM 1× TE buffer at pH 5.0 or 6.0 (3.0 mL) containing *hg*DNA at a concentration ranging between 50 ng/μL and 300 ng/μL. The medium was rotated at 100 rpm for 1 h at room temperature for the adsorption of *hg*DNA onto Fe_3O_4NP@pSiO_2 microspheres. At the end of this period, OPDA stock solution (i.e. 1.0 mL, 200 or 400 μM, 1× TE buffer at pH 5.0 or 6.0) was added to obtain an initial OPDA concentration of 50 or 100 μM within the reaction medium. The enzyme-mimicking reaction then continued at a rotating rate of 100 rpm at room temperature for 1 h in the presence of *hg*DNA adsorbed onto Fe_3O_4NP@pSiO_2 microspheres. This period was determined by preliminary runs as a sufficient time for obtaining a plateau absorbance for the reaction medium at 416 nm after separation of Fe_3O_4NP@pSiO_2 microspheres by an external magnet. To boost the absorbance signal obtained at the end of the reaction period, a concentrated HCl solution (3 N, 100 μL) was added into the reaction solution, and the absorbance was again measured at 460 nm after a period of 10 min.

3. Results and discussion

3.1 Characterization of Fe_3O_4NP@pSiO_2 microspheres

The SEM photographs of Fe_3O_4NP@pSiO_2 microspheres and their size and porous properties are presented in Fig. 1 and Table S1 of the Supporting Information, respectively. As seen in Fig. 1A, Fe_3O_4NP@pSiO_2 microspheres of 5.1 μm in average size were obtained with a narrow size distribution (i.e. coefficient of variation for size distribution (CV) <5%) (Table S1). The porous character of microsphere surface-containing Fe_3O_4 nanoparticles is indicated by the SEM photographs taken with high magnifications (Fig. 1B and 1C).

The proposed synthesis route provided in Fig. S1 allowed the immobilization of magnetic Fe_3O_4 nanoparticles with peroxidase-like activity in the microspheres with a bimodal pore-size distribution, including both mesoporous and macroporous compartments lying between 4 nm and 140 nm and possessing a mean pore size of 41 nm (Fig. S2A and Table S1). The peaks of Fe_3O_4 and SiO_2 phases were clearly observed in the X-ray diffraction spectrum of Fe_3O_4NP@pSiO_2 microspheres (Fig. S2B). Sufficiently high surface area (222 m^2/g, Table S1) originating from the mesoporous compartment of silica microspheres allowed the immobilization of a sufficiently high number of Fe_3O_4 nanoparticles onto the surface of the pores within the microsphere interior. Fe_3O_4NP@pSiO_2 microspheres exhibited superparamagnetic behavior with a satisfactorily high saturation magnetization (i.e. 28.3 emu/g) due to the magnetic Fe_3O_4 nanoparticles immobilized within porous silica matrix (Fig. S2C). The microspheres could be separated in ca. 3 s when an aqueous dispersion of microspheres (3 mL, microsphere concentration: 25 mg/mL) was exposed to an external magnet (inset photo in Fig. S2C).

To provide an idea of the presence of Fe_3O_4 nanoparticles in the porous silica microspheres, TEM images of Fe_3O_4NP@pSiO_2 microspheres and bare SiO_2 microspheres prepared by a similar synthesis protocol without the formation of magnetic Fe_3O_4 nanoparticles [24] are comparatively illustrated in Fig. S3. Fe_3O_4 nanoparticles immobilized in the porous silica microspheres were observed in the form of black regions (Fig. S3A), while a grey-dominant view was obtained from imaging the bare SiO_2 microspheres (Fig. S3B).

3.2. Peroxidase-mimetic activity of Fe_3O_4NP@pSiO_2 microspheres

The peroxidase-like activity of Fe_3O_4NP@pSiO_2 microspheres was investigated using bare Fe_3O_4 nanoparticles ca 15 nm in size (i.e. determined by TEM, data not shown) as the reference nanozyme in the presence of *hg*DNA at a concentration of 150 ng/μL. In these runs, *hg*DNA was first adsorbed onto both types of nanozymes, and their peroxidase activities were comparatively monitored against time after the addition of OPDA and H_2O_2 into the adsorption medium. The absorbance of the reaction medium was recorded at 416 nm after separating each nanozyme from the reaction medium by an external magnet at different reaction times. The variation of absorbance over time is given in Fig. 2A for both reaction media containing Fe_3O_4NP@pSiO_2 microspheres and bare Fe_3O_4 nanoparticles. The optical micrographs of aqueous dispersions of Fe_3O_4 nanoparticles and Fe_3O_4NP@pSiO_2 microspheres containing *hg*DNA are provided in Fig. 2B and C, respectively. No appreciable aggregation was observed with Fe_3O_4NP@pSiO_2 microspheres in the presence of *hg*DNA (Fig. 2C), while large aggregates could be easily seen in the optical micrograph of the aqueous dispersion of bare Fe_3O_4 nanoparticles containing *hg*DNA (Fig. 2B). The aggregation of bare Fe_3O_4 nanoparticles in the presence of *hg*DNA could be also observed by a non-homogeneous view of the aqueous dispersion including large aggregates in the liquid part

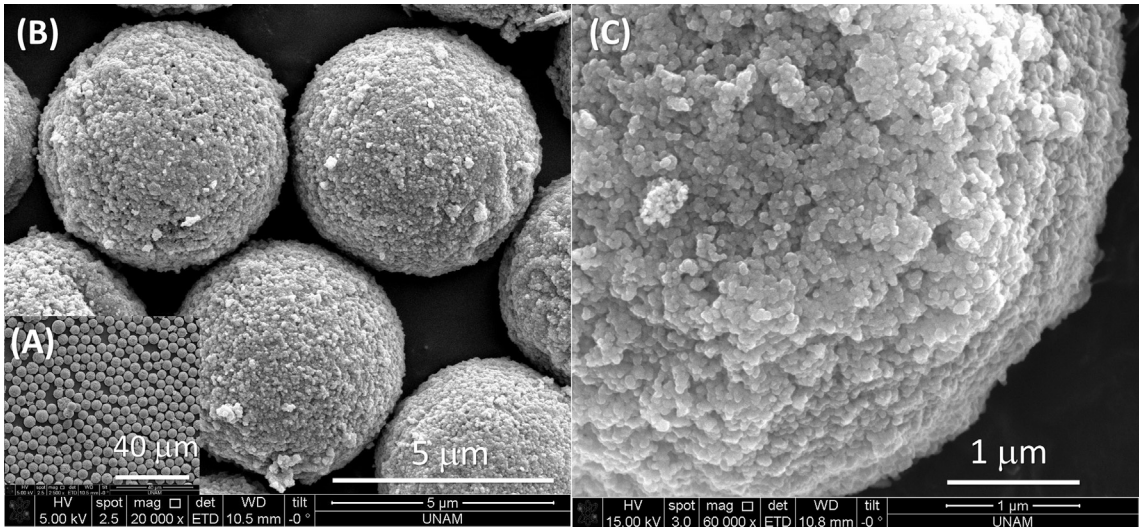

Fig. 1. SEM photographs of Fe_3O_4NP@pSiO_2 microspheres, Magnification: (A) ×2500 (B) ×20.000, (C) ×60.000.

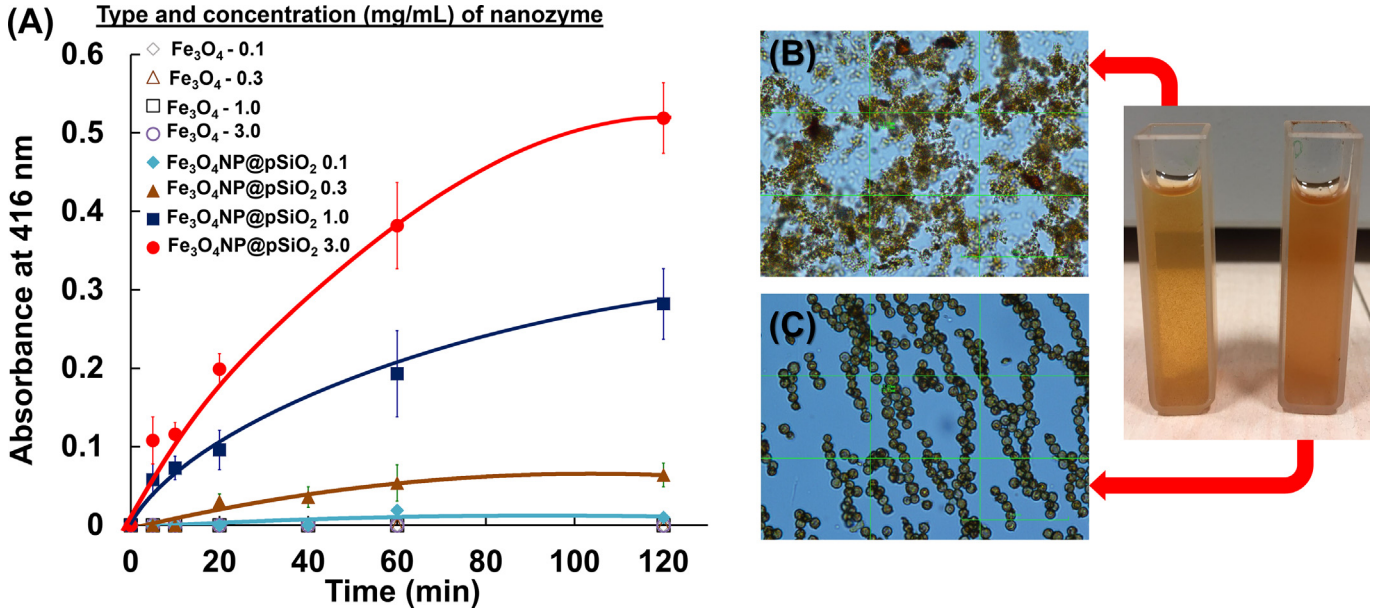

Fig. 2. (A) The variation of absorbance of reaction medium at 416 nm over time in the presence of *hg*DNA by using bare Fe_3O_4 nanoparticles or Fe_3O_4NP@pSiO_2 microspheres as the nanozyme. *hg*DNA concentration: 150 ng/µL (10.7 nM). The concentration of both nanozymes varied between 0.1 mg/mL and 3.0 mg/mL in a reaction volume of 4.0 mL at pH 5.0. OPDA concentration: 100 µM, 100 rpm, Room temperature. (B) The optical micrograph of the aqueous dispersion containing bare Fe_3O_4 nanoparticles (3 mg/mL) and *hg*DNA (150 ng/µL). (C) The optical micrograph of the aqueous dispersion containing Fe_3O_4NP@pSiO_2 microspheres (3 mg/mL) and *hg*DNA (150 ng/µL). Magnification: ×400.

and a precipitate that formed at the bottom of the tube (i.e. the left tube in the photo).

As seen in Fig. 2A, although the concentration of Fe_3O_4 nanoparticles was increased in the range of 0.1–3.0 mg/mL, almost zero absorbance was recorded at 416 nm with all reaction times up to 2 h for all Fe_3O_4 concentrations. This behavior indicated that no significant peroxidase activity was obtained using the bare Fe_3O_4 nanoparticles in the reaction medium containing *hg*DNA. The drastic decrease in the surface area of the nanozyme that was dependent upon the aggregation induced by the attachment of nanozyme particles onto the *hg*DNA chains and the prevention of the interaction between nanozyme and substrate by the *hg*DNA chains adsorbed onto the nanozyme particles were both evaluated as possible reasons for the loss of peroxidase-mimetic activity in the presence of *hg*DNA. These two factors are likely responsible for the use of DNA oligomers possessing limited chain lengths (i.e. the oligonucleotides mostly containing up to 30 bases) in the studies detailing the colorimetric determination of DNA using nanozymes in the form of underivatized or composite nanoparticles [4–17]. In a recent study, DNA methylation was assayed using genomic DNA via peroxidase-mimicking activity of mesoporous iron oxide nanoparticles [25]; however, the colorimetric detection was performed after binding of the antibody bound-nanozyme onto the genomic DNA immobilized on an electrode [25]. Specifically, genomic DNA was not present in the solution used as a detection medium containing iron oxide-based nanoparticles as the nanozyme [25].

For a given nanozyme concentration defined in terms of mass of nanozyme per volume of reaction medium, the number of particles per volume of reaction medium was extremely lower for

Fe_3O_4NP@pSiO_2 microspheres that were 5.1 μm in size compared to Fe_3O_4 nanoparticles ca. 15 nm in size. Given this, the interparticle binding of *hg*DNA molecules (i.e. binding of a single *hg*DNA chain onto more than one particle) and subsequent particle aggregation was substantially eliminated by the use of Fe_3O_4NP@pSiO_2 microspheres as the nanozyme. Additionally, in the case of Fe_3O_4NP@pSiO_2 microspheres, the adsorbed *hg*DNA molecules were predominantly located within the porous interior of the microspheres due to their extremely higher specific surface area that originated from mesopores and their macropores allowing the facile intraparticle diffusion of *hg*DNA. Hence, an appreciable peroxidase-like activity in the presence of *hg*DNA was obtained using the proposed nanozyme, by the elimination of particle aggregation.

The hydrodynamic size of *hg*DNA in the aqueous buffer media was determined in the absence and presence of chaotropic salt (Gu-HCl) by dynamic light scattering (Fig. 3 and Section 6 of Supporting Information). The hydrodynamic size of *hg*DNA increased as pH increased in the absence of chaotropic salt, and hydrodynamic size values of 370–820 nm were obtained in the pH range of 4.0–8.5. The aggregation observed with bare Fe_3O_4 nanoparticles in Fig. 2B likely occurred by linking of individual Fe_3O_4 nanoparticles, ca 15 nm in size with large *hg*DNA chains possessing an almost 45-fold higher hydrodynamic size at pH 5.0 (ca. 680 nm). The high particle number density of Fe_3O_4 nanoparticles in the aqueous medium is a factor that allows them to more easily interact with the *hg*DNA chains. The mean size of Fe_3O_4@pSiO_2 microspheres (5.1 μm), however, was almost 7.5 times higher with respect to the hydrodynamic size of *hg*DNA chains at the same pH. The bridging of large Fe_3O_4@pSiO_2 microspheres by smaller *hg*DNA chains should be much more difficult when the lower particle number density of Fe_3O_4@pSiO_2 microspheres in the aqueous medium is considered.

To the best of our knowledge, no study examining the colorimetric assay of a large biomolecule such as *hg*DNA by the use of a nanozyme in the nanoscale has been performed [4–17]. Previous studies have, however, examined silica based composite nanozymes that were synthesized by immobilizing noble metal nanoparticles on non-porous silica nanoparticles/nanostructures or into mesoporous silica nanoparticles possessing mean size values lower than 150 nm [14,26–28]. In a recent study, a composite nanozyme similar to Fe_3O_4NP@pSiO_2 microspheres was synthesized by the immobilization of "platinum nanoparticles with peroxidase-like activity, also accessible for substrate and DNA", within mesoporous silica nanoparticles that were 140 nm in size

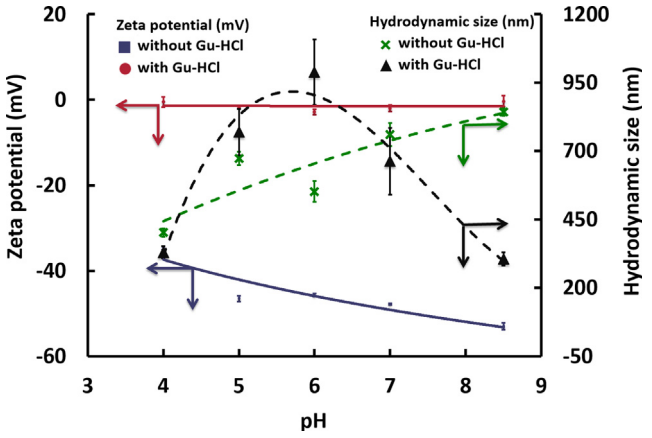

Fig. 3. The variation of hydrodynamic size and zeta-potential of *hg*DNA with medium pH. *hg*DNA concentration: 250 ng/μL.

[14]. Both the mean particle size and the mean pore size of this nanozyme were, however, designed according to the colorimetric detection of *ss*DNA oligomers up to 21 bases in length [14]. To perform colorimetric detection of a large biomolecule such as *hg*DNA, a nanozyme with a mean particle size in the micron-size range that provides a low particle number-density in the solution is necessary to prevent the extensive aggregation of nanozyme in the presence of *hg*DNA. Additionally, a much larger mean pore size and the presence of macropores are also necessary to make the immobilized enzyme-mimicking nanoparticles available for a large molecule like *hg*DNA within the porous silica microspheres. In our case, iron oxide nanoparticles exhibiting peroxidase-like activity were immobilized in the accessible form within monodisperse silica microspheres 5.1 μm in size that contained both macropores and mesopores.

Bare silica or magnetic silica nanoparticles/microspheres are also known as the most widely used sorbents for the isolation of various single stranded DNA or double stranded DNA (*ss*DNA or *ds*DNA) samples (i.e. oligonucleotides, their hybridized forms, plasmid DNA and genomic DNA) via solid phase microextraction protocols involving successive adsorption-elution stages [29–32].Typical DNA microextraction sorbents have been synthesized by the encapsulation of magnetic iron oxide nanoparticles into a nonporous or mesoporous silica shell [29–32]. In these sorbents, however, Fe_3O_4 nanoparticles were immobilized within a silica shell only to provide magnetic properties to the synthesized sorbent and not in the accessible form for the large biomolecules found in the liquid part of the isolation medium [29–32].

The synthesis method of Fe_3O_4NP@pSiO_2 microspheres made possible the tight immobilization of iron oxide nanoparticles with two separate functions (i.e. peroxidase mimicking activity and superparamagnetic behavior) on the large surface area of the mesoporous compartment of silica microspheres. Specifically, Fe_3O_4 nanoparticles were immobilized in the accessible form, both for the substrate and large biomolecules found in the solution within the monodisperse silica microspheres including both mesopores and macropores. This property highlights the superiority of monodisperse Fe_3O_4NP@pSiO_2 microspheres with respect to currently available "composite nanozymes and magnetic DNA purification sorbents" designed by using non-porous or mesoporous silica nanoparticles as the support material [29–32]. By considering the unique property of synthesized material in terms of aggregation behavior and peroxidase-like activity, "monodisperse-porous silica microspheres containing magnetic and enzyme-mimetic Fe_3O_4 nanoparticles in the accessible form" were evaluated as a new nanozyme with peroxidase-like activity for sensing of *hg*DNA via a colorimetric protocol.

The effect of pH on the peroxidase-like activity of nanozyme was studied in the absence of *hg*DNA using OPDA as the synthetic substrate (Fig. S4 of the Supporting Information). As seen in Fig. S4, the peroxidase-like activities at pH 4.0 and 5.0 were very similar and indicated the highest values. A slight decrease was observed in the peroxidase-like activity at pH 6.0. The effect of initial OPDA concentration on the peroxidase-like activity of Fe_3O_4NP@pSiO_2 microspheres was studied at pH 5.0 and 6.0, as those were determined as the pH values providing the highest equilibrium *hg*DNA adsorptions onto the microspheres (as will be shown in Fig. S5 of the Supporting Information) and also higher peroxidase-like activities. The variation of peroxidase-like activity of nanozyme with the initial concentration of OPDA is given in Fig. 4. K_m and r_{max} of the nanozyme were determined according to the Lineweaver–Burk plot and are given in Table S2 of the Supporting Information with the related correlation coefficients. As seen here, lower K_m and higher r_{max} were obtained for the reaction medium at pH 5.0 compared to those determined for pH 6.0.

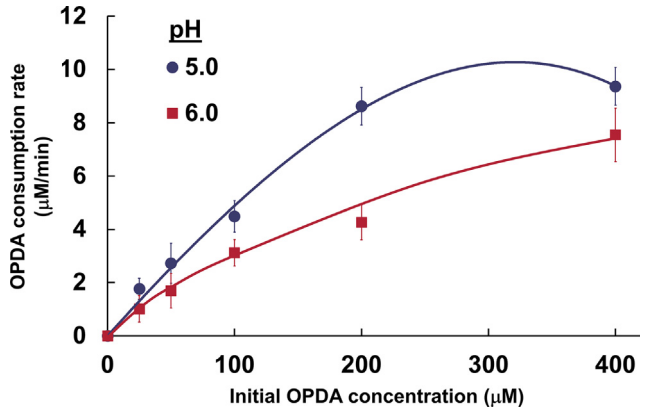

Fig. 4. The variation of peroxidase-like activity of nanozyme with the initial concentration of OPDA, Fe_3O_4NP@$pSiO_2$ microspheres: 2.5 mg/mL, H_2O_2 added: 2 μL, 50% w/w, 4 mL, 1× TE buffer at pH 5.0 or 6.0, 1 h, 100 rpm, Room temperature.

3.3. hgDNA adsorption desorption behavior of the proposed nanozyme

The highest equilibrium *hg*DNA adsorptions were obtained at pH 5.0 and 6.0 (Fig. S5 of the Supporting Information), which was in agreement with previous reports detailing similar silica-based sorbents utilized for DNA purification [24,29–32]. The effects of initial *hg*DNA concentration in the adsorption medium on equilibrium *hg*DNA adsorption at pH 6.0 and the desorption yield obtained in the absence/existence of chaotropic salt at pH 8.5 are provided in Fig. 5.

The negative charge of *hg*DNA significantly decreases in the pH range of 4.0–8.5 by the addition of chaotropic salt (Fig. 3). According to the mechanism proposed for DNA adsorption onto silica under chaotropic conditions, the chaotropic salt effectively shields the negative charge of DNA (i.e. Fig. 3) and also silica, and it weakens the repulsive electrostatic forces between them [33]. This presumably makes the DNA-silica interaction more favorable under acidic conditions, as the isoelectric points of DNA and silica are 5 and 1.5 ± 3.6, respectively [34]. The contribution of intermolecular hydrogen bonding between DNA and silanols to the driving force for adsorption was more effective in the absence of chaotropic salt [33,34]. Contrary to this behavior, the shielded intermolecular electrostatic forces and the dehydration effect were the factors controlling the adsorption conducted in the presence of the chaotropic salt [34]. On the other hand, the negatively-charged silica surface electrostatically repels the negatively charged phosphate backbone of *hg*DNA at pH 8.5, which is higher than the first silanol p*Ka* (4.5) and equal to the second p*Ka* (8.5) [34].

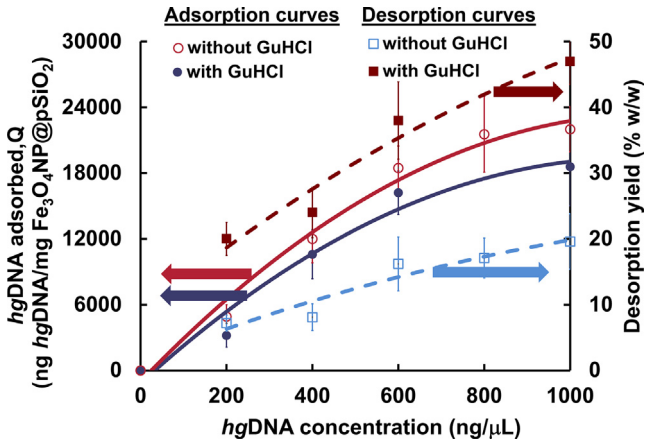

Fig. 5. The effect of initial *hg*DNA concentration on *hg*DNA adsorbed onto the nanozyme in the absence and presence of chaotropic salt (6 M GuHCl) and desorption yield, Adsorption conditions: Fe_3O_4NP@$pSiO_2$ microspheres: 2.5 mg/mL, 4 mL, 1× TE buffer at pH 6.0, 1 h, 100 rpm, Room temperature, Desorption conditions: 4 mL, 1× TE buffer at pH 8.5, 1 h, 100 rpm, Room temperature. Desorptions were applied after the adsorptions performed with or without chaotropic salt.

The higher desorption yields obtained after the adsorptions in the presence of chaotropic salt is explained by the effective removal of *hg*DNA adsorbed by means of shielded intermolecular electrostatic forces and the dehydration effect caused by the electrostatic repulsion forces activated at the desorption pH (i.e. 8.5) (Fig. 5). Specifically, the reduction of intermolecular hydrogen bonding between *hg*DNA and sorbent in the adsorption stage due to the high concentration of chaotropic salt is likely the reason for this behavior. Conversely, the electrostatic repulsion forces at pH 8.5 were less effective for the desorption of *hg*DNA adsorbed via intermolecular hydrogen bonding in the absence of chaotropic salt at acidic pH. Hence, lower desorption yields could be obtained after the adsorptions are performed in the absence of chaotropic salt.

Satisfactorily high equilibrium *hg*DNA adsorptions obtained in the presence of chaotropic salt and also satisfactorily high desorption ratios indicated that Fe_3O_4NP@$pSiO_2$ microspheres synthesized as a nanozyme could be also utilized as a magnetic sorbent for isolation of *hg*DNA via solid phase microextraction when the similar sorbents were considered [24,29–32]. This situation can likely be explained by the bimodal pore-size distribution of Fe_3O_4-NP@$pSiO_2$ microspheres. The mesopores provide sufficiently high surface area for parking of *hg*DNA molecules, and the macropores facilitate the diffusion of *hg*DNA molecules within Fe_3O_4NP@$pSiO_2$ microspheres during both adsorption and desorption.

3.4. Effect of hgDNA concentration on the peroxidase-like activity of nanozyme

In principle, *hg*DNA concentration can be determined according to the change of peroxidase-like activity proportional to the extent of *hg*DNA adsorbed onto a nanozyme. The highest peroxidase-like activity was obtained at pH 5.0 in the absence of *hg*DNA (Fig. S4), and the highest equilibrium *hg*DNA adsorption onto the nanozyme was observed at pH 6.0 (Fig. S5). Given these observations, the effect of *hg*DNA concentration on the peroxidase-like activity of nanozyme was examined at both pH 5.0 and 6.0. After determination of appropriate pH values for colorimetric determination of *hg*DNA, UV–Vis spectra of the solutions prepared with different *hg*DNA concentrations in the range of 50–300 ng/μL were recorded. Some of these are listed in Fig. S6 of the Supporting Information. As seen here, the absorbance maximum was obtained at a wavelength of 416 nm for the majority of the *hg*DNA concentrations at the studied pH. The effect of *hg*DNA concentration on the peroxidase-like activity of nanozyme is shown in Fig. 6. The absorbance of the reaction medium measured at 416 nm after the completion of colorimetric conversion in the presence of *hg*DNA was used in Fig. 6A. In these runs, 100 μM was selected as the maximum initial OPDA concentration, as the final absorbance of the reaction medium measured at 416 nm could be kept below 1.0 at this concentration. As indicated in Fig. 6A, a clear enhancement in the peroxidase-like activity of the nanozyme was observed at both pH values using the selected initial OPDA concentrations.

A similar enhancement in peroxidase-like activity was previously observed using bare Fe_3O_4 nanoparticles as a nanozyme with 3,3′,5,5′-tetramethylbenzidine (TMB) as the substrate in the presence of *ss*DNA oligomers 5–30 bases in length [15]. One explanation is provided by the theory previously proposed for the interaction of positively and negatively charged synthetic substrates, TMB and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic

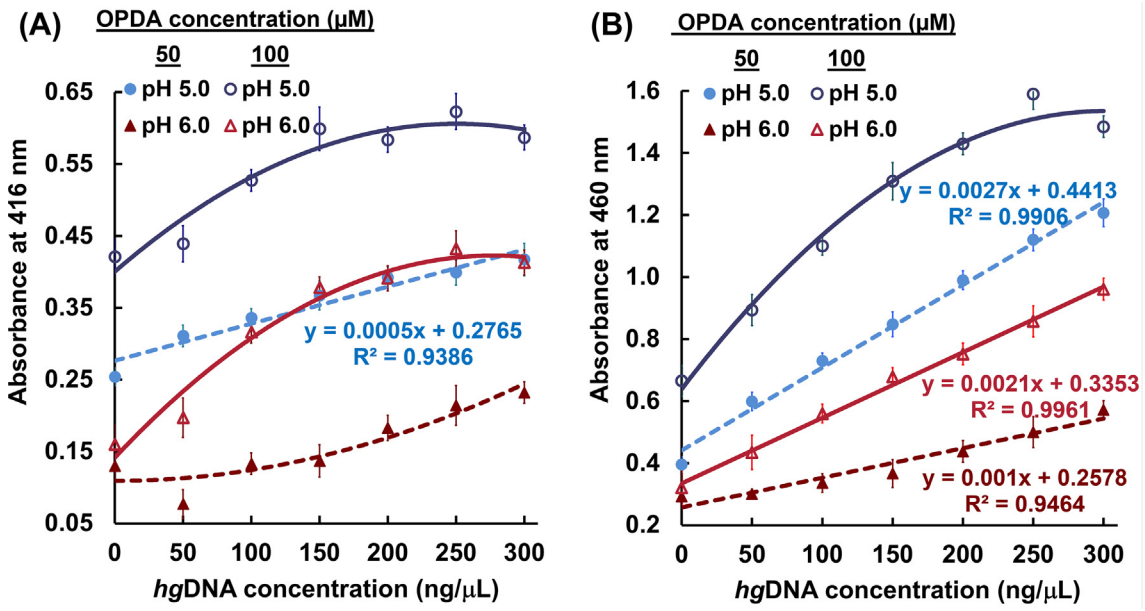

Fig. 6. The effect of *hg*DNA concentration on peroxidase-like activity of nanozyme. (A) The variation of absorbance at 416 nm at different *hg*DNA concentrations after completion of colorimetric conversion. *hg*DNA adsorption: 1 h, 100 rpm and room temperature, Fe_3O_4NP@pSiO_2 microspheres: 2.5 mg/mL, Colorimetric reaction: OPDA concentration: 50 and 100 μM, H_2O_2 added: 2 μL, 50% w/w, 4.0 mL, 1× TE buffer at pH 5.0 or 6.0, 1 h, 100 rpm, room temperature, (B) The variation of absorbance at 460 nm at different *hg*DNA concentrations by the addition of aqueous HCl to boost the colorimetric response after completion of colorimetric conversion.

acid) (ABTS), respectively, with the bare Fe_3O_4 nanoparticles in the presence of DNA oligomers 5–30 bases in length at pH 4.0 [15]. A clear enhancement was observed with the positively charged substrate (i.e. TMB) in the presence of *ss*DNA oligomer, and the peroxidase-like activity was inhibited when the negatively charged substrate (i.e. ABTS) was utilized [15]. In our case, OPDA has two p*Ka* values, where p*Ka*1 is less than 2 and p*Ka*2 is 4.47 [35]. Hence, OPDA should exist in the protonated form at the pH values studied (i.e. 5.0 and 6.0). This property should be the reason of its affinity for *hg*DNA. The *hg*DNA adsorbed onto Fe_3O_4NP@pSiO_2 microspheres may facilitate this adsorption and the subsequent interaction of OPDA with the Fe_3O_4 nanoparticles immobilized on the surface of the pores within the microspheres.

The absorbance of the reaction medium measured at 460 nm after the addition of aqueous HCl into the reaction medium to boost the colorimetric response after completion of the colorimetric reaction is given in Fig. 6B. Specifically, the absorbance maxima obtained by the completion of colorimetric conversion was shifted from 416 to 460 nm by the addition of aqueous HCl. As seen in Fig. 6B, the linear tendencies that could be utilized for estimation of *hg*DNA concentration in the range of 0–300 ng/μL (i.e. 0–21.5 nM) were obtained with OPDA concentrations of 50 μM at both pH and 100 μM at pH 6.0. The linear equations possessing satisfactorily high correlation coefficients were also included for these cases (Fig. 6B). The limit of detection (LOD) values were calculated using the linear equations describing the relationship between absorbance and *hg*DNA concentration both in the absence and presence of HCl. The lowest LOD value was determined as 16 ng *hg*DNA/μL (i.e. 1.1 nM) for the colorimetric determinations performed with the initial OPDA concentration of 50 μM at pH 5.0 by the addition of aqueous HCl.

The variation of visible region absorbance with changes in *hg*DNA concentration was monitored up to a *hg*DNA concentration of 300 ng/μL, as turbid solutions were obtained by the addition of aqueous HCl into the reaction medium containing *hg*DNA at concentrations higher than 300 μg/μL. Both the linearity range of 0.0–21.5 nM and the detection limit of 1.1 nM are comparable values to those found in the colorimetric assay studies performed using short-chain DNA oligomers using nanozymes (with particle sizes in nanoscale) such as Au nanoparticles, graphene, graphene/AuNP hybrid nanomaterials, single-walled carbon nanotubes, platinum nanoparticle loaded-mesoporous silica nanoparticles, and Fe_3O_4 nanoparticles [4–6,9–11,14,15].

The linearity of the relationship between absorbance and *hg*DNA concentration obtained at pH 5.0 was tested by either changing the salt concentration or by adding different biomolecules into the determination medium. As seen in Fig. S7A, no significant change occurred in either the slope or the intersection point of the straight-line obtained at pH 5.0 under increasing NaCl concentrations. The straight line obtained using KCl, however, exhibited a slightly lower slope with respect to colorimetric reaction medium containing no salt. Here, the salt concentration of 150 mM was selected as a representative value for blood level. In Fig. S7B, no significant change was observed in the slope and the intersection point of the straight-line obtained at pH 5.0 by the addition of certain small biomolecules such as amino acids (i.e. alanine and histidine), glucose, and glutathione. Here, the biomolecule concentrations were selected as the values close to the blood or plasma levels of the corresponding molecules. In the presence of macromolecules, however, the intersection point obtained at pH 5.0 decreased with RNA and increased with albumin. The linearity of the relationship between absorbance and *hg*DNA concentration was protected in all cases, and the slope of the straight line was nearly identical and was not affected by the type of the biomolecule in the determination medium (Fig. S7B).

4. Conclusion

No significant peroxidase-mimetic activity could be obtained using the bare Fe_3O_4 nanoparticles in the aqueous dispersion containing *hg*DNA due to the aggregation induced by bridging of Fe_3O_4 nanoparticles by the solubilized *hg*DNA chains. As a solution, a new kind of nanozyme that is resistant against aggregation and contains accessible Fe_3O_4 nanoparticles immobilized in monodisperse-porous silica microspheres 5.1 μm in size was synthesized. In a manner dependent upon the large particle size

providing low particle number density, the high mesoporous surface area providing a large parking area for *hg*DNA, and the presence of macropores allowing diffusion of *hg*DNA in the microspheres, *hg*DNA could be satisfactorily adsorbed onto the nanozyme without aggregation. An appreciable and stable spectrophotometric response originated from the peroxidase-like activity of the proposed nanozyme was achieved due to the presence of Fe_3O_4 nanoparticles tightly immobilized on the mesoporous surface of monodisperse-silica microspheres in the accessible form.

Based on these advantages, an appreciable enhancement in the peroxidase-like activity that could not be obtained by using the peroxidase-mimetic agents in nanoscale was first observed with the synthesized nanozyme in the presence of *hg*DNA. Based on this, a colorimetric assay for the determination of *hg*DNA concentration up to 300 ng/μL was developed by using the aggregation-resistant nanozyme. To the best of our knowledge, the present study is the first in which a large DNA molecule is detected using the peroxidase-like activity of a composite nanozyme synthesized in the form of porous particles in micron-size range. The same material could be used as a magnetic sorbent for isolation of *hg*DNA via solid phase microextraction. A nanozyme for colorimetric determination was also evaluated as magnetic, solid phase extraction sorbent for *hg*DNA for the first time.

The selection of silica-based microspheres for immobilization of Fe_3O_4 nanoparticles allowed the derivatization of synthesized nanozyme via well-known "*silane chemistry reactions*". This property provided considerable flexibility for covalent attachment of ligands sensitive to the biomolecules to be determined onto the synthesized microspheres. By utilizing more specific ligands, similar biological macromolecules (i.e. RNA, His-tagged proteins, histidine rich proteins, glycoproteins, phosphopeptides, etc.) can be identified by colorimetric methods based on the peroxidase-mimetic activity of proposed nanozyme.

Conflict of interest

There are no conflicts to declare.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2019.04.089.

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