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Preparation of iron oxide silica particles for Zika viral RNA extraction

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Abstract

In this work, a robust synthetic pathway for magnetic core preparation and silica surface coating of magnetic microparticles is presented. Silica-coated magnetic particles are widely used to extract DNA and RNA from various biological samples. We present a novel route for the synthesis of iron oxide silica particles (Fe_3O_4@Silica) and demonstrate their performance for extracting ZIKA viral RNA from serum. The iron (II, III) oxide (Fe_3O_4), magnetite core is first prepared by ammonia neutralization of ferrous and ferric chloride aqueous solution under argon, followed by the addition of citrate salt to stabilize the surface of the resultant magnetic nanospheres. After this one-pot, two-step synthesis, the magnetic nanospheres are consumed during silica coating by hydrolysis of tetraethoxysilane (TEOS) under alkaline condition. The final product is a sphere-like magnetic aggregate with a size range of 1−2 micron. By simply suspending the magnetic aggregates in guanidinium chloride solution, the silica surface can be prepared for RNA binding. The RNA extraction efficiency was evaluated by extracting ZIKA viral RNA from serum followed by a PCR-based assay. The data indicate excellent recovery of target RNA and removal of PCR inhibitors. This manufacturing procedure for the silica coated microparticles provides a low-cost, effective and ready for scale-up method whose performance is equivalent to commercial alternatives such as magnetic silica surface particles for DNA and RNA sample preparations. The cost of the clinical assays could be largely decreased due to the 100 fold reduction in cost by replacing the

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commercially available magnetic particles with the developed material for RNA extraction.

Keywords: Biomedical engineering, Nanotechnology, Materials science

1. Introduction

Magnetic nano or micro particles have received a great deal of attention in biological, medical, diagnostic and engineering areas for decades [1, 2] because of their outstanding magnetic properties, versatility in immunity separations, applications as MRI contrast agent, biosensor and the targeted drug delivery [3]. Of particular interest are superparamagnetic particles constructed from Iron (II, III) oxide (Fe_3O_4 – magnetite) [4, 5, 6, 7, 8]. Current reports indicate their potential in binding, extraction and purification of biomolecules including RNA, DNA, proteins, enzymes and organic small molecules; their use as MRI contrast agents; and as carriers of biomarkers and drugs [9, 10, 11, 12, 13]. The versatility of superparamagnetic particles derives from the combination of an iron oxide core (typically Fe_3O_4) with a variety of coating materials [14, 15]. Magnetic properties allow particles movement to be controlled by the external magnetic field. When the field is removed, superparamagnetic particles are no longer magnetized and have no magnetic memory [2, 16]. However, Fe_3O_4 is readily oxidized to hematite (Fe_2O_3), changing its magnetism from superparamagnetic to ferromagnetic, and reducing its saturation magnetization. In any case, it would worsening the magnetic properties. To avoid oxidation and to protect the metal core, natural and synthetic polymers and silica have been employed to coat the magnetic particles [17, 18, 19]. A wide range of chemical functional groups can be introduced on the coating surface to increase stability, wetting properties and binding flexibility for various applications. Chemical functionalization by amines, carboxylic acids, epoxy, and aldehydes is usually used to immobilize proteins, enzymes, RNA, DNA biomolecules on the surface via covalent linkages [20, 21].

In contrast of introducing functionality for covalent bonding, this report demonstrates the non-covalent binding of RNA molecules on the silica coating of Fe_3O_4 micro-particles. Advantages of non-covalent binding include (1) quick, simple release of the RNA molecules and easy removal of the Fe_3O_4@Silica particles after sample preparation, (2) yet allows a strong ionic binding between the beads' surface with the RNA molecules' phosphate backbone during purification. The sample preparation process requires a sorbent material or magnetic silica particle able to quickly bind to RNA molecules present in the sample fluid, followed by the application of an external magnetic field to isolate the RNA-bound particles from the sample fluid. After separation, the RNA molecules are desorbed from

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the magnetic particles or carriers for subsequent nucleic acid amplification reactions. In the present work, the Fe_3O_4 nanoparticles were coated with silica since rapid adsorption and desorption of RNA or DNA molecules was required [22]. Conventional methods for DNA and RNA separation typically employ ethanol or isopropanol precipitation [23, 24]. This extraction method limits the scale-down of sample size and workflow for high-throughput screening and diagnostic tests. Silica surface adsorption of DNA and RNA addresses these limitations. Although commercial magnetic silica particles are available from several sources, the novel technique to produce such particles reported in the present work is superior due to its simplicity, more straightforward preparation method, robust in preparation, ease of use and cost effectiveness than others reported. This process does not require special techniques and instruments for controlling the size of particle formation. And the range of particle size we obtained performs well in the binding application of the assays. We are capable of controlling the reaction ratio of tetraethoxysilane (TEOS) and other silylated components with iron (II, III) oxide particle seeds to adjust the surface functionality.

In this new method of preparation, iron oxide (Fe_3O_4) nanoparticles were prepared by a classical alkaline co-precipitation of ferrous and ferric chloride aqueous solutions under argon without surfactants (Eq. (1)).

$$2\ Fe^{3+}\ (aq) + Fe^{2+}\ (aq) + 8\ OH^- \rightarrow Fe_3O_4(s) + 4\ H_2O \quad (1)$$

2. Materials and methods

2.1. Reagents

To synthesize the Fe_3O_4@Silica for RNA extraction, the following chemicals of analytical reagent grade were received and used without further purification. Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), styrene, glycidyl methacrylate (GMA), divinylbenzene (DVB), azobisisobutyronitrile (AIBN), polyacrylic acid, guanidine hydrochloride (>99%) and ethanol anhydrous were obtained from Sigma Aldrich. Ammonia in water 28−30% w/w, sodium citrate tribasic dihydrate, nitric acid (90%) were obtained from BDH. Tetraethyl Orthosilicate (TEOS), 3-glycidyloxypropyltrimethoxysilane (GTMOS), (3-Aminopropyl)triethoxysilane (APTES) were purchased from TCI (USA). All aqueous solutions were prepared with deionized water obtained from a Milli-Q Integral system (resistivity = 18 MΩ cm, EMD Millipore). Zymo MagBinding® Beads was obtained from Zymo Research, USA for comparison in RNA binding. Dynabeads® MyOne™, M280 from Thermofisher were used as the microscopic reference sizing materials, employed at 800x magnification.

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2.2. Synthesis of Fe_3O_4@Silica from iron oxide nanoparticles (Fe_3O_4) as seeds

Fe_3O_4 nanoparticles were prepared by the basic co-precipitation method. 4.46 g (16.6 mmol) $FeCl_3 \cdot 6H_2O$ and 1.6 g (8.1 mmol) of $FeCl_2 \cdot 4H_2O$ were dissolved in 80 ml of deionized water at room temperature. The solution was degassed by bubbling with Ar for 5 min and magnetic stirring in conical flask. 10 ml of ammonia 28−30% w/w was added dropwise to the solution and stirred for another 10 min. at room temperature. Following with heating for 1 h at 90 °C, iron oxide magnetite (Fe_3O_4) formed was cooled down to room temperature and magnetically separated, washed with deionized water until pH 8 reached.

Fe_3O_4 particles formed in above method were treated with 2 M nitric acid for 5 min with stirrer mixing at room temperature, we believe this treatment can stabilize the Fe_3O_4 particle surface [25, 26]. After the reaction, the particles were washed with deionized water and magnetically separated until the pH reached ~2.5. Then, 10 ml aqueous solution of 0.5 M sodium citrate was added to the 30 ml particles suspension. The resulting solution was stirred for 1 h at room temperature to prepare the surface for silica coating. Finally, the citrate surface stabilized particles were magnetically recovered, washed thoroughly with deionized water and freeze-dried. Dried beads weighed 1.77 g (MW 231.53, 7.6 mmol); yield = 88%. Dynamic light scattering (DLS) and zeta potential measurements were obtained using a Malvern ZETASIZER NANO ZSP. The sample concentrations were adjusted to ~0.1% w/v particles in deionized water. The samples were sonicated in ultrasonic bath for 1 min and pre-equilibrated for 3 min at 25 °C prior to each measurement. Zeta potential measured was −28.5 mV. Scanning Electron Microscopy (SEM) analysis was obtained from FEI Helios Nanolab 660. SPION stabilization with citrate is a protocol inspired by Khosroshahi *et al* and Digigow *et al* [25, 26]. This 1-pot, 2-step procedure leads to citrate surface-stabilized Fe_3O_4 nanoparticles that can be stored in dry form at 4 °C.

To prepare the silica glass surface of the above citrate- Fe_3O_4 particles. Re-suspension and sonication of these dry particles in 10% v/v water in ethanol, followed by silica shell formation with hydrolysis of tetraethoxysilane (TEOS) under alkaline condition using 28−30% ammonia, results in silica magnetic particles that can be magnetically separated without further purification (Fig. 1). At the end, the silica coated magnetic microparticles were rinsed with ethanol and dried in vacuum. Dried microparticles weighed 3.4 g. The mass ratio of silica coating was found by the following Eq. (2):

$$Mass\ ratio_{\ (\text{silica coating})} = (Mass_{\ (\text{silica coated particle})} - Mass_{\ (\text{starting particles})})/ Mass_{\ (\text{silica coated particles})} \quad (2)$$

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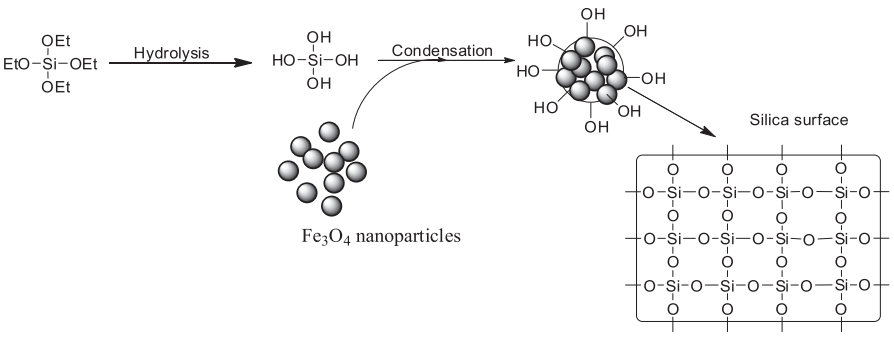

Fig. 1. Formation of Silica surface on Fe_3O_4 nanoparticles with TEOS.

The mass ratio of the silica coating to Fe_3O_4 was found to be around 55% averaged over multiple batches using a reactants ratio of TEOS:NP, 4:1 w/w. Zeta potential of this Fe_3O_4@Silica particles measured was −39.6 mV. Dynamic light scattering (DLS) analysis for sizing was recorded as below. Fig. 2 indicated that the Fe_3O_4@-Silica particles were 1.77 μm in diameter on average (Peak 1 = 1.75 μm, 91.1%; Peak 2 = 99.95 nm, 8.9%).

Different silica mass ratio were obtained by using different reactants ratio of TEOS:NP, ranging from 2:1, 4:1 to 6:1. Corresponding mass ratios of silica coating ranged from 45%, 55% and 62% were obtained. A mass ratio of TEOS vs starting particles NP of 1:1 was not pursued because of the possibility of incomplete silica enclosure on the Fe_3O_4 magnetic particles. And we observed that a higher silica

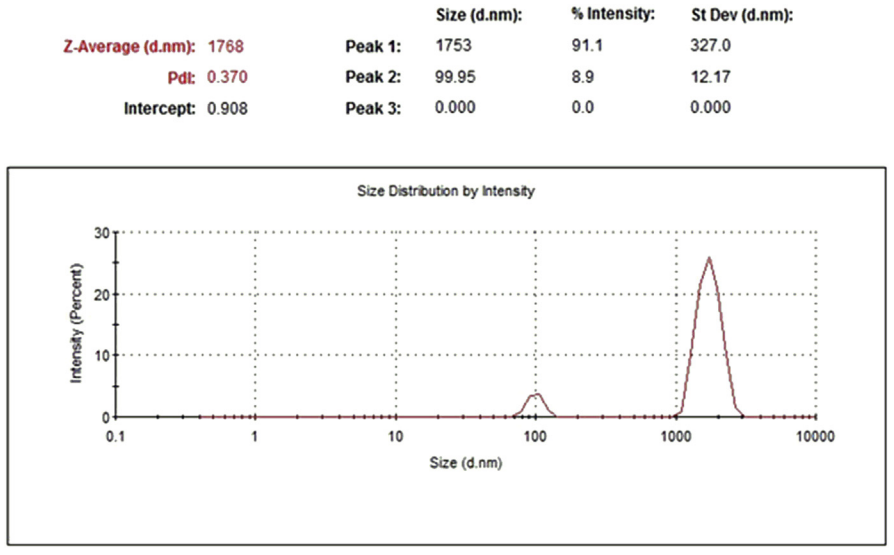

Fig. 2. Dynamic light scattering (DLS) size measurements of Fe_3O_4@Silica particles with 55% silica mass ratio (TEOS:NP, 4:1 w/w) was obtained using a Malvern ZETASIZER NANO ZSP. Z-average diameter = 1.77 μm, PDI = 0.370, Peak 1 = 1.75 μm (91.1%), Peak 2 = 0.099 μm (8.9%).

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mass ratio than 55% was not preferred as the additional weight of the silica shell resulted in a slower magnetic response of the coated Fe_3O_4@Silica particles.

2.3. Formulation of Fe_3O_4@Silica for RNA extraction in Zika assays

Preparation of 10% w/v of the Fe_3O_4@Silica in 1 M Gu.Cl buffer for Zika assays was done by suspension of 100 mg of the Fe_3O_4@Silica prepared above in 500 μl deionized water, then added with 2 M Gu.Cl buffer to make up to 1.0 ml final volume. Zeta potential of this formulated Fe_3O_4@Silica was measured as −4.84 mV.

2.4. Alternative methods for synthesis of Fe_3O_4@Silica from polymer seeds

Alternative methods to prepare Fe_3O_4@Silica beads were also tested; for example, polymerizing a mixture of (1) TEOS with γ-aminopropyl-triethoxysilane (APTES); (2) TEOS with (3-mercaptopropyl)trimethoxysilane (MPTMS); and (3) amino surface of magnetic polystyrene particles reacted with (3-glycidyloxypropyl)trimethoxy silane (GLYMO). In typical examples, in-house synthesized amine surface polymer microparticles with approximate 2 μm was used as seeds. 0.5 g of seeds were reacted with 0.2 ml 3-glycidyloxypropyltrimethoxysilane (GTMOS) for 1 h at room temperature in 5 ml ethanol, followed with 0.1 ml tetraethyl orthosilicate (TEOS) or a mixture of 0.1 ml tetraethyl orthosilicate (TEOS) and 0.2 ml (3-Aminopropyl)triethoxysilane (APTES) in 0.2 ml ammonia 28−30% w/w for 4 h at 90 °C (Fig. 3). After reaction, the magnetic particles were magnetically separated and washed with ethanol (5 times) with centrifugation at 3000 × g or separated magnetically. Zeta potential measured microparticles with silica surface formed from (TEOS:APTES, 2:1, v/v) = +39.2 mV microparticles with silica surface formed from TEOS only = +15.3 mV. This preliminary study on the ratio of TEOS:APTES relationship with surface Zeta potential demonstrates the fine tunable controls on the surface charges by the design of combination of silylating reagents used.

2.5. MiniLab ZNAA assay & samples preparation with Fe_3O_4@Silica

2.5.1. MiniLab ZNAA assay workflow

All necessary assay reagents and consumables were assembled in disposable, barcoded assay cartridges. Sample collection unit (SCUs) containing serum or capillary whole blood were inserted into assay cartridges (see next section) and the cartridges were inserted into the MiniLab. The sample-to-result fully-automated MiniLab

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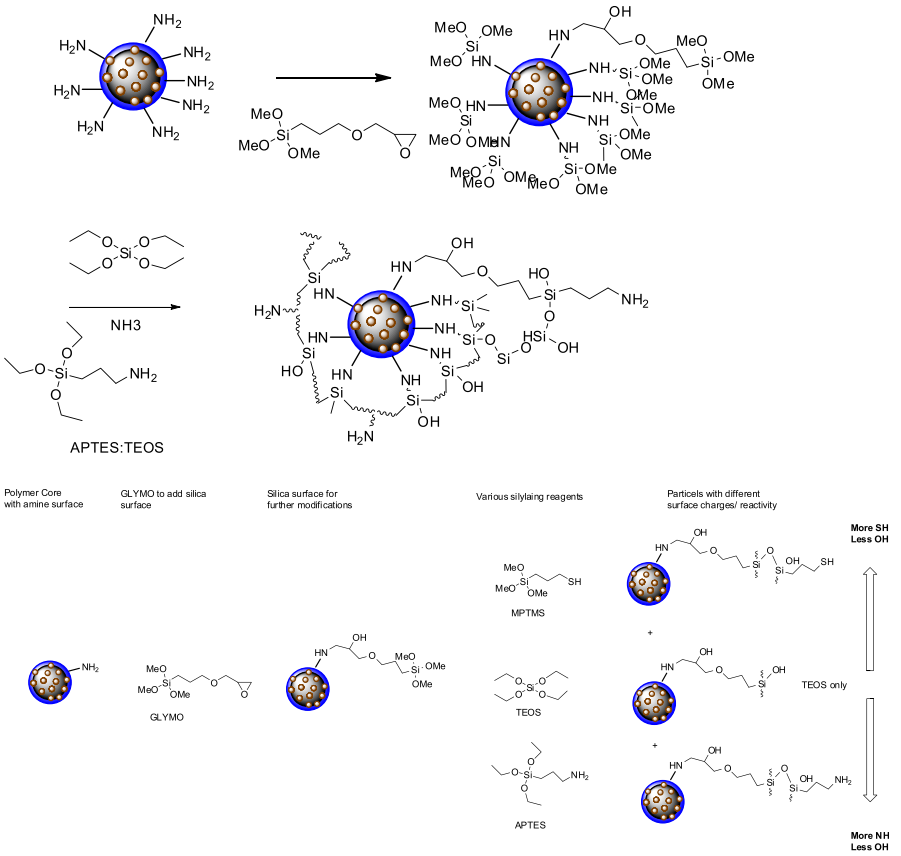

Fig. 3. Preparation of silica surface spherical monodispersed microparticles (polymer based), and the additions of variable MPTMS, TEOS, APTES for modified silica surface with different surface charges and reactivity.

extracted RNA from samples and performed nucleic acid amplification and detection. Reagents and consumables used for the assay were returned into the cartridge upon assay completion; the assay cartridge was then ejected and disposed of as biohazardous waste.

2.5.2. Blood sample preparation for the MiniLab ZNAA assay

Each SCU, comprised of two identical storage vessels, contained a total of 160 μl serum or capillary whole blood. As necessary, live ZIKV (strain PRVABC59, Centers for Disease Control, Atlanta, GA) was added into serum or capillary whole blood samples. ZIKV was added manually for serum samples or automatically within the MiniLab for capillary whole blood samples. The MiniLab also added MS2 bacteriophage to serum and capillary whole blood samples as a positive control for sample preparation/RNA extraction and thermal cycling-based amplification and detection.

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2.5.3. Automated RNA extraction from blood samples

Both serum and capillary whole blood samples were first centrifuged and then serum and plasma, respectively, were subjected to lysis, RNA capture onto Fe_3O_4@Silica beads (described in this article), washing, and then RNA elution into water.

2.5.4. Automated preliminary amplification

The liquid handling robot (LHR) within the MiniLab added 40 μl of extracted RNA to 60 μl of preliminary amplification master mix. Template-negative, DNase/RNase-free water was used in a separate, parallel reaction as a negative control. Reaction vessels were overlaid with mineral oil (Sigma, St. Louis, MO) and transferred by the LHR to a thermal-cycling module in the MiniLab to commence RT-PCR.

2.5.5. Automated isothermal amplification and detection

Primer pairs contained pair-wise complementary 5′ ends that resulted in amplicons containing 5′ overhangs. These overhangs facilitated the generation of concatemers, which are detectable with intercalating fluorescence dye. The Primer design is shown in supplemental methods. Three microliters of pre-amplified product (see above) for both sample and negative control were added by the MiniLab into separate wells containing 22 μl of isothermal reaction mix to detect ZIKV or MS2. The ZIKV DNA target amplicon, at 1×10^6 copies/ml, was used as a positive control for isothermal detection in a separate well. Assays were invalid if any controls failed.

3. Results and discussion

Although Fe_3O_4@Silica derived from polymer seeds pathway (Fig. 3) with well-defined spherical shape and monodispersed size as shown in Fig. 4 for the 800× magnification optical image comparison of Dynabeads M280, they require a longer synthetic pathway compared to silica surface beads prepared in Fig. 1. In addition,

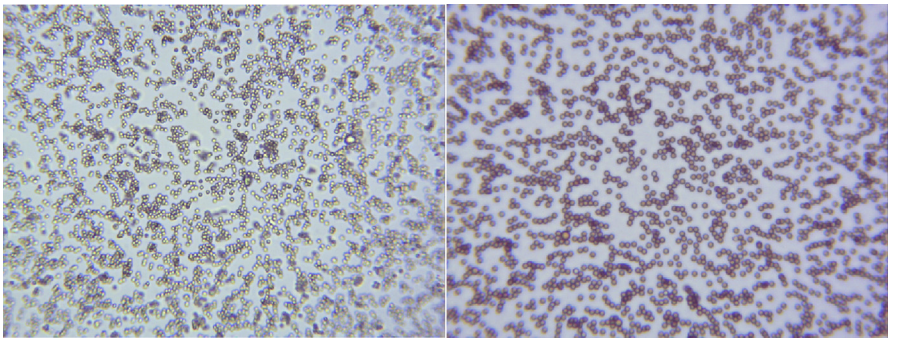

Fig. 4. 800× magnification of microscopic images to illustrate the well-defined spherical shape and monodispersed size: in-house silica coated polymer microparticles (*left*); reference 2.8−2.6 μm Dynabeads® (*right*).

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results generated from a comparison of beads in a nucleic acid assay indicated that magnetic particles with silica surfaces formed with TEOS provided comparable performance to commercially available Zymo product used for RNA separation from biological samples. Aggregated nanoparticles with citrate surfaces, Fig. 5(a), were observed under 800× optical microscope. After completing the coating step, the micro-particles Fig. 5(b) were less aggregated. To prepare these micro-particles for RNA or DNA binding, Particles showed in Fig. 5(b) were formulated in 1 M guanidinium chloride solution as a 10% w/v suspension mixture. Fig. 5(c) shows an image of commercial Zymo magnetic particles at the same magnification. This preparation method was further optimized to avoid aggregation if it is deemed to decrease the performance. Fig. 5(d) shows the well-defined silica-coated magnetic particles of 2 μm diameter, generated by alternative seed coating method described in Section 2.4. Fig. 6(a) and (b) show the Scanning Electron Microscopy (SEM) analysis of the silica surface iron (II, III) oxide (Fe3O4@Silica) particles described in the current work performed as RNA/DNA adsorption medium for sample preparations in PCR-based Theranos Zika assays. Surface morphology shows the nanoparticles fused together by Silica coating to yield the micron size particles. Fig. 6(a) confirms the optical image from Fig. 5(b), the particles generated with method described in Section 2.2 leads to non-spherical particles which is different from Fig. 5(d) particles synthesized by Silica coating from polymer seeds (Section 2.4).

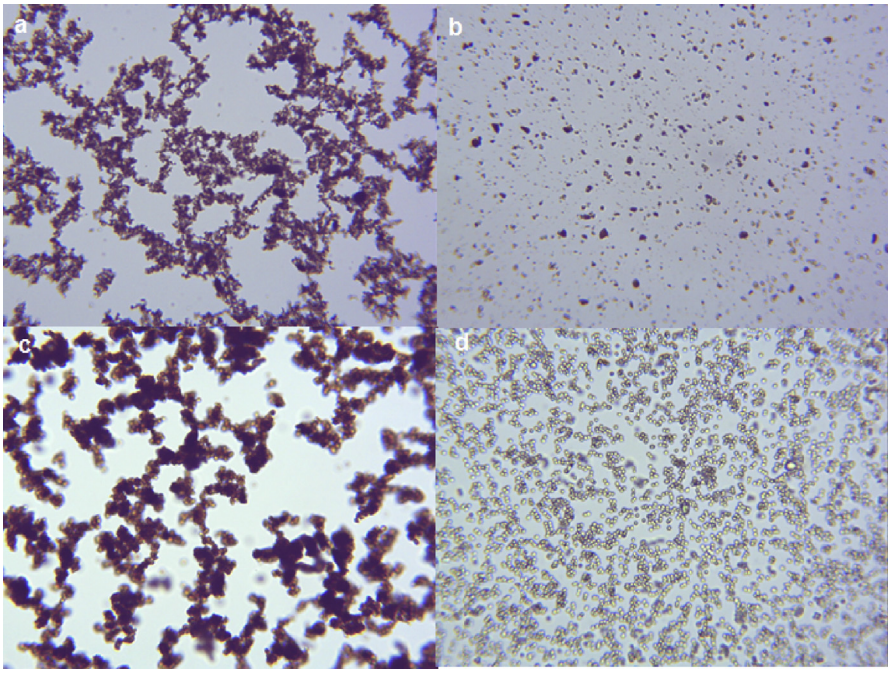

Fig. 5. (a) 800× optical microscope image of citrated Fe_3O_4. (b) Same magnification of TEOS functionalized silica surface beads used in this assay. (c) Commercial Zymo product. (d) Silica surface beads prepared by another method based on seed polymerization described in Section 2.4. Aggregation is observed in samples (a) and (c).

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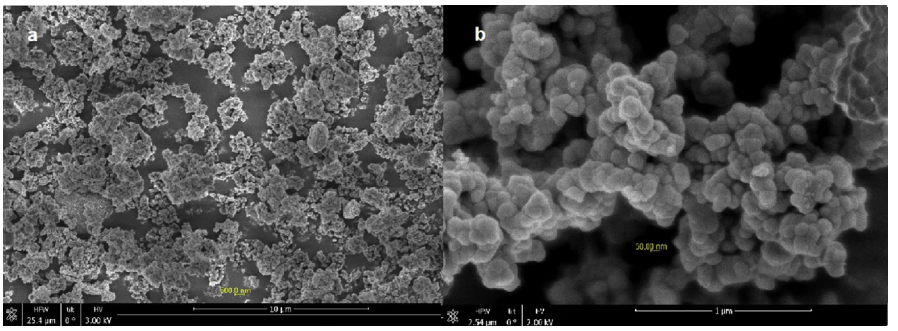

Fig. 6. (a) SEM analysis of Fe_3O_4@Silica with 500 nm & 10 μm scales. (b) SEM analysis of Fe_3O_4@-Silica with 50 nm & 1 μm scales.

The preparation described in Fig. 1 does not require polymerization of core and multiple coatings for surface functionality like other reported methods [17, 18, 19, 20, 21]. The simplicity and robustness of the method described here makes it very valuable for large-scale, low cost use in DNA and RNA separations, while providing comparable performance to commercially-available Zymo MagBinding® product. The micro-particles prepared by the current method are smaller in size and exhibit less aggregation relative to the commercial products. These two properties provide useful features: (1) smaller size provides same weight of particles containing higher surface area for RNA or DNA loading during sample preparation step; (2) less aggregation, on the other hand results in availability of higher loading areas for binding; (3) a well-defined spherical shape may not be an essential property for the particles made here when they are used as the sorbent material [25, 26]. Experimental results carried out in this work indicate functional performance is not compromised by the shape of the silica particles described here when compared to commercial magnetic micro-particles employed in the same applications. Zeta potential measurements show that the silica-coated particles have a −39.6 mV charge. The silica surface is negatively charged because of the deprotonation of silanol groups (SiO^-) [27]. Once the micro-particles were formulated with 1 M guanidinium chloride [28, 29], Zeta potential of the Fe_3O_4@Silica particles increased to −4.84 mV when measured in DI water. To demonstrate the functional performance of these silica surface magnetic particles in RNA extraction, Zika viral RNA extraction from plasma, followed by PCR-based studies, were conducted. The workflow of our straightforward Fe_3O_4@Silica particles preparation and using these to extract Zika viral RNA from available biological samples is described in Fig. 7.

Two sets of experiments were performed to establish the utility of the functionalized magnetic micro-particles in the automated detection of Zika virus RNA. The rapid detection of Zika virus has received increased international attention due to the 2015 outbreak in South America and its association with congenital microcephaly [30, 31]. The critical initial step in Zika virus RNA detection assays requires

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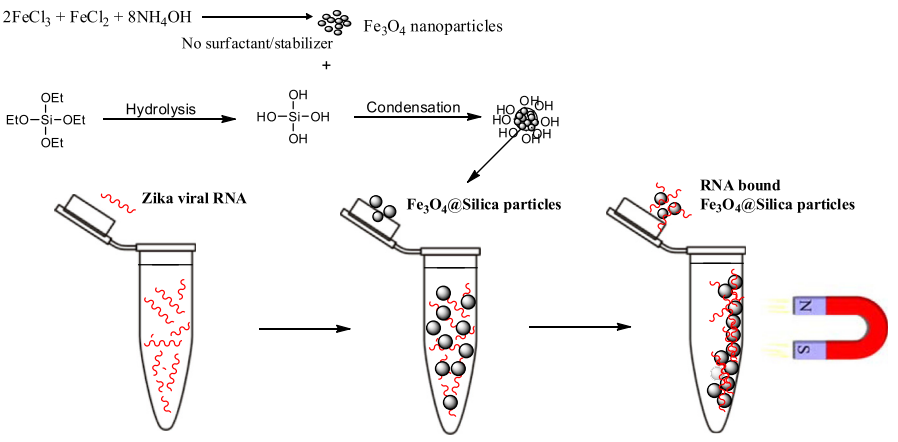

Fig. 7. Preparation of Fe_3O_4@Silica particles in 1-pot 2-step pathway and workflow of Zika viral RNA extraction with magnetic separation.

RNA extraction by either manual or automated methods [32]. The first set of experiments demonstrating the use of the magnetic micro-particles described in the current work for RNA extraction employed synthetic Zika RNA spiked into whole blood. These RNA samples were extracted and detected using Theranos miniLab equipment and a qualitative Zika virus nucleic acid amplification test. The second experiments used live Zika virus spiked into whole blood and detected using the same system. In addition, MS2 bacteriophage were spiked into the starting blood samples to serve as positive controls for sample preparation/RNA extraction and thermal cycling-based amplification and isothermal detection. The Theranos miniLab and Zika virus nucleic acid amplification test will be described in detail elsewhere (manuscripts in preparation).

Briefly, the Theranos Zika virus nucleic acid amplification tests were performed using single-use cartridges containing all necessary assay reagents and consumables that were processed on Theranos' automated, diagnostic platform ("miniLab") capable of sample preparation and processing. The miniLab contains an automated, multi-channel liquid handling system, centrifuge, thermal-cycler, isothermal heat block with fluorescence detection capabilities, and network functionality. In the miniLab, blood samples were first centrifuged, the resulting plasma was subjected to lysis, and RNA was captured on magnetic beads (commercial or in-house synthesized). Beads were collected and transferred by a sleeved magnetic rod. Samples were washed in commercial wash buffers, and RNA was subsequently eluted from the beads into DNase/RNase-free water.

Detection of Zika RNA was accomplished in the miniLab by a two-step amplification/detection process. A preliminary reverse-transcription and thermocycling amplification of the RNA target took place. This was followed by a proprietary fluorescence-based nested isothermal amplification and Zika nucleic acid (or

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Table 1. Summary of the inflection times determined using the commercial and prepared beads in this work.

	MS2	sZika	NTC
Zymo Commercial beads	15.9 (0.5)	15.2 (1.4)	NA
Beads prepared in this work	15.2 (0.7)	17.7 (2.6)	NA

Inflection times in min (standard deviation).

Table 2. The results of comparing the Zymo beads (n = 2) and the beads prepared in this work (n = 10).

	MS2	Zika	NTC
Zymo Commercial beads	16.6 (1.0)	13.2 (0.0)	NA
Beads prepared in this work	15.6 (0.78)	13.5 (1.0)	NA

Inflection times in min (standard deviation).

MS2) detection step. Relative fluorescence measurements were taken every minute, with inflection times (or threshold cycle, C_t) used to report the detection of Zika RNA and MS2 control RNA. Non-templated controls (NTCs) (controls without Zika or MS2 samples) were run and measured within the same assay cartridges; all NTC reactions were negative.

In the synthetic Zika (sZika) RNA experiments, 600 copies/mL of synthetic target were spiked into capillary whole blood samples which were then processed using either commercial microparticles (MagBinding® beads, Zymo Research, Irvine, CA) (n = 3), or the synthesized silica-coated Fe_3O_4 particles described in this paper (n = 6) (See Table 1).

The second set of experiments used live Zika virus spiked into whole blood samples. The virus was added at 250 copies/mL (See Table 2).

These two sets of experiments demonstrated comparable performance between the commercial micro-particles and the synthesized micro-particles prepared in this work in capture and extraction of Zika RNA followed by detection of Zika RNA using the Theranos miniLab and Zika virus RNA detection test.

4. Conclusions

In conclusion, the silica surface iron (II, III) oxide (Fe3O4@Silica) particles described in the current work performed as an outstanding alternative RNA/DNA adsorption medium for sample preparations in PCR-based assays. The silica coating of these particles prepared easily from reacting tetraethoxysilane with nano Fe_3O_4

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seeds by hydrolysis. Silica contents can be controlled effectively by the ratio of TEOS: NP, and the batch of Fe_3O_4@Silica with silica shell weighed about 55% of the total mass was selected for RNA adsorption studies. The particles dispersed well in aqueous and 1 M guanidinium chloride solution in 10% w/v ratio for final formulation and application to our miniLab Zika assay workflow. It was shown that mono-dispersity is not a critical material requirement for yielding performance in RNA extraction. Preparation of magnetic particles presented here features simple with short synthetic pathway. Finally, this method allows economical production at larger scale without compromising performance characteristics. Through this work it is concluded that this simple and cost effective method does not compromise the quality of RNA extraction.

Declarations

Author contribution statement

Alex H. F. Lee: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools and data; Wrote the paper.

Steven F. Gessert, Yutao Chen, Nikolay V. Sergeev, Babak Haghiri: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare the following conflict of interest: The authors of this paper are employees of Theranos Inc., the company producing “miniLab”, the equipment used to extract and detect RNA samples in this study.

Additional information

The subject matter of this publication is patent pending in at least the following: US Provisional Patent Application Ser. No. 62/513,999. Regarding regulatory status in the U.S., the Theranos miniLab system and the reagents described in this paper have not been cleared, approved or authorized by the FDA for diagnostic use. They are not for sale in the United States.

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