

Original Article

Stable Solid Magnetic Nanoparticles: With a Potential for Nucleic Acid Recovery and Delivery

S.R. Sreelekshmi, Susan Mani, S. Suresh Babu, Francis Boniface Fernandez*, P.R. Harikrishna Varma

Division of Bioceramics, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojappura, Thiruvananthapuram 695012, India

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Expanding scope of magnetic nanoparticles amongst various nucleic acid recovery methods is often questioned with the stability that it offers. Oxidation from magnetite state to maghemite state which is often accompanied by aggregation can affect their efficacy. Isolation of nucleic acid through conventional methods necessitates the need of precipitation, centrifugation and sophisticated equipment. Correspondingly, it initiates the outflow of aerosols into the working environment. Isolation assisted via magnetic nanoparticles can eliminate the spread of infection. High surface area possessed by iron oxide nanoparticles (IONPS) is an add-on property with its magnetic nature that enhances isolation from traces of nucleic acid. Present study involves surface modifications of IONPS using polyethyleneimine (PEI) and silicon alkoxide. This aims for an enhancement in stability of IONPS along with their binding and retrieval efficacy. Results validated using standard DNA confirmed a 70% retrieval efficacy for PEI coated IONPS. Silicon alkoxide surface modification offered improved life time and stability with a reduction in agglomeration of IONPS than PEI. Qualitative and quantitative analysis carried out confirmed the characteristics and stability of various IONPS and standard DNA. Automated DNA extraction can be seen as an extended goal for this technique.

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Introduction

Magnetic nanoparticles (MNPs) assisted nucleic acid retrieval techniques are opening up new avenues in diagnosis with the development of point of care diagnostic kits. An easy isolation and purification which is required for a quick, simple and robust method in the emerging field of diagnostics and medicine is offered by them. This also provides a high throughput method for isolation from diverse sources due to their ability to perform magnetically controlled aggregation, dispersion & purification. Retrieval using MNPs avoids column separation or vacuum filtration providing a device independent application. But the efficacy of MNPs have a linear dependence with its ability in retaining their magnetic nature for an extended time period. Aggregation, a consequence of strong dipole-dipole interaction reduces the homogeneous distribution of MNPs when kept in suspension form [1]. Similarly, interaction of MNPs with atmosphere results in oxidation, consequently switching its state from magnetite to maghemite. Correspondingly, its magnetic

nature shifts from paramagnetic to ferromagnetic upon this conversion which reduces saturation magnetization in turn reducing the magnetic property [2]. Nanoparticles also possess an advantage of high surface area to volume ratio when compared to macro and bulk particles. As a result, MNPs offers more reactive sites for DNA to bind with which varies increases with the reduction in particle size.

Stability of magnetic nanoparticles can be retained subjective to the coatings applied on to them. The surface coating creates a corona around the MNPs that can simultaneously increase their stable shelf life along with prevention of oxidation [1]. In addition to this, stability of nanoparticles is also achieved along with their protection against corrosion (acid and basic) [3].

We provide data on iron oxide nanoparticles (IONPS) that were synthesized by coprecipitation method. Further to have a comparative study of their stability and reliability with surface modified IONPS, they were modified with silica and polyethyleneimine. Silica coated IONPS assures an easy release of DNA from its surface as they bind non-electrostatically with nucleic acid [4]. This modification exhibits higher stability over a wide

* Corresponding author

E-mail address: francisbf@scimst.ac.in (Dr. Francis Boniface Fernandez)

range of pH in aqueous conditions and is more resistant towards biodegradation than organic coating materials like chitosan etc [5]. The robustness and simplicity offered by this method makes it very valuable for low cost, large scale DNA and RNA separations [2]. Thickness of silica shell can be controlled by varying TEOS concentration which can directly influence the charge of the nanoparticle [6]. PEI is an organic cationic polymer alkyl chain with primary, secondary and tertiary amines [7]. Positive charge provided by the protonation of single nitrogen atom present in every three atoms found in the backbone can enable an electrostatic interaction with the negatively charged backbone of DNA [8-10]. PEI has an ability to neutralise excess anionic colloidal charge under different pH conditions.

Among the commercially available magnetic particles, Mag-bind, MagJET, Magmax are matrices based on silica, glass, agarose, cellulose, polystyrene and silane. But all these MNPs are available in a suspension form in an appropriate buffer. The presence of which can reduce its shelf life as the buffer can retain its property only up to 3-6 months [11]. While this method enables the storage of dry IONPS which can be resuspended at the user end depending upon their requirements.

Materials and Methods

Ferric chloride FeCl_3 (Sigma Aldrich), ferrous chloride tetrahydrate $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (Sigma Aldrich), polyethyleneimine (branched, MW – 25000) PEI (Sigma Aldrich), sodium hydroxide pellets NaOH (S.D. Fine), δ -DNA (TaKaRa), tetraethylorthosilicate (TEOS) $\text{SiC}_8\text{H}_{20}\text{O}_4$ (Sigma Aldrich), ammonium solution NH_4OH (Avantor), agarose low EEO superior grade (Sisco Research Laboratories), cetyl trimethyl ammonium bromide (CTAB) $\text{C}_{19}\text{H}_{42}\text{BrN}$ (Sisco Research Laboratories).

Synthesis of IONPS

Co-precipitation is the synthesis technique used where ferric chloride, FeCl_3 (0.1M) and ferrous chloride tetrahydrate $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.1M) were dissolved in HCl in Fe_3O_4 stoichiometric ratio. The mixture is drop wisely added into 2 M NaOH upon continuous heating and stirring in the presence of nitrogen for 20 minutes. This is followed by dropwise addition of 25% ammonia in the required amount under continuous stirring for 30 minutes.



The black slurry obtained is further modified by cooling, centrifugation and peptization. IONPS is preserved at room temperature as a suspension in distilled water and lyophilization is carried out afterwards (herein after, this synthesis route is referred to as U and synthesized IONPS as U-IONPS).

Surface modification of IONPS

Modifications were done in order to evaluate the retrieval efficacy of DNA using different IONPS.

Synthesis of Silica coated IONPS

For the synthesis of iron oxide nanoparticles, required proportion of FeCl_3 and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ is mixed together in distilled water. This is followed by dropwise addition of 25% ammonia in the required amount under continuous stirring for 30 minutes. Solution heated for 1 hour without boiling is further cooled, centrifuged and peptized. An aqueous sodium citrate solution is added to IONPS. Continuous stirring is given for about 30 minutes followed by the magnetic separation of IONPS (Herein after, this synthesis route is referred to as S and synthesized IONPS as S-IONPS). The separated IONPS were added to 90% ethanol taken in a round

bottom flask (RB) under continuous stirring. The solution was heated at a temperature below boiling point. Dropwise addition of TEOS diluted with purified ethanol and ammonia is done. The suspension is continuously stirred and lyophilized (herein after, this synthesis route is referred to as S1 and synthesized IONPS as S1-IONPS). Same procedure is done S1-IONPS for an additional coating and is preserved (herein after, this synthesis route is referred to as S2 and synthesized IONPS as S2-IONPS).

Synthesis of PEI coated IONPS

FeCl_3 and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ are separately dissolved in deionized water in the presence of nitrogen. Both are mixed together to which 0.1M CTAB and 5 M ammonia were added. Continuous stirring was given for 40 minutes. IONPS separated magnetically were washed and stored in vacuum desiccator for drying. 5M NH_4OH is added drop wisely to 20% PEI solution prepared in distilled ethanol. The solution is heated up to 50°C. Required quantity of dried IONPS is added and stirred continuously for 30 minutes. The suspension is magnetically separated, washed and stored in vacuum desiccator for drying. The samples were preserved at room temperature (herein after, this synthesis route is referred to as P and synthesized IONPS as P-IONPS).

Retrieval of Nucleic acid using IONPS

2 mg of IONPS (all types) were taken and washed with TE Buffer. 5 μg of δ DNA (0.35 $\mu\text{g}/\mu\text{l}$) is added to Eppendorf's containing IONPS and kept idle for some time. IONPS are magnetically separated and washed in ethanol. 20 μl of autoclaved water is added to DNA bound IONPS for elution and is kept in water bath maintained at 60°C. The IONPS are magnetically separated preserving the supernatant containing eluted DNA. The purity and concentration of DNA is analyzed using Nanodrop Spectrophotometer.

Physical Studies

The particle size and charge is determined using Dynamic Light Scatterer, DLS (Zetasizer Nanoseries, Nano ZS, Malvern Instruments). The concentration of the sample is adjusted to 0.1% w/v particles in distilled water. Before analysis, samples are sonicated to ensure uniform distribution.

Morphological Studies

The surface morphology of magnetic nanoparticles is analysed by scanning electron microscope, SEM (FEI, Quanta). IONPS suspended in distilled water is dropped onto a glass plate after sonication. Glass plate is then coated with Au/Pd material using sputter coater (E1010, Hitachi). This will ensure the conductivity to carry charged electrons across the surface.

For transmission electron microscope, TEM analysis, IONPS dispersed in distilled water after sonication is dropped onto the formvar coated TEM grid made of copper. Grid is allowed to dry. A magnified image of the sample is obtained with the help of electron beam ejected out of electron gun at a very high voltage (Hitachi H-7650).

Chemical Studies

Fourier transform infrared spectroscopy (FT-IR, Thermo Nicolet 5700 spectrometer (USA) gives information about various functional groups and components present in the sample. Spectrometer is operated in DRIFT mode. KBr powder (IR Grade) is mixed along with sample against KBr background. The sample is scanned between a wavelength region 4000 cm^{-1} - 400 cm^{-1} (mid IR Region) with 64 number of scans and 4 cm^{-1} resolution.

Energy dispersive X-ray spectroscopy (EDS) gives surface elemental compositions (FEI, Quanta). This microanalysis technique is accompanied with SEM. The X-Rays emitted from the sample during SEM analysis provides necessary information regarding samples elemental composition.

X-ray diffraction (XRD) provides the crystallinity and phase of samples (Bruker D8 Advance, Germany). Including their size, crystal structure, composition etc. Cu-K α radiation at a current of 30mA and voltage of 40KV is used for scanning. X-ray diffraction occurs when they collide with the sample depending up on the arrangement of planes in them. XRD pattern is a curve of 2θ v/s intensity; θ being the angle of diffraction. 2θ ranges from 20-80° with a speed rate of 2°/minute at a step size of 0.02 degree.

Magnetic studies

Vibrating sample magnetometer (VSM) having 10^{-6} emu sensitivity with 1 second averaging is used. Magnetic field up to 100kOe with field sweeping from -100kOe to +100kOe was used to determine saturation magnetism at 300K (Multipurpose Broad Band Probe with ATM (SmartProbe BB(F)-H-D 5mm – AZ: PH3723-500), broad band inverse probe with ATM (BBI Broad Band Inverse Probe H-BB-D 5mm – Z: PH3162-500)

DNA retrieval

To determine the purity and quantity of DNA retrieved, Nanodrop spectrophotometer (NanoDrop™ 2000/2000c) is used. This is a full spectrum UV-Vis microvolume spectrophotometers. They require samples less than 0.5 μ l which is dropped directly on the optical measurement surface of the instrument. Concentration and purity of DNA sample is the ratio of absorbance at 260 nm and 280 nm. Generally accepted ratio for pure DNA is 1.8 and 1.8-2.2 for pure nucleic acid. Between 0.4ng/ μ l and 15000ng/ μ l is the detection range of nanodrop spectrophotometer.

Luminescent image analyzer

Luminescent image analyzer (LAS 4000 Fuji Film, Japan) provides the fluorescence image of the gel after run. Gel upon illumination by different filters provides images using multipurpose CCD camera. EtBr intercalated with DNA shows enhanced fluorescence when illuminated with 312nm and 365 nm filter (UV region) at an exposure time is adjusted to 1/30th and 1/15th of a second.

Results

DLS and zeta potential

U-IONPS were found to be monodispersed with a particle size of 166 nm as per the DLS measurements whereas 743.7 nm and 669 nm for P-IONPS and S2-IONPS respectively. At pH=7, zeta potential of different IONPS were measured as +22.1 mV for U-IONPS, +43.4 mV for P-IONPS, -50.3 mV for S1-IONPS and -44.7 mV for S2-IONPS.

Morphological studies

SEM micrographs in figure 1 depicted the size of IONPS as 146nm for U-IONPS, 88 nm for P-IONPS, 372nm for S-IONPS, 394.5nm for S1-IONPS. IONPS were found to be clustered for S2-IONPS. TEM micrographs in figure 2 depicted the size of IONPS as 37.38 nm for U-IONPS, 64.24 nm for S-IONPS and 340.86 nm for S2-IONPS. The XRD pattern of U-IONPS (figure 3a) possess characteristic peaks of Fe₃O₄ at $2\theta = 29.878^\circ, 35.454^\circ, 42.891^\circ, 53.589^\circ, 57.015^\circ$ and 62.651° corresponding to (2 2 0), (1 1 0), (4 0 0), (4 2 2), (5 1 1) and (2 1 2) planes respectively. This exactly matches with the pattern of magnetite (pdf number – 01.071.6336 Magnetite). XRD pattern of P-IONPS (figure 3b) possess characteristic peaks of Fe₃O₄ at $2\theta = 30.251^\circ, 35.639^\circ$ and 43.220° corresponding to (220), (110) and (400) planes respectively. Broad and less intense peaks are seen between 10° and 20°, 40° and 50°

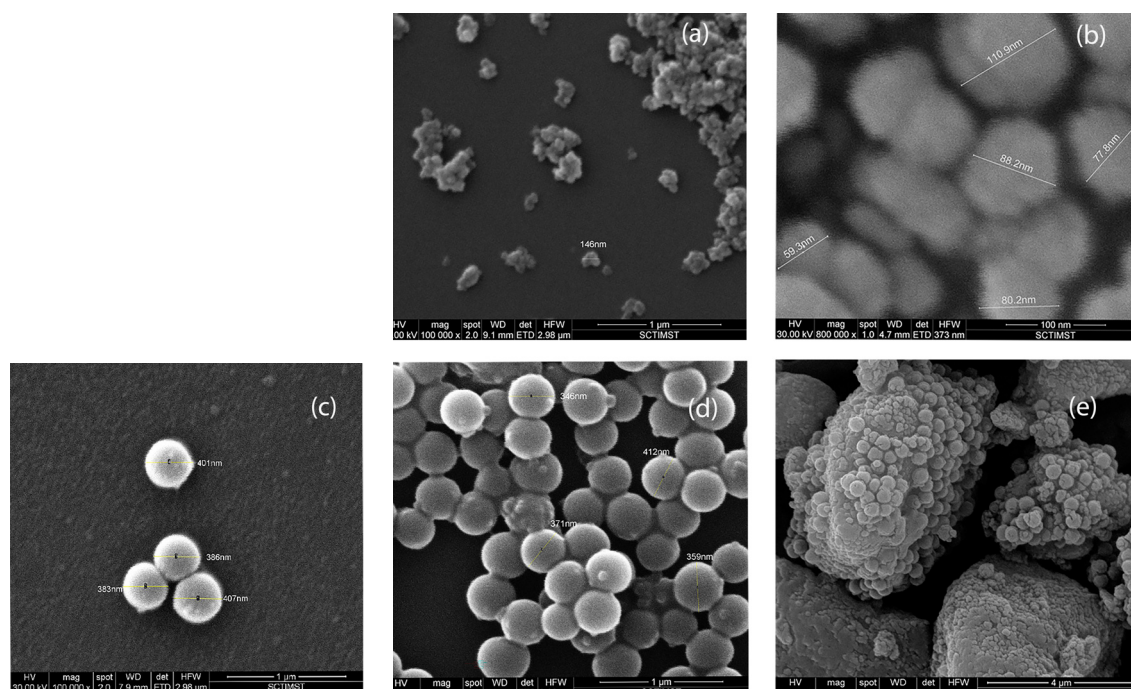


Figure 1: Effect of surface modifications on IONPS as depicted in SEM micrographs for various IONPS

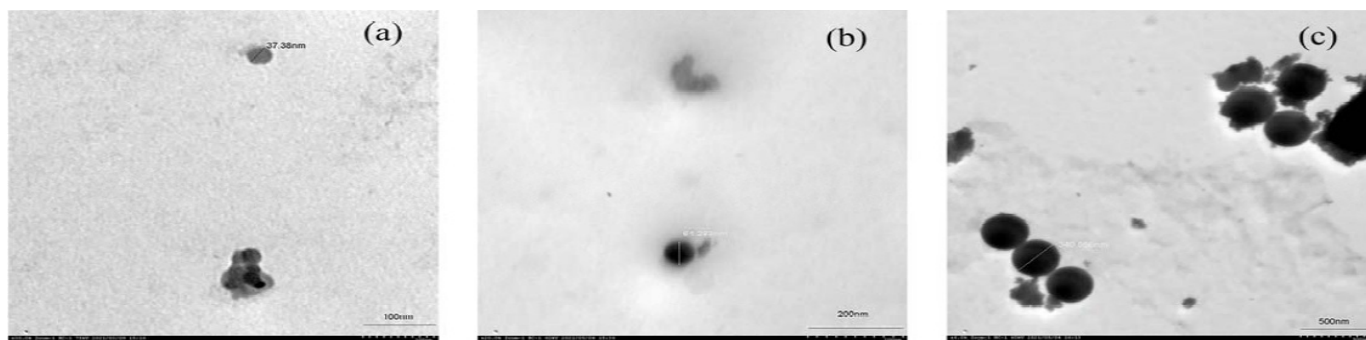


Figure 2: TEM micrographs of IONPS (a) U-IONPS (b) S-IONPS (c) S2-IONPS

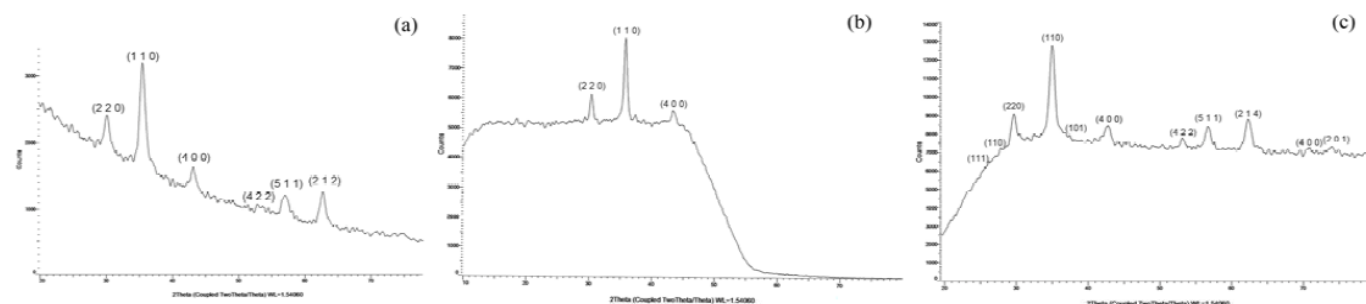


Figure 3: XRD Pattern of (a) U-IONPS, (b) P-IONPS, (c) S2-IONPS

while the XRD pattern of S2-IONPS (figure 3c) possess of characteristic peaks of Fe_3O_4 at $2\theta = 29.657^\circ, 34.990^\circ, 42.873^\circ, 53.124^\circ, 56.693^\circ$ and 62.303° corresponding to (220), (110), (400), (422), (511) and (212) respectively and characteristic peaks of silicon oxide at $2\theta = 26.161^\circ, 28.588^\circ, 37.384^\circ, 70.888^\circ, 73.963^\circ$

corresponding to (111), (110), (101), (400) and (201) respectively. This corresponds to peaks of magnetite (pdf number - 01.071.6336) and silicon oxide (pdf number - 00.013.0026).

From the Debye Scherrer's equation the size of the U-IONPS was found to be 102 nm, for S2-IONPS 103.8 nm and for P-IONPS 181.6 nm.

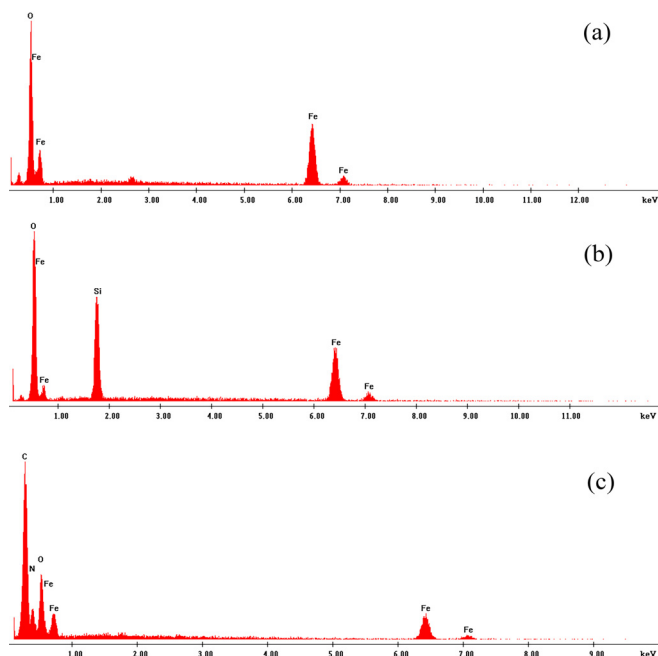


Figure 4: EDS Analysis to confirm the surface modifications on IONPS (a) U-IONPS, (b) P-IONPS, (c) S1-IONPS

EDS pattern

EDS pattern shows only the presence of Fe (Iron) and O (Oxygen) peaks at figure 4a. This ensures chemical purity of U-IONPS. The presence of silicon peaks (figure 4b) along with Fe and O peaks indicates the silica coating of S1-IONPS. Presence of C, O and N peaks in EDS pattern of P-IONPS (figure 4c) along with Fe peaks indicates the PEI coating.

FTIR Spectrum

FTIR spectrum of U-IONPS shows the characteristic band around 582 cm^{-1} and 557 cm^{-1} due to Fe-O stretching vibration (figure 5a) in Fe_3O_4 . In figure 5b, along with the characteristic band around 577 cm^{-1} due to the absorption of Fe-O, other characteristic bands around 1109 cm^{-1} corresponding to asymmetrical stretching vibrations of Si-O-Si and around 799 cm^{-1} corresponding to Si-O stretching vibrations were found. Thereby indicating the presence of silica coating on IONPS.

The FTIR pattern of P-IONPS shows additional characteristic bands apart from 543 cm^{-1} due to Fe-O vibration in Fe_3O_4 (figure 5c). The characteristic bands around 1397 cm^{-1} due to C-N stretching and around 2848 cm^{-1} due to C-H bond stretching present in PEI. The bands between 1553 cm^{-1} and 1646 cm^{-1} are due to NH_2 bending present in PEI. This confirms the presence of PEI coating on the IONPS.

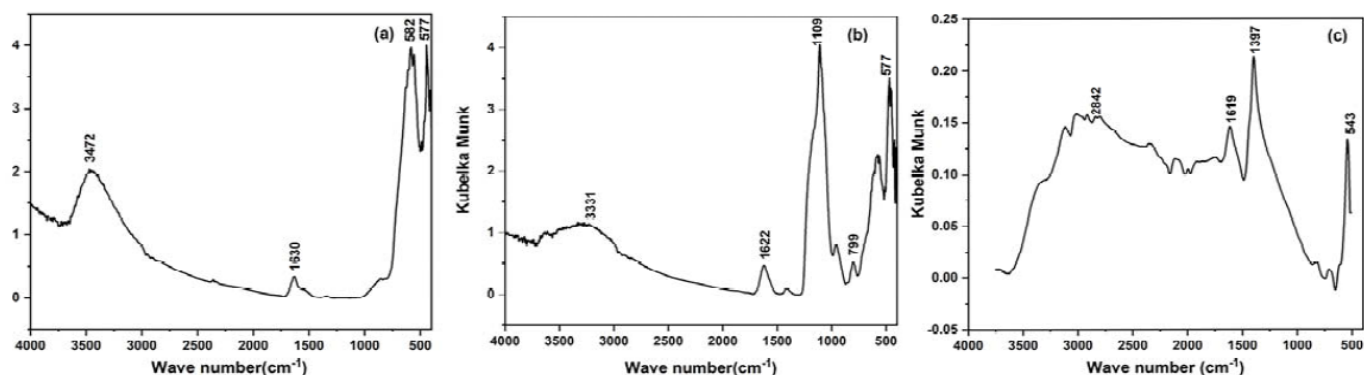


Figure 5: IR Spectrum of (a) U-IONPS, (b) S1-IONPS, (c) P-IONPS

VSM Analysis

A strong magnetic response in M-H curve is observed for all samples with an increase in magnetic field (figure 6). A sharp increase in magnetic field is seen within the range of 0 to 30kOe after with saturation is reached. Here, saturation magnetism of U-IONPS can be taken as a reference, 62 emu/g which varies upon coating. Maximum saturation magnetism (M_s) is observed for P-IONPS, 70 emu/g. Silica coating decreased M_s value to 42 emu/g and 40 emu/g for S1-IONPS and S2-IONPS respectively. Our sample exhibits zero residual magnetism and coercive force as seen from the graph i.e. no magnetism is retained in the material once external magnetic field is removed.

DNA Retrieval using IONPS

Nanodrop spectroscopic analysis confirmed the ability (table 1) of the coated and uncoated IONPS to retrieve nucleic acid and retrieval efficacy was calculated.

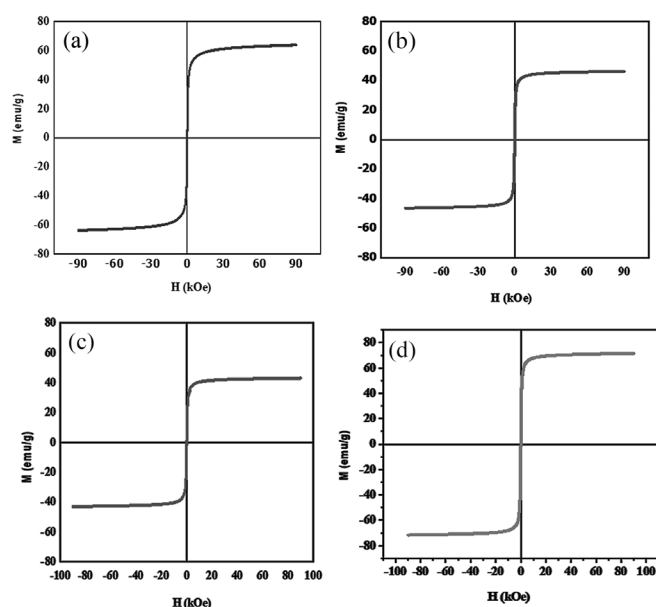


Figure 6: Magnetic responses of Superparamagnetic IONPS (a) U-IONPS, (b) P-IONPS, (c) S1-IONPS, (d) S2-IONPS

Discussion

IONPS offers numerous advantages in nucleic acid retrieval techniques. Isolation of nucleic acid through conventional methods necessitates the need of precipitation, centrifugation and

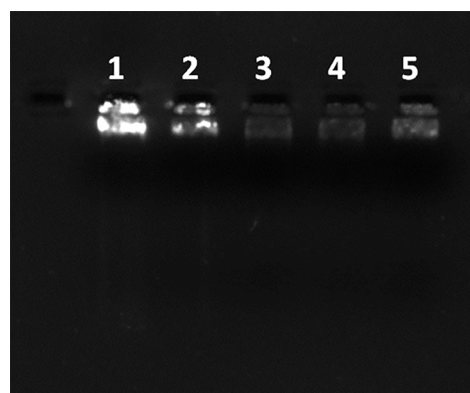


Figure 7: Agarose Gel Electrophoresis (1%) image of λ DNA retrieved using IONPS. Lane 1 control [λ DNA], Lane 2 U-IONPS alone, Lane 3 S1-IONPS, Lane 4 S2-IONPS, Lane 5 P-IONPS

Table 1: Concentrations of λ DNA retrieved using different IONPS

IONPS	Initial concentration (ng/ μ l)	Retrieved concentration (ng/ μ l)	Retrieval Efficacy (%)
U-IONPS	246.00	131.00	53.25
P-IONPS	259.40	232.50	89.63
S1-IONPS	246.00	116.00	46.75
S2-IONPS	246.00	81.30	33.05
After 3 months			
U-IONPS	214.00	22.30	10.42
P-IONPS	350.00	144.00	41.14
S1-IONPS	214.00	93.50	43.69
S2-IONPS	214.00	49.40	23.08

sophisticated equipment while magnetic nanoparticle assisted retrieval techniques are quick, simple and robust. DNA adsorbed onto them can be simply eluted in water. At the same time, their performance is hindered due to their inability to hold its property in long run. Agglomeration of IONPS can directly influence their shelf life and will reduce its nucleic acid binding capacity. Higher the surface charge of IONPS, greater will be its shelf life. As the surface charge increases agglomeration is also reduced as equal charges gets repelled. The magnetic property possessed by the IONPS also need to be sufficient for magnetic separation.

These stability issues can be minimized by surface modifications to an extent by making IONPS in a buffer free format. Commercially available IONPS are suspended in proprietary buffer (Mag-bind, MagJET, Magmax).

U-IONPS synthesized here bear a positive charge which can initiate the adsorption via electrostatic interaction (phosphate backbone in DNA is negatively charged). The efficacy of nucleic acid isolation could be enhanced with the increase of positive charge as retrieval efficacy has a direct effect on positive charged IONPS.

Electrostatic interaction can be promoted by inducing positively charged moieties onto the IONPS [12] like PEI. This contributed to more positive charge on the IONPS in turn enhancing the electrostatic attraction [13]. Density of charge imparting amino groups in PEI can directly influence to the amount of DNA adsorbed [12]. The peaks corresponding to PEI were seen as broad and less intense peaks (figure 3b) due to the amorphous nature of PEI.

Surface modification of IONPS with PEI, showed that its retrieval efficacy increased up to 1.6 fold times as that of U-IONPS (table 1). PEI modification witnessed an increase in its magnetic properties as well (figure 6b). This has reduced agglomeration of molecules and increased the reactive site of IONPS. Though P-IONPS exhibit higher efficacy than U-IONPS, but they showed a shorter shelf life (table 1).

In order to address this stability issue, IONPS were modified with silicon alkoxide. In spite of the negative charge possessed by silicon alkoxide coating and increased agglomeration due to it, they exhibited an enhanced resistance towards degradation. Thereby, ensuring their stability and increased shelf life in a wider range of pH. IONPS shares a covalent bond with silicon alkoxide which strengthens the coating. Their bind with DNA is via non electrostatic bonding [13]. This agrees with the findings of Lee et al and Bertolucci et al [2] where he has examined the adsorption of DNA onto silicon alkoxide coated IONPS that takes place through intermolecular hydrogen bonds. Hydrogen bonds can occur between the unwound nucleotides and silica surface [14]. In addition to that, prevention of oxidation from magnetite to maghemite is also achieved. Surface modification by silicon alkoxide helps allows for further modification if required as the silanol groups can bind with other functional groups.

The results showed an enhanced stability of S1-IONPS when compared with both U-IONPS and P-IONPS (table 1). At the same time, silicon alkoxide coating on the surface of IONPS inhibits the penetration of external magnetic field into the sample (figure 6). This reduction in magnetization can also be considered as a way to confirm silicon alkoxide coating onto the IONPS [15]. Similar to the results obtained by M. Abbas et al. as the number of silicon alkoxide coatings on the IONPS, its magnetization decreased. They found a considerable decrease in the saturation magnetization when the external magnetic field varied from -15kOe to +15kOe at 300K

which is comparable to the results obtained here [16]. Silicon alkoxide coating did not exhibit any change in the retrieval efficacy. Comparable to the observation made by [17] gelation of silica resulted in formation of clusters which subsequently reduced specific surface area of IONPS (figure 1). Due to reduced reaction site of IONPS, DNA could not bind effectively with them.

As the literature says, magnetic properties of IONPS depends on their particle size and also on the coatings made on them [15]. The M-H magnetic responses curve of different IONPS synthesized by coprecipitation method confirms their magnetic property (figure 6). Superparamagnetism is the magnetic property shown by ferromagnetic materials when their size reduces down to single domain. This agrees with the fact that, size of the particles synthesized are in the nano-regime. As magnetization curve showed no hysteresis behavior, superparamagnetic nature of IONPS is proved. Wallyn, Anton, and Vandamme (2019) studied the nature of IONPS, their report can be correlated to confirm the superparamagnetic nature of synthesized IONPS. Neither remanence nor coercivity was observed in any of the samples.

Both the coatings were successful in the prevention of IONPS agglomeration [13]. The strong dipole-dipole interaction which exist between IONPS that results in agglomeration is avoided here (figure 1, figure 2). Large positive and negative charges for IONPS ensures greater stability. As the magnitude of charge increases, more is the repulsive force that exist between IONPS. This repulsion will prevent the particle agglomeration.

These IONPS synthesized is stored in dried form. When IONPS are suspended in buffer, oxidation will be initiated and the magnetic state of IONPS will shift from magnetite to maghemite in turn reducing its saturation magnetization. The charge of IONPS varies as the pH of buffer changes. This can possibly affect their retrieval efficacy [11]. This instability can be reduced when nanoparticles are stored in stable powdered form. The properties possessed by the IONPS are retained on resuspension. There by extending their flexibility in utilization, storage and application based on users needs.

Conclusion

In summary, we have validated the properties of iron oxide nanoparticles (IONPS) synthesized by co-precipitation method, further modified using silicon alkoxide and polyethyleneimine. Qualitative and quantitative analysis were carried out. Finding indicated that nucleic acid retrieval efficacy and saturation magnetization was higher for P-IONPS compared to other samples but they exhibit shorter shelf life. Surface modified IONPS using silicon alkoxide showed consistency in their retrieval efficacy and showed longer shelf life. Here, the storage of synthesized IONPS in solid form is an added advantage. Evaluation and optimization of parameters to increase the retrieval efficacy is also carried out. This strategy offers a magnetic nanoparticle mediated nucleic acid recovery limiting aerosol with better retrieval efficacy. This technique can be extended for automated DNA extraction.

Future Scope

As silicon alkoxide coating offers more sites for binding it enables further modifications. This can increase its acceptance in nuclei acid retrieval techniques. The retrieval efficacy and shelf of S1-IONPS can be compared with other silicon coating agents apart from TEOS.

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