Ultrasound assisted chitosan coated iron oxide nanoparticles: Influence of ultrasound irradiation on the crystallinity, stability, toxicity and magnetization of the functionalized nanoparticles

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ABSTRACT

Due to unique reaction conditions of the acoustic cavitation process, ultrasound-assisted synthesis of nanoparticles has attracted increased research attention. In this study, we demonstrate the effect of ultrasonic irradiation on the crystallinity, stability, biocompatibility, and magnetic properties of chitosan-coated superparamagnetic iron oxide nanoparticles (CS-SPIONs). CS solution and colloidal suspension of SPIONs were mixed and sonicated using an ultrasonic probe of 1.3 cm tip size horn, frequency (20 kHz), and power (750 W). Different samples were sonicated for 1.5, 5, and 10 min with corresponding acoustic powers of 67, 40 and 36 W, and the samples were denoted S1.5, S5, and S10, respectively. The samples were characterized using X-ray diffractometer (XRD), Energy dispersive X-ray (EDX), Transmission electronic microscope (TEM), Fourier transform infrared spectroscopy (FTIR), Zeta sizer, and vibrating sample magnetometer (VSM). Cell cytotoxicity and cell uptake were investigated with human embryonic kidney 293 (HEK-293) cells through MTT assay and Prussian blue staining, respectively. The sharp peaks of the XRD pattern were disappearing with an increase in the sonication period but a decrease in acoustic power. EDX analysis also demonstrates that atomic and weight percentages of the various elements in the samples were decreasing with an increase in the sonication period. However, the Zeta potential (ζ) values increase with an increase in the sonication period. The saturation magnetization (Ms) of the S1.5 before and after the coating is 62.95 and 86.93 emu/g, respectively. Cell cytotoxicity and uptake of the S1.5 show that above 70% of cells were viable at the highest concentration and the longest incubation duration. Importantly, the CS-SPIONs synthesized by the sonochemical method are non-toxic and biocompatible.

1. Introduction

Sonochemical method is a non conventional and efficient route of fabricating, functionalizing, and doping iron oxide nanoparticles [1–4]. In addition, it has been used to synthesize other nanomaterials which include gold [5], silver [6], and platinum [7]. The process generates several unique conditions such as acoustic cavitation process with temperature, pressure, and cooling rate of 5000 K, 2000 atm, and 1010 K/S, respectively. In addition to the localized hot spot generated by the sonochemical process, shock waves and microjets are produced [8]. These unique reaction conditions are the main advantages of the sonication process over the conventional techniques [9]. Recently these unique conditions have been exploited to rapidly and effectively graft functional groups onto the surface of superparamagnetic iron oxide nanoparticles (SPIONs) [10]. SPIONs have considerably gathered much research attention due to their promising biomedical, and related applications [11–15]. SPIONs in particular magnetite (Fe3O4) nanoparticles have superlative properties...
which include relatively least toxicity, high magnetic susceptibility, and superparamagnetic behavior [16–18]. However, one of the major disadvantages of SPIONs is the agglomeration of the particles owing to their high surface energies [19,20]. To overcome this limitation, surface modifications of SPIONs with biocompatible materials are one of the reported strategies [21–25]. Chitosan (CS) is a natural biopolymer with comprehensive biological and chemical properties which has been used to coat the surface of SPIONs [26,27]. Structurally, CS consists of amino and hydroxyl functional groups that enable CS to easily graft to the surface of SPIONs. Thereby, CS enhances the stability, biocompatibility, and biodegradability of SPIONs [28,29].

Many works have reportedly coated CS on the SPIONs via co-precipitation, thermomixer incubation, stirring, solvothermal, and reverse microemulsion methods for various biomedical applications including drug delivery, contrast agents for magnetic resonance imaging (MRI), biosensor, enzyme, and protein immobilization, and cancer chemotherapy [30–34]. Unlike these methods which are laborious, time-consuming, and costly by requiring multiple reagents, several works have reportedly used facile and effective sonochemical methods to produce CS-SPIONs [35–37]. Successful functionalization of moieties on nanomaterials by the sonochemical method requires precise control and manipulation of several reactions and operational parameters such as sonication time, ultrasound frequency, energy input, and power [38,39]. These conditions can allow a rapid, facile, and effective method of grafting biomolecules on SPIONs [40]. However, to the best of our knowledge, no work has studied the effect of ultrasonic irradiation on the physicochemical properties, cellular uptake, and biocompatibility of CS-SPIONs synthesized by the sonochemical method. In this work, the influence of acoustic cavitation on the crystallization, stability, magnetization, toxicity, and biocompatibility of the CS coated SPIONs is reported. Herein, SPIONs are prepared through co-precipitation method. Subsequently, CS solution was mixed with a colloidal suspension of SPIONs and sonicated at different sonication periods of (1.5, 5, and 10) min with corresponding ultrasonic power of (67, 40, 36) W, respectively. Physicochemical analyses of the functionalized SPIONs show that at a lower sonication period but higher acoustic power crystalline CS-SPIONs with enhanced saturation magnetization samples are produced.

2. Materials and method

Ferric chloride hexahydrate (FeCl₃·6H₂O, ≥98%), ferrous chloride tetrahydrate (FeCl₂·4H₂O, 98%), low molecular weight chitosan, Phosphate Buffer Saline (PBS, pH 7.4), Potassium hexacyanoferrate (II) trihydrate (K₂Fe(CN)₆·3H₂O), and Eosin Y dye were purchased from Sigma-Aldrich. Concentrated ammonium hydroxide (NH₄OH, 28–30%) was obtained from Merck. All chemicals were used as received without any purification. Ultrasonic irradiation was done using a Vibra-Cell probe with a 1.3 cm tip size horn, frequency (20 kHz), and power (750 W).

The human embryonic kidney 293 (HEK-293) cell lines were acquired from American Type Culture Collection (USA). Metabolic dye [4,5-dimethylthiazol-2-yl] – 2,5-diphenyl tetrazolium (MTT), and dimethyl sulfoxide (DMSO) were acquired from Thermo Fisher Scientific (Massachusetts, USA) and Bio Basic Inc. (Ontario, Canada), respectively. Dulbecco’s modified Eagle’s medium (DMEM), Fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-ethylenediamine tetraacetic acid (Trypsin-EDTA) were purchased from Gibco (Massachusetts, USA).

A. Synthesis of SPIONs: Bare SPIONs were synthesized by a modified co-precipitation method. Ferric (Fe³⁺, 1.33 g) and Ferrous (Fe²⁺, 0.6 g) salts were separately and completely dissolved in (40 ml) of deionized (DI) water for two minutes with sonication. The iron solutions were mixed in a 100 ml three-neck round-bottom flask and mechanically stirred with a glass stirrer at 700 rpm under inert nitrogen gas to maintain the inert atmosphere. Next, 12.5 ml of ammonium hydroxide (NH₄OH) was added drop-wise to the iron mixture and simultaneously heated up to 70 ± 3 °C for 20 min. The resulting SPIONs were subsequently separated from unreactive impurities using a permanent external magnet. The sample was washed three times with DI water and re-dispersed in DI water. The purified aqueous colloidal suspension of SPIONs was instantly utilized.

B. Synthesis of CS coated SPIONs: CS solution was prepared with 35 mg of CS powder which was dissolved in 100 ml aqueous acetic acid (0.35 M). Three samples (S₁, S₅, and S₁₀) of CS-SPIONs were produced by adding 2 ml of the prepared CS solution to 25 ml colloidal suspension of SPIONs and sonicated without an iced bath (heat dissipation) for different sonication periods of 1.5, 5, and 10 min and powers of 67, 40, and 36 W respectively. The ultrasonic output power for each period was measured and calculated by Eq. (1) [41]. The results were left for 24 h. S₁, S₅, and S₁₀ were purified through centrifugation at 10000 rpm for 15 min at room temperature.

\[
\text{Power (W)} = \frac{E (J)}{T (s)}
\]

Where E is the consumed energy in Joules (J), and T is the reaction period in seconds (s).

S₁ = 6045/90 = 67 W.
S₅ = 12030/300 = 40 W.
S₁₀ = 21619/600 = 36 W.

C. Characterization: To evaluate the structure, morphological properties, and sizes of the produced samples, X-ray diffraction (XRD), PANalyticalX’pert PRO MRD PW 3040 with Cu-Kα radiation (λ = 1.54060Å), field emission scanning electron microscopy (FESEM) (FEI Nova NanoSEM 450), transmission electron microscope (TEM) (Zeiss Libra 120 at 100 kV), and ImageJ software were employed. Fourier transform infrared spectroscopy (PERKIN ELMER system 2000 FT-IR) was used to determine the surface functional groups of CS-SPIONs. Energy dispersive X-ray (EDX, analyzer Oxford Instruments X-Max) was deployed to identify elements present in the as-synthesized CS-SPIONs. The Zeta potential (stability) of the nanoparticles was measured via dynamic light scattering (DLS, ZETA SIZER Nanoseries Model ZEN 3600, Malvern Instruments). The magnetic properties of the samples were evaluated using a vibrating sample magnetometer (VSM, LAKESHORE MODEL 7404) at room temperature.

D. Assessment of Cytotoxicity: In vitro cytotoxicity of CS coated SPIONs was examined in HEK-293 cells using the metabolic dye MTT assay. HEK-293 cells were cultured and grown in DMEM, supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics (penicillin/streptomycin) at 37 °C in 5% CO₂ with 95% humidify. The assay was performed in a 96-well plate with the density of 1 x 10⁴ cells per well in 100 μl of complete growth medium and incubated at 37 °C in 5% CO₂. Cell seeding for 24 h, 100 μl of culture media containing five different concentrations (100, 200, 300, 400, and 500 μg/ml) of sample S₁, S₅ was added to each well and incubated for 24, 48, and 72 h, respectively. Six replicate wells were used for each control and treated concentration per micro-plate. After each time point, 30 μl MTT (2.5 mg/ml in PBS) was added to each well and the plates were incubated for 4 h at 37 °C in 5% CO₂. Afterward, the medium was removed, the DMSO (100 μl/well) was added for dissolving the crystals and the plates were incubated for 5 min. Then, the spectrophotometric absorbance of the samples was measured using the micro-plate reader (ELx800, BicTek Instruments, USA) at 570 nm wavelength.

The percentage viability of cells was evaluated using Eq. (2a):

\[
\text{Cell viability} = \frac{\text{Absorbance(sample)}}{\text{Absorbance(control)}} \times 100
\]

E. Cell uptake of CS-SPIONs: In order to measure the uptake of the produced nanoparticles by HEK-293 cells, Prussian blue staining was utilized. HEK-293 cells were incubated with various concentrations (0, 50, 100, and 200 μg/ml) in 24-well plates at a density of 10⁵ cells/well
(n = 3) for 24 h. After incubation, cells were washed twice with PBS, fixed with 4% formalin (PBS-buffered formalin), and incubated for 20 min at room temperature. To stain intracellular iron, equal volumes of HCl (2.4 M) and K$_4$Fe(CN)$_6$·3H$_2$O (0.24 M) were mixed to prepare the Prussian blue solution and incubated with the fixed cells for 30 min at room temperature. Afterward, the fixed cells were washed 2–3 times with autoclaved distilled water and counter-stained with Eosin Y dye for 30 s. Consequently, the cells were washed again with autoclaved distilled water 2–3 times and placed on a microscope to visualize and observe the cellular uptake of the nanoparticles.

3. Results and discussion

3.1. XRD analysis

The effects of different sonication periods on the crystalline structure and purity of the CS-SPIONs (S$_{bare}$, S$_{1.5}$, S$_{5}$, and S$_{10}$) are shown in Fig. 1.

The peaks correspond to the JCPDS database (Ref. Code 01-075-0033) of the Fe$_3$O$_4$ phase. Comparatively, the XRD peak intensity of S$_{bare}$ and S$_{1.5}$ are high as observed in Fig. 1(a and b). Both samples showed cubic inverse spinel crystal structure [42]. Using the diffraction planes (hkl), seven prominent characteristic peaks of Fe$_3$O$_4$: (220), (311), (400), (422), (511), (440) and (533) were observed in S$_{bare}$ and S$_{1.5}$. Like the S$_{bare}$, the XRD result of S$_{1.5}$ shows that the sonication conditions did not cause phase change in both samples.

However, broadening of the main peaks and a decrease in intensities were observed in the XRD spectra of S$_{5}$ and S$_{10}$ as illustrated in Fig. 1(c and d). A shift in the d-spacing values of (220) and (311) planes is observed in both samples. The inter-planar spacing (d-spacing) values were calculated according to Eq. (2b).

\[
d_{hkl} = \frac{\lambda}{2 \sin \theta}
\]

where hkl are Miller indices, \(\lambda\) is the wavelength of X-ray for CuK\(\alpha=1.5406\)Å, and \(\theta\) is the Bragg’s angle in degrees [43].

In addition, two peaks (422) and (533) in S$_{5}$ and three peaks (400), (422), and (533) in S$_{10}$ did not appear. These indicate that the crystallinity of the two samples was affected by the increase in the sonication period and the decrease in the acoustic power. This finding suggests that an increase in the ultrasonic period with an increase in the intense heating condition affects the crystalline structure of nanoparticles. This shortcoming can be minimized via heat dissipation, i.e. irradiating the mixtures under an iced bath environment [10].

3.2. Surface charge analysis

It is well known that the SPIONs are susceptible to agglomeration due to their high energy and large surface area. A large absolute Zeta Potential (\(\zeta\)) value implies colloidal stability of the particles [44]. Generally, ± 30 mV is the common distinct criterion between stable and unstable colloidal suspensions. Particles with higher values are considered stable [45]. \(\zeta\) values between ± 40 to ± 60 mV indicate excellent colloidal stability [46]. Experimental works show that the sonochemical route is one of the influential techniques that can break down large
particle clusters into smaller ones or even discrete. This can extend stability (dispersibility) of aqueous media for a long duration [47,48]. As shown in Fig. 2, the $\zeta$ values of the $S_{\text{bare}}$, $S_{1.5}$, $S_5$, and $S_{10}$, are 35.9, 48.7, 48.9, and 56.9 mV, respectively. The results indicate that the samples are very stable [23,31]. The values increase with increase in the sonication period. However, the $\zeta$ values of $S_{1.5}$ and $S_5$ are approximately the same. The increase in $\zeta$ values of the sonicated samples can probably be due to: 1) the ultrasonic irradiation increases the amount of positively charged amino group of CS that binds to the SPIONs or the electrostatic repulsion between the formed positive ($-\text{NH}_3^+$) charge on CS-SPIONs surface [23,49]. 2) The acoustic cavitation process of the ultrasonic irradiation can influence the stability of the colloidal solutions through rapid grafting of the CS to the SPIONs while also preventing the agglomeration of the clusters [50].

### 3.3. Elemental analysis (Fe, O, C and N)

The EDX spectra were utilized for quantitative analysis of elements present in $S_{\text{bare}}$ and CS-coated SPIONs ($S_{1.5}$, $S_5$, and $S_{10}$). The corresponding elemental peaks in $S_{\text{bare}}$ were due to Fe, O, and C elements, while CS-SPIONs exhibited additional N and C peaks. The presence of N in the spectra of $S_{1.5}$, $S_5$, and $S_{10}$ confirms the presence of CS on the SPIONs surface. In the $S_{\text{bare}}$, C denotes contaminants which can be due to impurities from the substrate used during sample preparation. The atomic percentage and weight concentration of elements present in the samples are displayed in the upper right corner of Fig. 3a–d. It is important to observe here that only N’s atomic and weight percentage concentrations are further discussed because the element represents the binding of the terminal amino functional group of CS on the SPIONs. The atomic percentage of N in $S_{1.5}$, $S_5$, and $S_{10}$ are 1.5, 1.29, and 0.75, respectively. The weight percentages are 0.64, 0.56, and 0.32, respectively. The atomic and weight percentage of N in $S_{1.5}$ are higher than $S_5$ and $S_{10}$. The results show the number of N decreases with an increase in the sonication period but decreases in acoustic power. This shows that most CS grafted on the SPIONs at the 1.5 min sonication period.

Based on the XRD, zeta potential, and EDX results, $S_{1.5}$ is the best and most preferable sample. This sample is used for further analyses such as FTIR, TEM, FESEM, VSM, cellular uptake, and cytotoxicity.
3.4. FTIR spectra

The successful grafting of CS on the SPIONs is confirmed using FTIR spectra as shown in Fig. 4. The absorption peak around 3276 cm\(^{-1}\) is observed in all three spectra corresponding to the (–OH) group. The peak around 3276 cm\(^{-1}\) and 1640 cm\(^{-1}\) are attributed to stretching and bending vibrations of the OH group absorbed due to water molecules (O–H) on the SPIONs surface, respectively. In the IR spectrum of S\(_{\text{bare}}\) (Fig. 4A), the energy absorbed at 680 cm\(^{-1}\) is due to the metal oxide (M–O) vibration, which is the Fe-O bond of the iron oxide [51]. Although, it is previously reported that the characteristic \(\nu_1\) and \(\nu_2\)-absorption band for Fe-O appeared at 590 and 445 cm\(^{-1}\), respectively [52], like the findings of Sureshkumaret al., the Fe-O band appeared at 686 cm\(^{-1}\) in the S\(_{\text{bare}}\) and S\(_{1.5}\) in Fig. 4 (A and C), shifted to 680 and 639 cm\(^{-1}\), respectively. In the CS spectrum, Fig. 4 (B), the absorption peak at 1638 cm\(^{-1}\) can be referred to the vibrational bending of N–H [53] which is the peak of the primary terminal amine group (–NH\(_2\)) [54]. In Fig. 4B, the peaks at 1454 cm\(^{-1}\) and 1417 cm\(^{-1}\) match with C–H bending. The characteristic peaks at 2845 cm\(^{-1}\) and 2987 cm\(^{-1}\) appear as the stretching of the alkyl C–H group in CS and the sharp peak at 1015 cm\(^{-1}\). The stretching of C-O in CS appeared at 1045 and 1087 cm\(^{-1}\). This can be related to the alcoholic primary group. In comparison with the CS spectrum (Fig. 4B), the peak at 1638 cm\(^{-1}\) is shifted to 1642 cm\(^{-1}\) in the IR spectrum of S\(_{1.5}\) (Fig. 4C) due to amide absorption which corresponds to high intensity. The broad absorption peak is found to be relatively larger (shrink). This increase in absorption intensity can be explained by the fact that the hydrogen of the amino-functional group in CS forms a strong hydrogen bonding with the oxygen in SPIONs [55].

Moreover, the dip due to the N–H bending of the terminal amine group in S\(_{1.5}\) (Fig. 4C) is overlapped with the O–H dip at 1642 cm\(^{-1}\). The other dips that appeared in the spectrum correspond to the stretching and bending of C–H and stretching of C–O in the primary alcoholic group of the CS. The energy absorbed at 680 cm\(^{-1}\) that is assigned to the Fe-O band S\(_{\text{bare}}\) is shifted to 639 cm\(^{-1}\) in the S\(_{1.5}\). The shift is due to the binding of the functional amine group to the SPIONs. The other peaks which appeared in the IR spectrum of S\(_{\text{bare}}\) (Fig. 4A) are due to overlapping. The FTIR results indicate the successful attachment of CS onto the SPIONs surface. These findings further demonstrate the ability of ultrasonic irradiation to rapidly graft the amino group in CS onto the nanoparticles within a minimum sonication period of one and half minutes.

3.5. TEM analysis

The TEM micrograph of the S\(_{\text{bare}}\) and S\(_{1.5}\) are shown in Fig. 5 (A and B), respectively. As illustrated in Fig. 5 (B), like S\(_{\text{bare}}\), the morphology of S\(_{1.5}\) nanoparticles maintained the spherical shapes surface modification. As shown in the inset of Fig. 5 (A and B), the S\(_{\text{bare}}\) and S\(_{1.5}\) have average particles diameter of 10.9 and 11.1 nm, respectively. The increase in the size of S\(_{1.5}\) can be associated with the grafting of the CS on the SPIONs [56]. In addition to the findings of Yang et al., [57] which recently demonstrated that the use of ultrasonic irradiation of different power ranges between 0 and 500 W can decrease the size of nanoparticles exponentially with an increase in ultrasonic power, our result shows at minimum sonication period mono-dispersed CS coated SPIONs with a narrow size distribution can be prepared as shown in Fig. 5 B.

3.6. FESEM observation and EDX mapping

The morphology of the CS-SPIONs was analyzed using FESEM. As shown in Fig. 6, the coated nanoparticles are spherically shaped and...
exhibit a uniform surface. Images of elemental mapping for the sample $S_{1.5}$ are presented in Fig. 7. Fe, N, C, and O elements are evenly distributed across the sample. This further confirms the presence of CS on the surface of SPIONs.

3.7. Magnetic properties

The magnetization results of $S_{\text{bare}}$ and $S_{1.5}$ are presented in Fig. 8. Both samples exhibit superparamagnetic behavior with no remanence and zero coercivity. The saturation magnetization ($M_s$) of $S_{\text{bare}}$ and $S_{1.5}$ at room temperature are found to be 62.95 and 86.93 emu/g, respectively. The results indicate that the sonochemical assisted functionalization process has no decline impact on the magnetic properties of SPIONs. Instead, the grafting process enhanced the $M_s$ of the nanoparticles. The enhancement can probably be attributed to: 1) The proportion of magnetite vs. maghemite ($\gamma-\text{Fe}_2\text{O}_3$) in the core of CS-SPIONs [58]. The $\text{Fe}_3\text{O}_4$ nanoparticles have higher $M_s$ and magnetic susceptibility compared to $\gamma-\text{Fe}_2\text{O}_3$ [59]. The uncoated ferrofluid can easily oxidize to $\gamma-\text{Fe}_2\text{O}_3$ due to exposure to air [60], thereby affecting the $M_s$ of $S_{\text{bare}}$. However, rapid and instant coating of the SPIONs with CS layer retained the magnetite phase of the $S_{1.5}$ with a high $M_s$ value. 2) The refinement of the SPIONs during the grafting process by the acoustic cavitation process is another aspect that might enhance the magnetization. Recent studies have revealed that the ultrasonic irradiation’s power and time can influence the purity, particle size, and particle refinement of the products [57,61]. Although CS is a diamagnetic material which supposed to affect the $M_s$ of the CS coated SPIONs, our result shows that the ultrasonic irradiation influenced the final $M_s$ of the as-synthesized samples. However, this is an ongoing project further research is needed to establish the role of ultrasonic irradiation in influencing the $M_s$ of the CS coated SPIONs.

Table 1 compares our results with similar works based on the sonochemical method. Our results show that at a minimum sonication period of 1.5 min and power of 67 W, highly stable, crystalline, and enhanced magnetic CS-SPIONs can be synthesized. Unlike other reports that prepare CS-SPIONs at higher sonication period, our result shows minimum sonication period is required to produce highly magnetic CS coated SPIONs.

3.8. Cell viability

The cytotoxic effect of CS-SPIONs on HEK-293 cells is evaluated via MTT assay at different concentrations (100 to 500 $\mu$g/ml) and different incubation times (24 to 72 h). The results are presented in percentage cell viability. In the MTT assay, the enzyme activity in the mitochondria is detected by the reduction of MTT tetrazolium salts, which only occurs in living cells [62]. As presented in Fig. 9, the examined CS coated SPIONs had no obvious adverse effect on cell viability (no toxicity) at the doses used and incubation times. The viability of HEK-293 cells is reduced by increasing the concentrations of the nanoparticles and incubation times. However, the cell viability percentage was more than 70% at all concentrations and different incubation periods. Previous reports have demonstrated that nanoparticles with the viability of cells at 70% and above can be used as biocompatible nanomaterials [63]. This result confirmed that the CS-SPIONs synthesized by the sonochemical method were found to be non-toxic to HEK-293 cells. So, the samples have the potential used for biomedical applications.
3.9. Cell uptake of CS-SPIONs

Fig. 10 (a–d) illustrates the Prussian blue staining of the HEK-293 cellular uptake. S1.5 is incubated at various concentrations (50 μg/ml to 200 μg/ml) for 24 h. It can be seen clearly from the first concentration of 50 μg/ml that the cytoplasm of the cells is stained blue. The blue color of the cytoplasm increases with an increase in the concentration of the nanoparticles. However, the cytoplasm of the control group is stained

Table 1
Synthesis and characteristic comparison of the previous works on CS-SPIONs using sonochemical methods.

| Coated method | Coating reaction time | Particle size (nm) | ζ-potential (mV) | Saturation magnetization \(M_s\) (emu/g) | Ref. |
|---------------|-----------------------|-------------------|------------------|----------------------------------------|-----|
| Bare SPIONs   |                       | 15.1              |                  | 51.68                                  |     |
| Sonochemical  | 30 min                |                   |                  | 49.96                                  | [35]|
| Sonochemical  | 20 min                |                   |                  | 68.80                                  | [36]|
| Sonochemical  | 30 min                | 30                | High stability   | 25.60                                  |     |
| Sonochemical  | 1.5 min               | 11.1              | 48.7             | 40.00                                  | [37]|
| Sonochemical  | 1.5 min               |                   |                  | 62.95                                  |     |
| Sonochemical  | 1.5 min               |                   |                  | 86.93                                  | Present study |

Fig. 9. Viability of HEK-293 cells after (24–72 h) incubation times with different concentrations (100–500 μg/ml) of S1.5. Data are presented as the mean ± SEM using Two-way ANOVA (multiple comparisons). Statistically, all the groups are highly significant compared to their control groups (\(p < 0.001\)).

Fig. 10. Prussian blue staining of the HEK-293 cells uptake of S1.5 at different concentrations (a) control, (b) 50 μg/ml (c) 100 μg/ml, and (d) 200 μg/ml for 24 h of incubation.
red, Fig. 10(a). The results indicate that the intracellular uptake (internalization) of the nanoparticles is dose-dependent. It is critical to understand that the cellular uptake of nanoparticles is considered for various biomedical applications, specifically in MRI, magnetic hyperthermia, and drug delivery. A previous study investigated that the concentration of nanoparticles and incubation period are the two main factors that affect the properties of the cellular uptake [64]. In our present study, we have demonstrated that the majority of cellular uptake of thermia, and drug delivery. A previous study investigated that the intracellular uptake of the CS-SPIONs occurs at the highest concentration within 24 h of incubation and more than 90% of the cells were viable at 200 μg/ml. This result implies the biocompatibility of the CS SPIONs produced with 1.5 min sonication period.

4. Conclusion
This study presents the influence of ultrasonic irradiation on the crystallization, stabilization, functionalization, magnetization, and biocompatibility of CS coated SPIONs. The results indicate that CS can be grafted onto the SPIONs with a minimum sonication period of 1.5 min and a maximum power of 67 W. In addition, Prussian blue staining of HEK-293 cells incubated with the highest concentration of 200 μg/ml confirmed the presence of the nanoparticles in the majority of the cells. Besides, the viability of HEK-293 cells was more than 70% after being exposed to the CS-SPIONs with the highest concentration and incubation time. This indicates the as-synthesized CS-SPIONS is biocompatible.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References
[1] F. Dang, N. Enomoto, J. Hojo, K. Enpuku, Sonochemical synthesis of monodispersed magnetic nanoparticles by using an ethanol-water mixed solvent, Ultrason. Sonochem. 16 (2009) 649–654, https://doi.org/10.1016/j.ultsonch.2008.11.003.
[2] M.d. Nazrul Islam, L.e. Van Phong, J.J. Jeong, CheolGi Kim, A facile route to sonochemical synthesis of magnetic iron oxide (Fe3O4) nanoparticles, Thin Solid Films 519 (23) (2011) 8277–8279.
[3] Z.R. Stephen, C.J. Dayringer, J.J. Lim, R.A. Revia, M.V. Halbert, M. Jeon, A. Bakhtavatsalam, R.G. Ellenbogen, M. Zhang, Approach to Rapid Synthesis and Functionalization of Iron Oxide Nanoparticles for High Gene Transmission, ACS Appl. Mater. Interfaces. 8 (2016) 6520–6528, https://doi.org/10.1021/acsami.6b01883.
[4] M.A. Almessiere, Y. Slimani, A.D. Korkmaz, N. Taskhandi, M. Sertkol, A. Baykal, S. Ayyanaar, C. Balachandran, R.C. Bhaskar, M.P. Kesavan, S. Aoki, R.P. Raja, S. Laurent, D. Forge, M. Port, A. Roch, C. Robic, L. Vander Elst, R.N. Muller, Functionalization of Iron Oxide Nanoparticles for High Gene Transfection, ACS Nano 5 (2011) 2058–2064.
[5] S. Ayyanaar, C. Balachandran, R.C. Bhaskar, M.P. Kesavan, S. Aoki, R.P. Raja, S. Laurent, D. Forge, M. Port, A. Roch, C. Robic, L. Vander Elst, R.N. Muller, Functionalization of Iron Oxide Nanoparticles for High Gene Transfection, ACS Nano 5 (2011) 2058–2064.
[6] V. Vinoth, J.J. Wu, A.M. Asiri, S. Anandan, Sonochemical synthesis of silver nanorods, J. Phys. Chem. B. 106 (2002) 3848–3854, https://doi.org/10.1021/jp0155050.
[7] B.K. Sedipo, A.A. Aziz, Recent advances in synthesis and surface modification of superparamagnetic iron oxide nanoparticles with silica, J. Magn. Magn. Mater. 416 (2016) 275–291, https://doi.org/10.1016/j.jmmm.2016.05.019.
[8] G. Kandasamy, D. Maity, Recent advances in superparamagnetic iron oxide nanoparticles (SPIONs) for in vitro and in vivo cancer nanotheranostics, Int. J. Pharm. 496 (2015) 191–218, https://doi.org/10.1016/j.ijpharm.2015.10.058.
[9] A. Ali, H. Zafar, M. Zia, I. ul Haq, A.R. Phull, J.S. Ali, A. Hussain, Synthesis, characterization, applications, and challenges of iron oxide nanoparticles, Nanotechnol. Sci. Appl. 9 (2016) 49–87, https://doi.org/10.1166/ntsa.2016.99886.
[10] Q. Li, C.W. Kartikowati, S. Horie, T. Ogi, T. Iwaki, K. Okuyama, Correlation between particle size/domain structure and magnetic properties of highly crystalline Fe3O4 nanoparticles, Sci. Rep. 7 (2017) 8984, https://doi.org/10.1038/s41598-017-09982-0.
[11] S. Murep, M.F. Hansen, C. Frandsen, Magnetic interactions between nanoparticles, Beilstein J. Nanotechnol. 1 (2010) 182–190, https://doi.org/10.3762/bjnano.1.22.
[12] W. Ling, M. Wang, C. Xiong, D. Xie, Q. Chen, X. Xu, Q. Yi, L. Xiao, Synthesis, surface modification, and applications of magnetic iron oxide nanoparticles, J. Mater. Res. 34 (2019) 1828–1844, https://doi.org/10.1557/jmr.2019.129.
[13] M. Balas, C.S. Gobanu, C. Burtse, M.S. Stan, E. Bezirtzoglou, D. Predoi, A. Dinischiotu, Synthesis, characterization, and toxicity evaluation of dextran-coated iron oxide nanoparticles, Metals (Basel). 7 (2017), https://doi.org/10.3390/met7020063.
[14] J. Ge, M. Zhai, Y. Zhang, J. Bian, J. Wu, Biocompatible Fe 3 O 4/chitosan scaffolds with high magnetism, Int. J. Biol. Macromol. 128 (2019) 406–413, https://doi.org/10.1016/j.jbiomac.2019.01.077.
[15] M. Khalikhli, K. Rostamizadeh, S. Sadighian, F. Khoeini, M. Naghibi, M. Hamidi, The impact of polymer coatings on magnetite nanoparticles performance as MRI contrast agents: A comparative study, BARU, J. Pharm. Sci. 23 (2015), https://doi.org/10.1016/j.rspcr.2015.04.014.7.
[16] N. Mohamad Nor, A. Kurabz Razak, S.C. Tan, R. Noordin, Properties of surface functionalized iron oxide nanoparticles (ferrofluid) conjugated antibody for lateral flow immunoassay application, J. Alloys Compd. 538 (2012) 100–106, https://doi.org/10.1016/j.jallcom.2012.05.053.
[17] M.Z. Iqbal, W. Ren, M. Saeed, T. Chen, X. Xu, Y. Yu, J. Zhang, L. Zhang, A. Li, A. Wu, A facile fabrication route for binary transition metal oxide-based Janus nanoparticles for cancer theranostic applications, Nanomaterials. 11 (2021) 1–21, https://doi.org/10.3390/nano11120063.
[18] I.Y. Kim, S.J. Seo, H.S. Moon, M.K. Yoo, I.Y. Park, B.C. Kim, C.S. Cho, Chitosan and its derivatives for tissue engineering applications, Biotechnol. Adv. 26 (2008) 1–21, https://doi.org/10.1016/j.biotechadv.2007.07.009.
[19] S. Ayyanaar, C. Balachandran, R.C. Bhaskar, M.P. Kesavan, S. Aoki, R.P. Raja, S. Laurent, D. Forge, M. Port, A. Roch, C. Robic, L. Vander Elst, R.N. Muller. Magnetic iron oxide nanoparticles: Synthesis, stabilization, vectorization, physicochemical characterizations and biological applications, Chem. Rev. 108. (2008). 2064–2110. 10.1021/cr078123x.
[20] S. Shen, Y. Yu, G. Fan, G. Chen, Y. min Jin, W. Tang, W. Jia, The synthesis and characterization of monodispersed chitosan-coated Fe3O4 nanoparticles via a facile one-step solvothermal procedure for superparamagnetic iron oxide nanoparticles, Nanoscale. 4 (2012) 1–8, https://doi.org/10.1039/c1nr10644e.
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