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A simple way to synthesize tartaric acid, ascorbic acid and their mixture coated superparamagnetic iron oxide nanoparticles with high saturation magnetisation and high stability against oxidation: Characterizations and their biocompatibility studies

Fatmahan Özel[a,1], Oznur Karaagac[a,1], Esra Tokay[b,2], Feray Köçkar[b,2], Hakan Köçkar[a,*,1]

[a] Department of Physics, Balikesir University, 10145 Balikesir, Turkey
[b] Department of Molecular Biology and Genetics, Balikesir University, 10145 Balikesir, Turkey

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ABSTRACT

A simple way to synthesise biocompatible superparamagnetic iron oxide nanoparticles coated with ascorbic acid, tartaric acid and a mixture of ascorbic acid and tartaric acid is proposed. The iron oxide nanoparticles were synthesized and coated with the surfactants in one step by co-precipitation method at room temperature in air atmosphere. The samples have the characteristic (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1), (4 4 0) peaks of cubic spinel structure of iron oxide. Fourier Transform Infrared Spectroscopy analysis revealed that the nanoparticles were coated with the surfactants. The mean physical sizes of core obtained from the transmission electron microscopy images were 7 ± 2 nm. Magnetic measurements by vibrating sample magnetometer indicate that all samples are superparamagnetic at room temperature. And, the saturation magnetization, M_s of coated nanoparticles was around 62 emu/g. The effectiveness of the coating on the surface of nanoparticles against oxidation is also an important parameter. The coating agents successfully protected the core against oxidation since the M_s stayed almost stable (~62) emu/g by 12 weeks and then decreased to ~57 emu/g by 48 weeks. It can be concluded that coated superparamagnetic nanoparticles have high saturation magnetization and high stability against oxidation. Furthermore, the MTT assays were also carried out for cytotoxicity of various concentration of coated nanoparticles by using Hep3B, Saos-2 and HUVEC cell lines. From the MTT assay results, it is observed that the coated nanoparticles are non-toxic for all types of cells investigated and therefore can be biocompatible for potential biomedical applications.

1. Introduction

Magnetic nanoparticles (especially magnetite, Fe_3O_4 and maghemite, γ-Fe_2O_3) have attracted much attention due to their unusual chemical and physical properties different from the bulk forms [1]. Because of these properties they have a lot of application such as catalysis, magnetic data storage, ferrofluid technology, energy storage and environmental applications, and they also have found widespread use in biomedical research [2–4]. Superparamagnetic nanoparticles have been exploited for labelling and separation of DNA, proteins, bacteria and various biological species, as well as used in magnetic resonance imaging (MRI), guided drug delivery, and hyperthermia treatment of cancer [5–8].

For biomedical applications, magnetic properties of the nanoparticles must be preserved long last and the nanoparticles must be non-toxic for biological environment. In order to achieve these specialities, magnetic nanoparticles have been coated with various capping materials [9–13]. Capping agents are also effective on controlling the size and shape of the nanoparticles during the synthesis procedure [14,15].

Ascorbic acid (AA) is a biocompatible surfactant and is used in many studies to coat magnetic nanoparticles. AA coated nanoparticles can be used in biological applications especially for MRI [16–18]. Xiao et al. [16] and Gupta et al. [17] synthesized superparamagnetic Fe_3O_4 nanoparticles and they examined the potential of the nanoparticles as

* Corresponding author.
E-mail addresses: fatmahanzel@hotmail.com (F. Özel), karaagac@balikesir.edu.tr (O. Karaagac), esratokay@balikesir.edu.tr (E. Tokay), fkockar@balikesir.edu.tr (F. Köçkar), hkockar@balikesir.edu.tr (H. Köçkar).
[1] Address: Balikesir Universitesi, Fen Edebiyat Fakultesi, Fizik Bolumu, 10145, Cagis Yerleskesi, Balikesir, Turkey.
[2] Address: Balikesir Universitesi, Fen Edebiyat Fakultesi, Molekuler Biyoloji ve Genetik Bolumu, 10145, Cagis Yerleskesi, Balikesir, Turkey.

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negative contrast agent for MRI. Also, tartaric acid (TA) is a biocompatible surfactant. There are studies about TA coated magnetic nanoparticles and their possible usage for MRI contrast agent [19,20].

Various synthesis routes have been employed to produce magnetic nanoparticles such as co-precipitation, microemulsion, hydrothermal method, thermal decomposition and sonochemical approach [21]. Magnetic properties of these nanoparticles strongly depend on their size and surface properties. Therefore, for the synthesis of nanoparticles with the desired properties, it is important to select the appropriate method and the surfactant. The co-precipitation method is the simplest and most efficient chemical pathway to synthesize naked and coated superparamagnetic iron oxide nanoparticles [1]. Besides, hydrothermal treatment is one of the successful ways of growing crystals for nanoparticles [22]. For the synthesis of AA and TA coated nanoparticles, generally hydrothermal method is used in the literature [16,23–25].

In this study, the surface of magnetic nanoparticles was coated with biocompatible surfactants: AA and TA in order to make the nanoparticles appropriate for biological applications. The nanoparticles coated with AA, TA and a mixture of AA + TA were synthesized by hydrothermal process combined with co-precipitation method. The precipitation was performed at room temperature in air enviroment. The hydrothermal process was applied right after the co-precipitation. This method is quite simple and a lot of product can be obtained in one go. Results showed that coated superparamagnetic nanoparticles have high saturation magnetization and high stability against oxidation. It is also seen from the in vitro cytotoxicity experiments that the coated nanoparticles are non-toxic and biocompatible for biomedical applications.

2. Experimental

2.1. Synthesis procedure

Ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$ Merck > 99%) ferric chloride hexhydrate ($FeCl_3{\cdot}6H_2O$ Merck > 99%) salts and ammonium hydroxide (NH_4OH Merck, 25% of ammonia) were used for the synthesis of iron oxide nanoparticles. L(+)Ascorbic acid(Carlo Erba, 99%) and L-(+)-Tartaric Acid (Sigma-Aldrich, ≥99.7) were used for the coating agents. All chemicals were of reagent grade and used without further purification. The nanoparticles were synthesized using co-precipitation method, and then hydrothermal treatment was applied to the samples.

Co-precipitation: Iron oxide core nanoparticles (IONs) were synthesized according to the procedure in [26]. First, NH_4OH (25%) was added to 25 ml of an aqueous solution containing a total 75 mmol of $FeCl_2{\cdot}4H_2O$ and $FeCl_3{\cdot}6H_2O$ at molar ratio of 1:1 due to the synthesized in air atmosphere. The molar ratio of Fe^{2+}:Fe^{3+} is chosen to be 1:1 due to the fact that the synthesis takes place in air environment, for further information [26]. The solution was stirred for 2 min at 700 rpm and iron oxide nanocrystals were obtained. Iron oxide nanoparticles obtained after this step were called as naked iron oxide nanoparticles (naked IONs). For AA coated iron oxide nanoparticles obtained by co-precipitation (C-AA-IONs), 200 mM AA solution was added to the solution right after the nanoparticles were formed at the end of 2 min and the reaction was continued under stirring at 1000 rpm for 15 min. Since AA may prevent the crystallization of nanoparticles, it is added to the system after the formation of the nanoparticles. Thus, it is not effective in the process of magnetic core formation and the only function of AA in this synthesis is surface coating. The C-AA-IONs were collected by a magnet and washed many times with distilled water to remove the remaining free surfactants and then dissolved in water. To obtain powder form of the nanoparticles for the characterizations, the sample was dried at 60 °C. The same process was repeated for TA and AA + TA coated iron oxide nanoparticles. Samples C-TA-IONs and C-AA + TA-IONs were obtained by using TA (600 mM) and TA + AA (300 mM TA and 300 mM AA), respectively. Fig. 1 shows the dispersions of the coated nanoparticles in water and the collection of them by a magnet.

Hydrothermal treatment: In order to obtain hydrothermally synthesized AA coated IONs, 15 ml of the co-precipitated solution was transferred to the Teflon-sealed autoclave. The autoclave was kept at 155 ± 5 °C for 12 h and cooled naturally after the reaction. Thus, the sample H-AA-IONs was obtained by using hydrothermal combined co-precipitation method. The product was collected by magnet and washed with distilled water to obtain dispersion. And the washed sample was dried at 60 °C in the oven to get powder form. Samples H-TA-IONs and H-AA + TA-IONs were prepared by using the hydrothermal combined co-precipitation as described above by applying 155 ± 5 °C for 12 h. Another sample H-AA + TA-IONs-36 was synthesized at 155 ± 5 °C for 36 h.

2.2. Characterizations

The phase purity and phase structure of the samples were investigated by the X-ray powder diffraction (XRD) technique, using a PANalyticalX'Pert PRO X-ray diffractometer in the range of 2θ between 20 and 80° by using CuK_α (λ = 1.5406 Å) radiation. The crystal diameters of the nanoparticles were calculated by using the Scherrer equation [27]:

$$D_{XRD} = \frac{0.9\lambda}{\beta cos\theta} \quad (1)$$

Where, λ is the wavelength, β is the width at half-height of the maximum of the peak and θ is the diffraction angle. The particle sizes were calculated by using the most intense peak (3 1 1) in the patterns of the samples. For the analysis of the Fourier transform infrared (FT-IR) spectra, the powder samples were mixed with KBr powder to obtain pellets and then recorded on a Perkin-Elmer spectrometer. Transmission electron microscopy (TEM) images were taken by an FEI Tecnai G2 F30 model scanning electron microscope. The samples were prepared by placing one drop of a dilute suspension of iron oxide nanoparticles in water on a carbon coated copper grid. The physical particle sizes were manually counted and calculated from the TEM images by using ImageJ programme after taking the TEM pictures of nanoparticles. Magnetic properties of powder samples were measured on an ADE EV9 model vibrating sample magnetometer (VSM). ± 20 kOe was applied in 1 Oe intervals at room temperature.

2.3. Biocompatibility

In vitro biocompatibility studies of the samples naked IONs and H-AA + TA-IONs-36 were tested by MTT assay with different particle concentrations. We applied MTT assay in two cancer cell lines; Hep3B (Human Hepatoma Carcinoma Cells), Saos-2 (Human Osteosarcoma Cells) and a healthy model: HUVEC (Human Umbilical Vein Endothelial Cells) cells.

Preparation of Cells: All cells were cultured in DMEM(Gibco) containing 5 mM L-Glutamine (Sigma) and 10% fetalcalf serum (FCS) (Gibco) in a humidified incubator at 37 °C and 5% CO_2. Both cells were harvested by the use of trypsin and were re-suspended in the fresh complete medium before plating.

Treatment of cells with nanoparticles and Cell cytotoxicity Assay (MTT): The cells were plated out in 96-well plates with a density of 5×10^4 per well. After 24 h of incubation period for attachment of the cells, serial dilutions of C-AA + TA-IONs and naked IONs were added to the culture medium. MTT assays were carried out to quantify the cytotoxicity of various concentrations of nanoparticles 10 to 500 μg/mL coated with sample AA + TA while untreated cells were used as a control. At the end of the incubation (48 h), above medium was removed, and samples were treated with 20 μL of MTT (5 mg/mL) solution and incubated for 4 h. Then, isopropanol containing 0.004 M HCl (200 μL) was added to these samples to dissolve the formazan crystals. Thermo-microplate reader was used to measure the absorbance at a

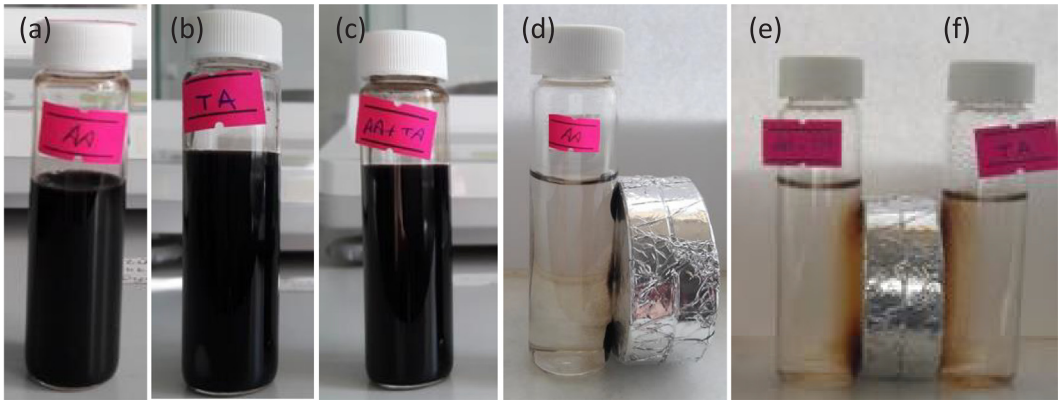

Fig. 1. Photos of the dispersions of (a) C-AA-IONs, (b) C-TA-IONs, (c) C-AA + TA-IONs, and photos of magnetic separation of them (d) C-AA-IONs, (e) C-AA + TA-IONs, (f) C-TA-IONs using a magnet.

wavelength of 550 nm [28]. All samples were evaluated in triplicate and the percent cell viability was determined by using the formula as Percentage of cell viability = (OD of Sample/OD of Control) × 100.

3. Results and discussion

The structural and magnetic characterizations of the naked IONs, coated nanoparticles synthesized by co-precipitation and by hydrothermal treatment were made besides the biocompatibility tests.

3.1. Structural analysis

XRD measurements were carried out to characterize the crystalline structure of the samples. The XRD patterns of the samples C-AA-IONs and H-AA-IONs were given in Fig. 2a and b, respectively as example since all samples had the same core structure. The samples have the characteristic (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1), (4 4 0) peaks of cubic spinel structure of magnetite (JCPDS no.019-0629) or maghemite phase (JCPDS no. 039–1346) as seen in the figure. No impurities were detected in the patterns. The crystal size of the sample C-AA-IONs was 8.5 nm whereas the size of the sample H-AA-IONs was 9.6 nm.

The presence of the coating agents on the surface of the nanoparticles was proven by the FT-IR spectroscopy analysis. The FT-IR spectrum of pure AA, TA and the samples obtained by co-precipitation were presented in Fig. 3. A series of AA bands (Fig. 3a) at 3519 cm^{-1}, 3404 cm^{-1}, 3306 cm^{-1} and 3209 cm^{-1} are attributable to OH vibrations. The bands at 1750 cm^{-1} and 1664 $cm^{-1}$are assigned to C=O and C=C bonds, respectively [16]. In the spectrum of TA (Fig. 3b), a broad band including 3404 cm^{-1} and 3325 cm^{-1}peaks is attributed to O–H stretching and the band at 1731 cm^{-1} to C=O stretching vibration [29]. The flexing vibration of hydroxyl group (–OH) can be observed between 3100 cm^{-1} and 3600 cm^{-1}. This band may result from the absorbed H_2O molecules on the surface or –OH group of TA or AA. For

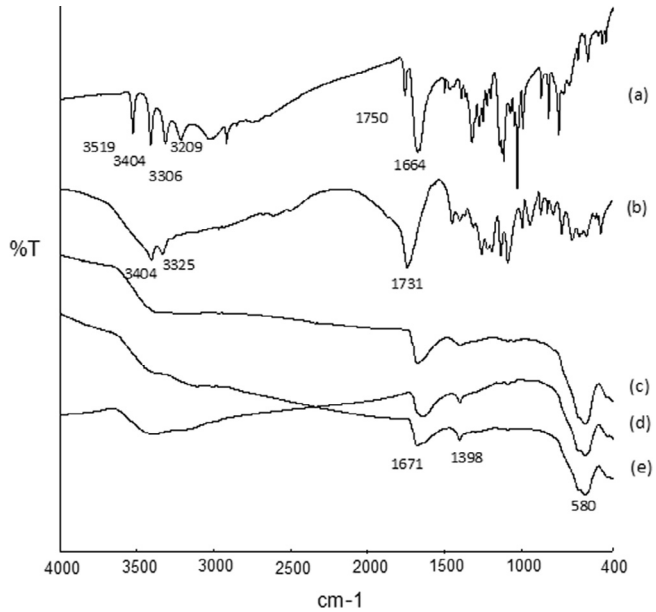

Fig. 3. FT-IR spectra of the (a) AA, (b) TA and the samples (c) C-AA-IONs, (d) C-TA-IONs, (e) C-AA + TA-IONs.

the coated nanoparticles (Fig. 3 c-e), a strong and broad absorption band were observed at around 580 cm^{-1} corresponding to the Fe-O bond of iron oxide [16,25]. Two peaks at 1625 cm^{-1} and 1398 cm^{-1} appears indicating that TA or AA is bound to the surface of nanoparticles [16,17,24,25]. It is thought that the expansion of the band around 1625 cm^{-1} is due to the absorbed water. C=O band which comes out at 1671 and 1625 cm^{-1} indicates that the O atom coordinated to the Fe on the surface of nanoparticles. The absence of C=

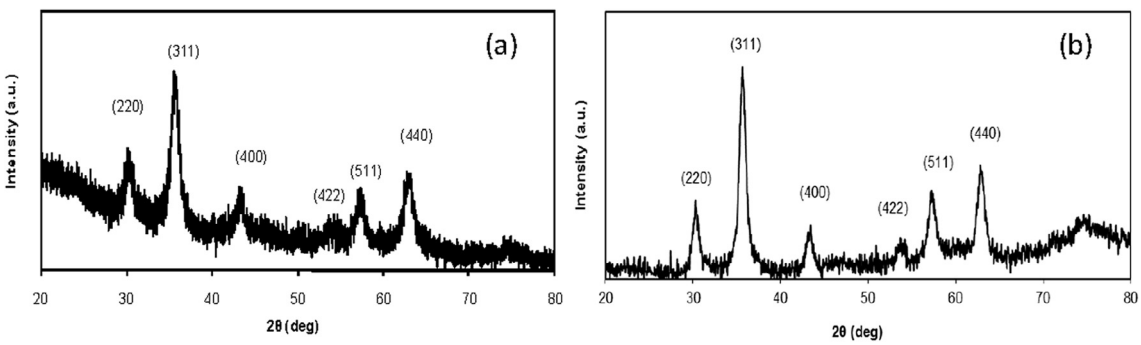

Fig. 2. XRD patterns of (a) the sample C-AA-IONs and (b) the sample H-AA-IONs.

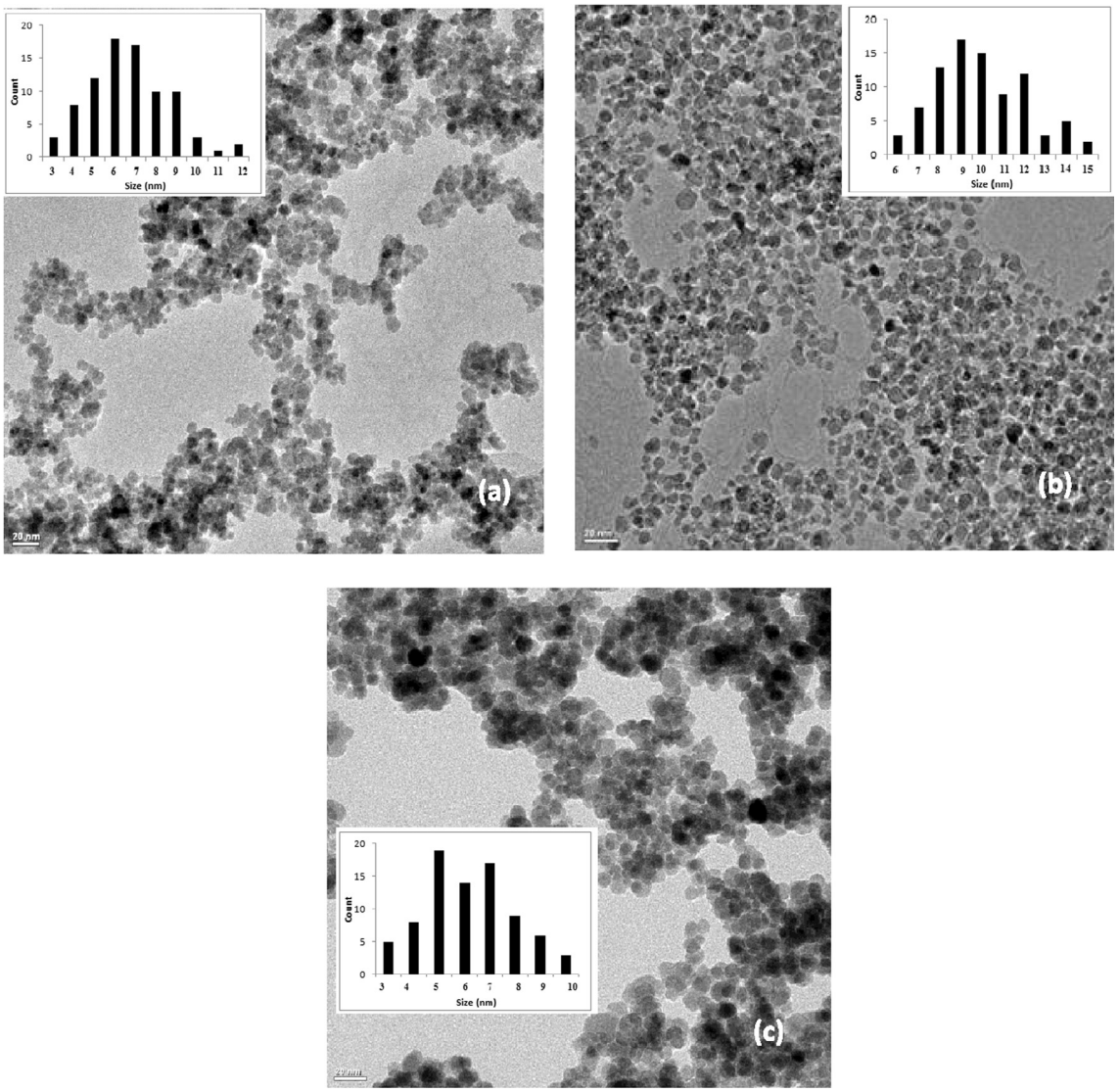

Fig. 4. TEM images of the samples (a) C-AA-IONs (b) C-AA + TA-IONs and (c) H-AA-IONs.

O bands at about 1700 cm^{-1} confirms that free AA and TA removed with the extraction. The nanoparticles were properly coated with the surfactants (AA and TA).

TEM images of the coated nanoparticles were taken to observe the morphology and size and shown in Fig. 4. The images of samples show nearly spherical shaped particles. And, the mean physical sizes of the samples C-AA-IONs and C-AA + TA-IONs (Fig. 4a and b, respectively) obtained from the TEM images were same and 7 ± 2 nm. The possible reason is that the surfactant was added right after the formation of the nanoparticles, therefore the same size of the nanoparticles for the samples were obtained during the co precipitation. The TEM images of the sample H-AA-IONs (the sample obtained after the hydrothermal treatment of C-AA-IONs at 155 ± 5 °C for 12 h) were shown in Fig. 4c. The mean physical size of sample H-AA-IONs 10 ± 2 nm.

3.2. Magnetic analysis

Fig. 5 shows the magnetization curves of the samples synthesized by co-precipitation (Fig. 5a) and hydrothermal treatment (Fig. 5b). Magnetic measurements indicate that all samples are superparamagnetic at room temperature. Saturation magnetization, M_s of the samples C-AA-IONs, C-TA-IONs and C-AA + TA-IONs were 62.6 emu/g, 62.4 emu/g and 60.8 emu/g, respectively (see Table 1). The coated nanoparticles reached the M_s at about 9500 Oe. Since the surfactants were added only as a capping agents right after the crystallization of the nanoparticles, no effect on the growth of the nanoparticles was observed and thus high M_s values were obtained compared to the literature which these coated nanoparticles synthesized using co-precipitation [17]. Table 1 represents the core sizes and M_s values of the coated nanoparticles in the literature and in our study. Gupta et al. [17] synthesized dehydroascorbic acid coated nanoparticles having an average size of ~ 6 nm using co-precipitation method at 90 °C. And, the M_s value of nanoparticles was 36 emu/g. The M_s values in our study is also higher than the values in other studies which used hydrothermal synthesis [16,23–25]. Xuan and co-workers [24] synthesized AA coated magnetite nanoparticles using hydrothermal approach and they obtained nanoparticles with the average size of 5.2 nm and with the M_s of 5.2 emu/g. Yan and co-workers [25] obtained TA coated nanoparticles using hydrothermal synthesis and the M_s of the coated nanoparticles with the average size of less than 10 nm and 13.5 nm were 29.6 emu/g and 40.1 emu/g, respectively. Xiao et al. [16] synthesized ultra-small superparamagnetic Fe_3O_4 nanoparticles (5.1 nm) under hydrothermal conditions. And, the AA was used as the chemical reducing agent and capping agent and the M_s of the nanoparticles was 47.0 emu/g. Feng et al. [23] synthesized AA coated nanoparticles (about 5 nm) using hydrothermal route and M_s was found to be 40.0 emu/g. In our study,

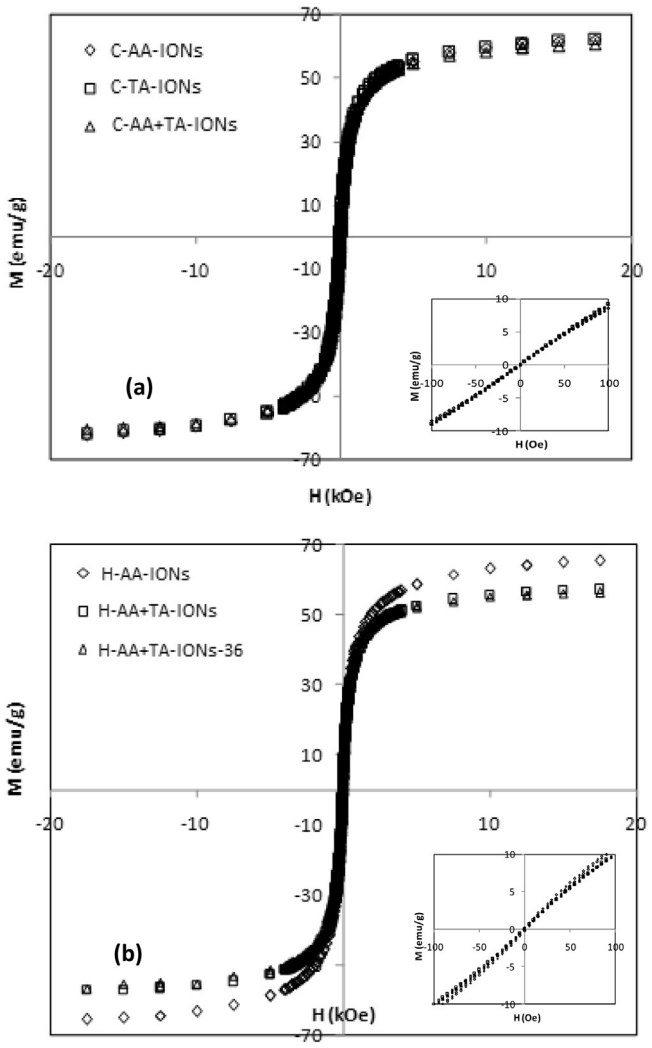

Fig. 5. Magnetization curves of the samples synthesized (a) by co-precipitation method and (b) by hydrothermal process. (Inset shows magnetization curves between −100 Oe and +100 Oe.).

Table 1
List of the findings in the studies investigated AA and/or TA coated iron oxide nanoparticles and our results.

Coating materials	Synthesis methods	D(TEM) nm	M_s emu/g	Reference
AA	Co-precipitation	7 ± 2	62.6	This study
TA	Co-precipitation	–	62.4	This study
AA and TA	Co-precipitation	7 ± 2	60.8	This study
AA	Hydrothermal method	10 ± 2	65.3	This study
AA and TA		–	57.5	This study
AA	Hydrothermal method Co-precipitation	6	36	Gupta et al. [17]
AA	Hydrothermal method	5.2	5.2	Xuan et al. [24]
TA	Hydrothermal method	~10	29.6	Yan et al.[25]
		13.5	40.1	
AA	Hydrothermal method	5.1	47	Xiao et al. [16]
AA	Hydrothermal method	~5	40	Feng et al. [23]

the M_s of coated nanoparticles (~7 nm) obtained by co-precipitation technique is ~62 emu/g. The M_s of the coated nanoparticles are considerably high compared with the ones synthesized in the literature using co-precipitation method and hydrothermal treatment [16,17,23–25]. Hydrothermal treatment was also applied as a second step and the effect of hydrothermal processing on magnetic properties of the coated nanoparticles was investigated in our study. The magnetization curves of the samples are shown in Fig. 5b. The M_s value changed from 62.6 emu/g to 65.3 emu/g for the samples C-AA-IONs and H-AA-IONs after the hydrothermal treatment at 155 ± 5 °C for 12 h. The mean size was also changed from 7 ± 2 nm to 10 ± 2 nm. A slight increase in particle size causes a small increase in the M_s was observed due to the hydrothermal treatment. And, with the hydrothermal treatment of sample C-AA + TA-IONs at 155 ± 5 °C for 12 h and 36 h, the M_s decreased from 60.8 emu/g to 57.5 and 56.7 emu/g, respectively. No significant increase in the M_s values of the samples was observed with hydrothermal treatment.

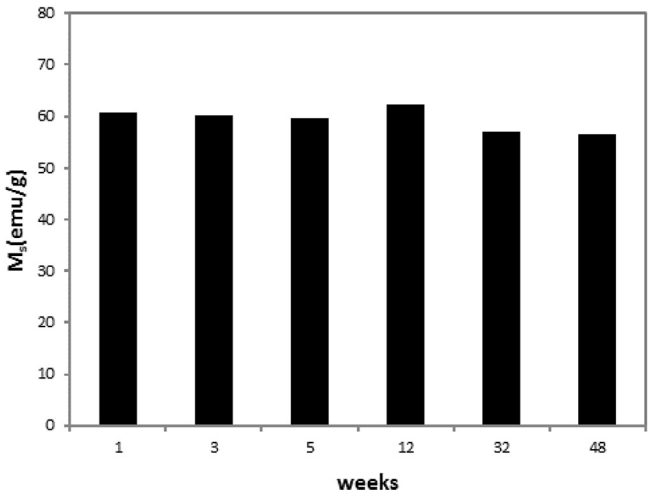

Fig. 6. Saturation magnetisation, M_s of the sample C-AA + TA-IONs measured during forty-eigth weeks.

To investigate whether the coating agent has an effective protection against oxidation, the sample C-AA + TA-IONs was kept in air environment and magnetic properties were measured during 48 weeks. The M_s values were presented in Fig. 6. In the figure, the M_s values were stable and were ~62 emu/g for first 12 weeks. After 12 weeks, the M_s decreased to ~57 emu/g and stayed almost still by 48 weeks. It can be concluded that coating agents (TA and AA) successfully protected the core against oxidation for a long time for biological applications. No significant change is observed between the M_s values of co-precipitated and hydrothermally treated samples. Besides, the M_s value of sample C-AA + TA-IONs is stable over 48 weeks. This sample may be a good candidate for biomedical applications. Thus, co-precipitated sample C-AA + TA-IONs was selected for the cytotoxicity experiments.

3.3. Biocompatibility

For biological studies, coating is required for keeping the particles apart and preventing agglomeration [30,31]. The cytotoxicity of the naked IONs and coated C-AA + TA-IONs was studied with two cancer cell lines (the Saos-2 and Hep3B) and a healthy cell line (HUVEC) by MTT assay for 48 h. The cytotoxicity effect on the cell growth was examined at different concentration (10, 100 and 500 μg/mL) and shown in Fig. 7a-c. As seen in the figures, the naked IONs and sample C-AA + TA-IONs were found to be non-toxic although there is a small amount of cell loss in the Saos-2 (Fig. 7a). Due to the colour of of the samples at the high concentration (500 μg/mL), over-loaded in the % cell viability were also observed in Fig. 7b-c. It is considered that these samples exhibited an excellent biocompability profile using in cancer therapy system. These coated IONs may have the potential to be used in drug delivery systems and MRI contrast agents.

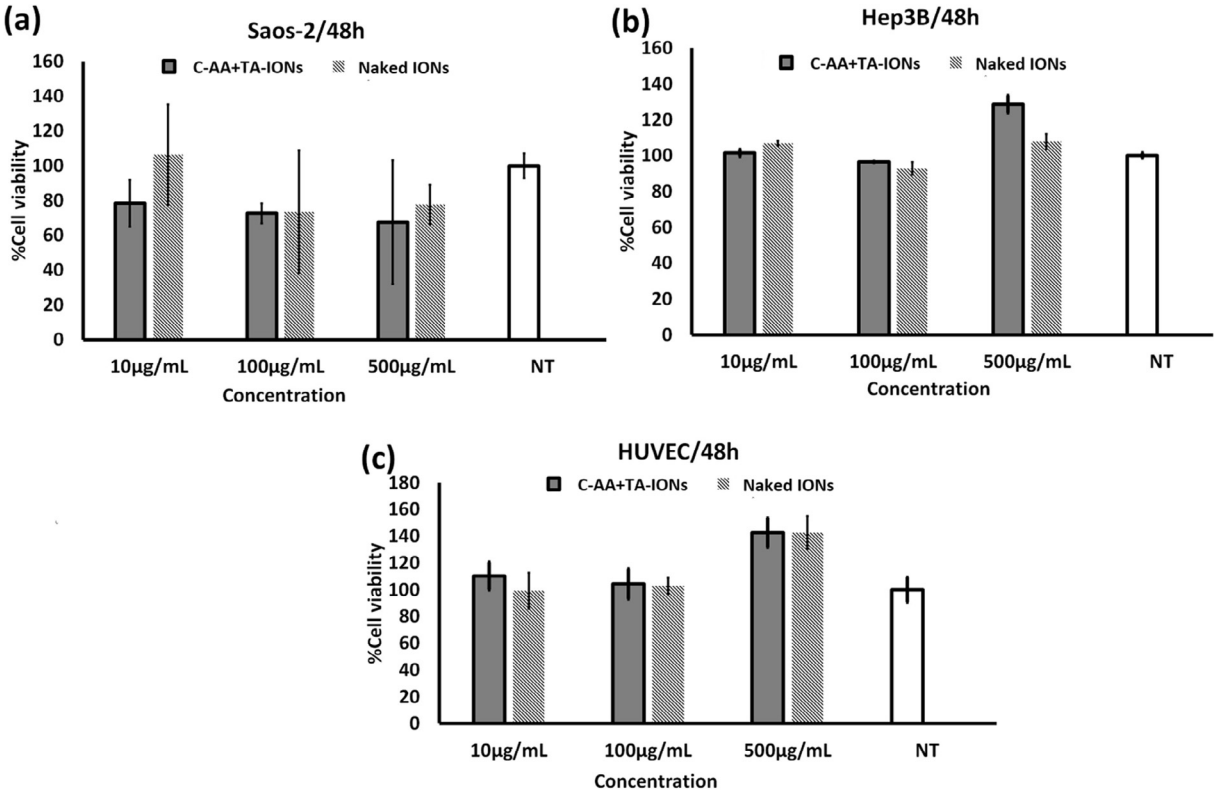

Fig. 7. Cytotoxic effect of naked IONs and sample C-AA + TA-IONs. %Cell viability was applied on (a) Saos-2 cells (Human osteoasarcoma cell line) (b) Hep3B (Human Hepatoma Cell line) (c) HUVEC (Human Umbilical Cell line). NT represents non-treated groups.

4. Conclusions

A simple way to synthesize biocompatible superparamagnetic IONs coated with AA, TA and a mixture of AA and TA is presented. The IONs were synthesized and then coated with surfactants in one step by co-precipitation method at room temperature in air atmosphere. This method is quite simple and a lot of product can be obtained in one go. The samples have the structure of iron oxide and the mean physical sizes of core were obtained as 7 ± 2 nm. All samples were superparamagnetic and the M_s were found to be around 62 emu/g. The IONs showed high crystallinity and therefore M_s of the samples are quite high. Stability of the M_s over time was measured and stable M_s values were observed for 48 weeks. The coated IONs found to have high and stable M_s values. The toxicity of the nanoparticles was evaluated with MTT method. The percentage cell viability values are presented in order to discuss the biocompatibility of the nanoparticles. With MTT analysis, it is found that coated nanoparticles had no toxic effect for all cell types: Hep3B, Saos-2 and HUVEC. Due to these properties, iron oxide nanoparticles possess great potential for biomedical application.

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