Trends in Biomaterials and Artificial Organs
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Evaluation of Doxorubicin Loaded Gelatin Coated Iron Oxide Nanoparticles for Drug Delivery and Magnetic Hyperthermia for Anti-Cancer Treatment

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Received: *18 February 2021*
Accepted: *26 April 2021*
Published online: *23 February 2022*

Keywords: *cytotoxicity, doxorubicin, genipin, hyperthermia, iron oxide nanoparticles*

The contribution of magnetic nanoparticles to the field of cancer treatment has recently created an immense impact with various modalities. In the present work we report the fabrication of magnetic iron oxide nanoparticles (IONPs) coated with a biocompatible natural polymer gelatin, followed by cross-linking of the adsorbed model drug, doxorubicin (DOX) on the surface of the nanoparticles using a natural cross-linker genipin. The particles were characterized by evaluating the zeta potential, VSM, hyperthermia and X-ray diffraction. To investigate the morphology of the IONPs, field emission scanning electron microscopy (FE-SEM) was used. Functional and elemental group was evaluated by FTIR. The drug loading efficiency and drug release kinetics was studied, whereas the toxicity of gelatin coated IONPs was evaluated by MTT assay using MG-63 osteosarcoma cells. The nanoparticles shows good super paramagnetic properties exhibiting a potential hyperthermia effect with enhanced drug release on increase in temperature. The cell viability assay displayed that gelatin coated IONPs are non-toxic thus making it a potential candidate suitable for application in anti-cancer treatment.

Introduction

In the present circumstances, the contribution of magnetic iron oxide nanoparticles (IONPs) in treatment of cancer is being researched very extensively. IONPs has exhibited potential in drug delivery [1], cell-targeting [2], hyperthermia [3], magnetic resonance imaging [4], magnetic assays[5,6], and theranostic applications. Apart from low toxicity effect by these particles in the human body, its unusual magnetization property, termed 'superparamagnetism' which is exhibited under the influence of a magnetic field wherein these particles with high saturation magnetization, undergo small vibrations in an attempt to align itself to the field direction reaching a high temperature in a very minimal time. This serves the need to generate heat inside the human body at a localized area beyond the temperature tolerable for cancer cells without hampering the surrounding normal cells, leading to destruction of tumour cells alone [7]. Moreover these particles do not retain their magnetic field after removal of the externally applied field which allows the systemic circulation within the body.

The targeted drug-delivery principle by which the accumulation of the specific drug at a particular site is controlled using an external magnetic field, is also an area where iron oxide based ferrofluids or nanoparticles have been conjugated with a drug, and used as a nanocarrier for systemic or localized delivery [8,9]. Even though the administration of certain polymer coated superparamagnetic nanoparticles have been clinically approved [10], the toxicity, safety, agglomeration, cellular alterations like oxidative stress and degradation issues associated with respect to internalization of the particles within cells and their fate after drug release is still under study.

The common challenge faced in the biomedical application of the iron oxide nanoparticles is the surface coating, which is expected to be non-toxic and biocompatible with enhanced selective drug delivery at the specific site, for which proteins are tagged on them guiding to the particular binding site [11,12]. Many natural and synthetic polymers have been used to coat the surface of the

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particles using various polymers like poly(lactic co-glycolic acid) (PLGA) [13,14], poly (ε-caprolactone) (PCL) [15], poly(ethylene glycol) (PEG) [16], collagen [17], gelatin [18], alginate [19], polyvinyl alcohol (PVA) [20], Pluronic-127 [21], silk fibroin, dextran, silicon, citric acid and many more. These coatings prevent precipitation and agglomeration under the physiological condition promoting easier transport of the drug carrier to the targeted site.

Gelatin is collagen-derived polymer and is used in a wide range of biomedical and pharmaceutical applications for its easier water solubility, biocompatibility and degradation features [22, 23]. The coating of the iron oxide particles during synthesis itself prevents oxidation of the sample and combines with the drug molecules. Doxorubicin (DOX) is one of the most common drugs used in chemotherapy for treatment of breast cancer, bone cancer, liver cancer, neuroblastoma, and several solid tumors and lymphomas [25]. The cross-linking of the drug and the polymer plays an important role in the efficient drug conjugation, drug release and degradation of the nanocarrier. In view of this condition, instead of using the common cross-linking agents like formaldehyde or glutaraldehyde which leaves residue causing toxicity to the cells, an attempt is made to cross-link using a natural agent genipin. It is a natural cross-linker extracted from fruit of *Gardenia Jasminoides* [26], which is water soluble, executes the linking between polymers through covalent coupling. It also exhibits less toxicity, delayed degradation, anti-inflammatory and anti-angiogenesis properties for potential biomedical applications when compared to the other usual cross-linkers like glutaraldehyde [2]. Genipin has already been used for crosslinking various combinations of gelatin, chitosan, collagen, silk fibroin and other polymers for applications like hydrogel-based drug delivery systems [28], polymeric micelles [29], drug loaded microspheres[30], gels and nanoparticles [31] which has a beneficial impact on the release profile of a nanocarrier used, improving the targeting effect [32].

Recently, the cancer activity of drug-loaded magnetic nanoparticles loaded with a model drug has been studied with peg coated IOPs loaded with Dox for the effective inhibition of HeLa cells showing a high efficiency of cell uptake and retention[33] .Super paramagnetic calcium ferrite has been coated to vanillin modified chitosan as a hybrid drug carrier of curcumin drug and investigated for biocompatibility studies as reported elsewhere[34] while the research group along Qiu et al. [35] has demonstrated the vascular endothelium permeability to increase by the action of IONPs causing a magnetic force to partially disrupt the endothelial adherent junctions which could result in enhanced drug uptake within the cells. The biomedical use of APTES (3-aminopropyltriethoxy silane) coated functionalized to iron oxide and subjected to the sequential reactions to 2-bromoisobutyrate as an Atom Transfer Radical Polymerization(ATRP) initiator for further polymerization [36] has been a hybrid platform for a smart drug delivery and synergistic properties leading to better biological stability and circulation time. Also core-shell model of poly acrylic acid (PAA) coated cobalt ferrite for a magnetic nano-system has depicted good biocompatibility without renal cytotoxicity and have been administered intramuscularly to Albino mice with encouraging results for magnetic guided drug delivery and image guided therapy [37]. Thus a substantial increase in the reports on magnetic nanoparticles have given significant development of the nanocarriers based drug delivery systems which is still an expansive process being researched to manage the different drawbacks yet to be addressed.

In this investigation, gelatin coated IONPs with adsorbed drug (DOX), cross-linked with genipin is studied to analyse the drug release profile and its biocompatibility on osteosarcoma cancer cell lines (MG-63).

Materials and Methods

Iron (III) chloride hexahydrate, Ferrous (II) sulphate heptahydrate and Sodium hydroxide (NaOH) pellets was obtained from Sisco Research Laboratories (SRL) Pvt.Ltd., Mumbai-India. Gelatin type A, Doxorubicin Hydrochloride (DOX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Genipin was purchased from Sigma-Aldrich, Mumbai, India. Phosphate buffer saline (PBS), fetal bovine serum (FBS), 1X penicillin and streptomycin antibiotic solution, Trypsin EDTA, and Dulbecco's modified eagle medium (DMEM) were purchased from Hi Media Laboratories, Bangalore India. MG-63 osteosarcoma cell lines were obtained from NCCS, Pune, India. Deionized water was used in all the steps of the experiments.

Synthesis of gelatin coated iron oxide nanoparticles

Gelatin coated iron oxide nanoparticles (G-IONPs) were synthesized by the co-precipitation of iron salts at high alkaline pH condition and subsequently coated with the gelatin solution, slightly modified from the work reported elsewhere [38]. Briefly, 5ml of 1M NaOH was added drop wise to precipitate the iron ion solution taken in the molar ratio of 0.1M of Fe^{2+} and 0.2M of Fe^{3+} added in the presence of 10ml of 1%(w/v) of gelatin dissolved in a solution during continuous magnetic stirring at 80ºC until reaching a pH of 11. After maturation time of 30 minutes, the black coloured precipitate was magnetically separated and washed thoroughly, followed by brief sonication and then freeze-dried to obtain the black powder.

Drug loading and cross-linking

The resultant powder after lyophilization was re-dispersed in water with continuous stirring to which the water-soluble model drug doxorubicin (DOX) was added. The aqueous solution of DOX (2mg/ml) was prepared and added to the G-IONPs dispersion at pH 7.4 with vigorous mixing and was incubated at 25ºC for 12 hours under vigorous shaking in dark condition. The excess free drug unattached was removed by dialysis against water for 1hour. In sterile phosphate-buffered saline (PBS), stock of 1% genipin solution was prepared and kept at room temperature (RT). From stock solution the cross-linker genipin (20µg/ml) was added to the drug combined nanocarrier dispersion of magnetic iron oxide nanoparticles (G-IONPs-DOX) and again incubated under shaking condition for 2 hours, followed by mild washing and storage at 4ºC until further use.

X-ray diffraction analysis

The X-ray diffraction analysis study was done to determine the phase formation and crystalline nature of the prepared gelatin coated nanoparticles using the instrument, XRD D8 Advance Bruker diffractometer (Germany) using Cu K radiation taken in the 2θ range of 20-80º.The sample was placed in a sample holder and diffraction patterns were obtained by scanning with a scan step of 0.01p and a speed of 1p/min. The peak position and relative intensities were matched to the reference JCPDS number.

Fourier transforms infrared spectroscopy

The Fourier transforms infrared spectra for confirmation of functional groups were recorded between the ranges 4000-400 cm^{-1} using SHIMADZU CROP -IR Affinity-1 instrument. The sample was taken in a small quantity and compacted with KBr mixture to form pellets which were mounted for analysis and the spectra were obtained.

Vibrating sample magnetometer analyses

Magnetic properties of the coated sample were measured using a

commercial Vibrating Sample Magnetometer (VSM) Lakeshore model 7404 and the hysteresis loop measured with the range from -15 to +15 KOe of applied magnetic field at room temperature.

Scanning electron microscope analysis

The morphological structure of the IONPs nanocarriers was examined using a scanning electron microscope ZISS-EVO 18 (Carl Zeiss), Germany. The sample powder was sputter coated with a thin layer of platinum and mounted on a carbon adhesive tape for surface studies.

Surface charge

To obtain the zeta potential (æ-potential) values of the colloidal solutions before and after drug loading and cross-linking each solution was measured using a particle analyzer (HORIBA SZ-100 Nanoparticle analyser) at 659nm wavelength, temperature at 25± 2°C and at an angle 173° to its incident beam.

In vitro drug release

The *in-vitro* release profile was studied by performing the experiment in phosphate buffer saline (PBS) with acidic pH 6 and pH 7.4 at two different temperatures 37°C and 42°C. In order to determine the amount of DOX released. 10ml PBS was added to 0.1mg of the drug loaded particles and stirred continuously for a pre-determined period. After fixed time intervals, 2ml aliquots of the suspension was collected for UV-visible spectrophotometer analysis at 495 nm, followed by immediate supplementation of fresh PBS to maintain the constant volume.

In vitro cytotoxicity and cell viability

The cell viability study of the G-IONPs and G-IONPs-DOX nanocarriers was carried out by MTT assay using MG63 osteosarcoma cells. MTT assay measures cell-proliferation through the reduction of a tetrazolium salt (MTT) by the mitochondria of viable cells to an insoluble formazan product. The cell culture medium used was Dulbecco's Modified Eagles Medium (DMEM), with a supplement of 10% fetal bovine serum (FBS) and 1% antibiotic solution (penicillin and streptomycin). The cells were seeded at a density of 1 x 10^4 cells per well in a 96 well plate and incubated for 24 hours at 37°C and 5% CO_2 atmosphere to permit cell attachment. After 24 hours of incubation, the media in each well was replaced with fresh complete media containing concentrations of particle suspension ranging from (200 to 1000µg/mL) and cultured for the next 24 hours. Control experiments were carried out using only cells in the culture medium at the required condition.20µl of MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) was added into the 96 well plate and after 4 hours of incubation time, DMSO (100µL) was added to each of the wells to dissolve the formazan crystals. The produced colour amount is directly proportional to the viable number of cells.

Finally, the absorbance was measured using an Elisa plate reader at 570nm and the cell viability was calculated using the formula:

% Cell viability = (Abs of sample/Abs of control well) x 100

Hyperthermia experiment

The time-dependent calorimetric studies was done using an induction heating unit (4.2-kW Ambrell Easy Heat 8310) to evaluate the heating profile and measure the rise in temperature of gelatin coated particles using an alternating magnetic field.1ml of the particle suspension was taken at different concentrations and dispersed using ultra-sonication in water. The sample was placed in the middle of the heating coil with 9 turns and 6cm length. The frequency and amplitude of the field were fixed constant at 316kHz and 30kA/m, respectively. The temperature rise of each sample was recorded by a sensitive fiber thermometer.

Statistical analysis

The data were analyzed using a two-sample t-test in Excel. Values were considered statistically significant where differences between groups were p≥0.05 and with p≥0.01 were considered highly significant.

Results and Discussion

Synthesis of IONPs by the co-precipitation of iron salts is a frequently used method. Moreover the fabrication of polymer coated magnetic nanoparticles have been used routinely in nanomedicine applications as they provide sufficient colloidal stability and acts as a physical barrier on the surface of the

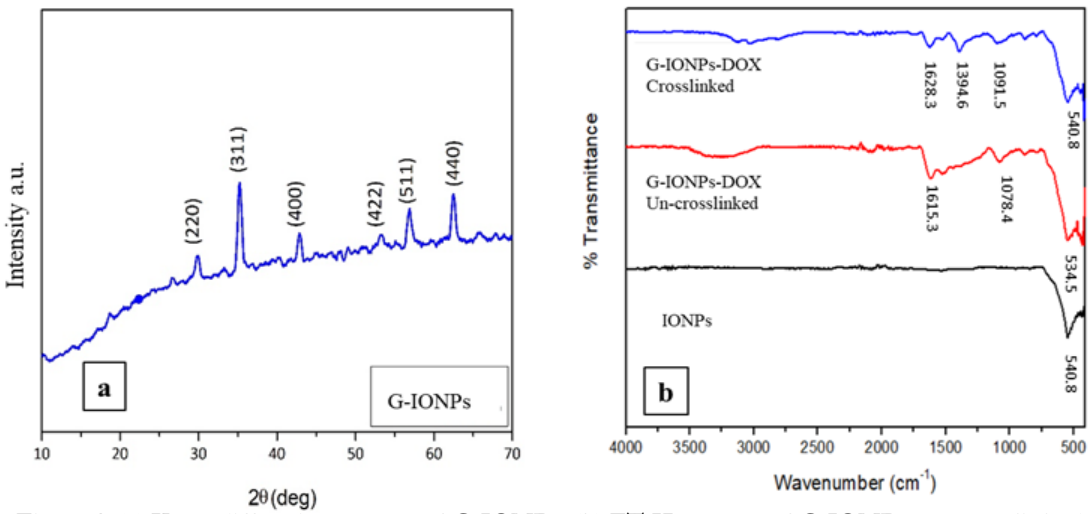

Figure 1: (a) X-ray diffraction pattern of G-IONPs, (b) FT-IR spectra of G-IONPs, un-crosslinked and crosslinked G-IONPs-DOX

nanoparticles increasing stearic stabilization in suspensions [39]. The Fe_3O_4 NPs coated with gelatin(G-IONPs) were synthesized by adding a basic solution(pH 8-12) to the iron precursor solution(Fe^{2+}/Fe^{3+}) at 0.5 molar ratio in the presence of gelatin solution as reported previously for the production of magnetite nanoparticles [40]**.** The addition of the basic solution to increase the pH results in increased IONPs leading to enhanced hydrolysis process [41]. The coating of gelatin on the IONPs was due to the electrostatic interaction between the positively charged Fe_3O_4 and the carboxyl group of gelatin [42] in order to form an encapsulated magnetic core. In the experiment G-IONPs were crosslinked with genipin rather than using traditional method of using gluteraldehyde (GTA) solution as cross-linking agent. GTA solution has the potential to have fast and effective cross-linking reaction but however show cytotoxicity if residual GTA is left. From GTA crosslinked material, the residual GTA is treated with glycine solution to inactivate the aldehyde group. However, it can still affect the cell growth on exposure of GTA aldehyde group due to removal of glycine molecules during washing process [43]. Genipin, a natural crosslinking agent compared to aldehyde and epoxy cross linkers possesses lower toxicity. Genipin in PBS solution introduces a nucleophilic agent that starts crosslinking reaction with itself resulting in an oligomeric crosslinking agent that creates intermolecular binding, with no residual left to cause cell toxicity [44]. Additionally, pH 7.4 of PBS results in potent crosslinking activity [45].

X-ray diffraction peaks observed showing the nature of G-IONPs is shown in figure 1(a) The characteristic diffraction peaks at 2θ observed correspond to the assigned crystal plans (220), (311), (400), (440) and (511) of the spinel structure phase of magnetite (Fe_3O_4), JCPDS No. 82-1533). Additionally the other phases of iron oxide that could be present due to oxidation like haematite, ferric hydroxide has not been identified [46]. It is observed there is a slight broadening of peaks and amorphous nature depicted by the sample due to presence of polymer coating on the particles which is predominantly corresponded to the reduction in crystallite size. The Scherrer's formula has been used to calculate the crystallite size where the mean crystallite size has been found to be 45nm comparable to the previous reports [47,48]. Figure 1(b) shows the FT-IR spectra with the organic functional groups and molecular binding of gelatin coated (G-IONPs) and drug loaded samples (G-IONPs-DOX). The peak observed at 1523 cm^{-1} confirms the presence of primary amine in the gelatin molecule of N-H bending vibration and the peak at 1445 cm^{-1} represents genipin, in addition to the amine band due to C=N stretching is confirmed at 1628.3 cm^{-1}. The N-H stretching is seen at 1515 cm^{-1} with a slight shift confirming the electrostatic attraction between gelatin molecules and iron oxide present [49]. The first band seen at around 500 -540 cm^{-1} corresponds to the Fe-O bond absorption confirming the tetrahedral site of Fe^{3+} ions. The colloidal solution seen in figure 2 (a) shows stability and exhibits its magnetic property in the presence of an external magnetic field figure 2(b). The FE-SEM images seen in figure 2(c) shows the morphological structure of the genipin cross-linked nanocarriers to display a slight slightly spherical structure with the average size to be around 60 nm but is seen in the form of agglomerated clusters. The agglomeration in nanoparticles with a larger surface-to-volume ratio is due to their high surface energy with relation toward their stability. Due to which greater aggregation is seen in smaller particles owing to the lesser energy barriers which in turn reduces the surface area of magnetic nanoparticle. The addition of gelatin inhibits the excessive growth of nuclei and thus reduces the particle size resulting in formation of fine nanoparticles [50]. Moreover according to previous reports it has also been confirmed that the pH values also directly influence the size and shape corresponding to the nucleation and crystal growth [51,52] reporting that the optimum pH should be more than 10 which is consistent to the present work. The zeta potential values of gelatin coated iron oxide nanoparticles before (figure 3(a)) and after (figure 3(b)) cross-linking of the drug observed to be -18.4 and -13.7 mV that relates to the colloidal stability of the particles and the conjugation of the drug on the particles facilitated by electrostatic attraction. The DOX molecules have neutral charge at pH 7.4 and negative charge in an alkaline condition [16] due to which it attaches to the slightly negative charged gelatin molecules in water and addition of genepin binding to the amine groups results in further decrease of zeta potential.

The measurements of the magnetic field obtained from the hysteresis curve from a vibrating sample magnetometer (VSM) is the magnetization plot as seen in figure 3(c) which depicts the magnetization (M) versus applied magnetic field (H), where the dox-loaded particles (G-IONPs-DOX) nanocarriers display a saturation magnetization (M_s) value around 37 emu/g exhibiting no remanance effect from the hysteresis loop [53], which is slightly lower than the reported literature values of bulk iron oxide

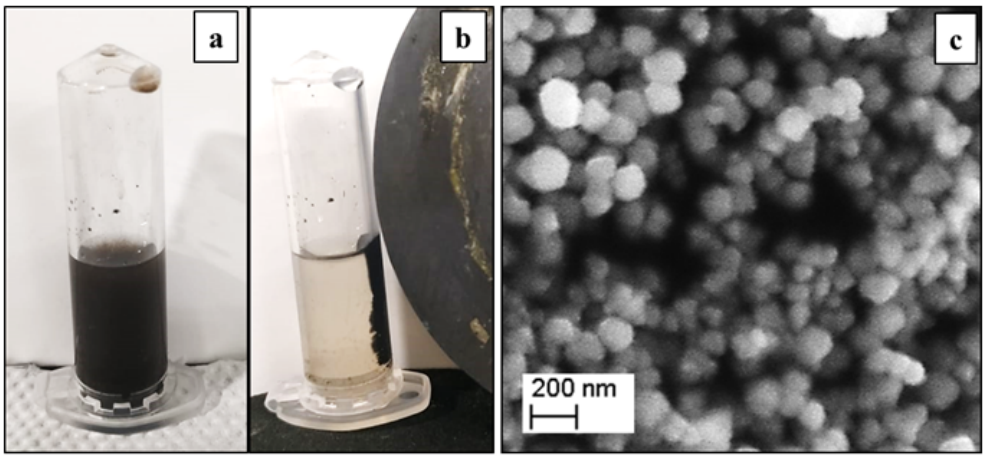

Figure 2: Colloidal dispersion of the G-IONPs in the absence (a) and presence (b) of magnetic field. (c) Fe-SEM image of the synthesised G-IONPs

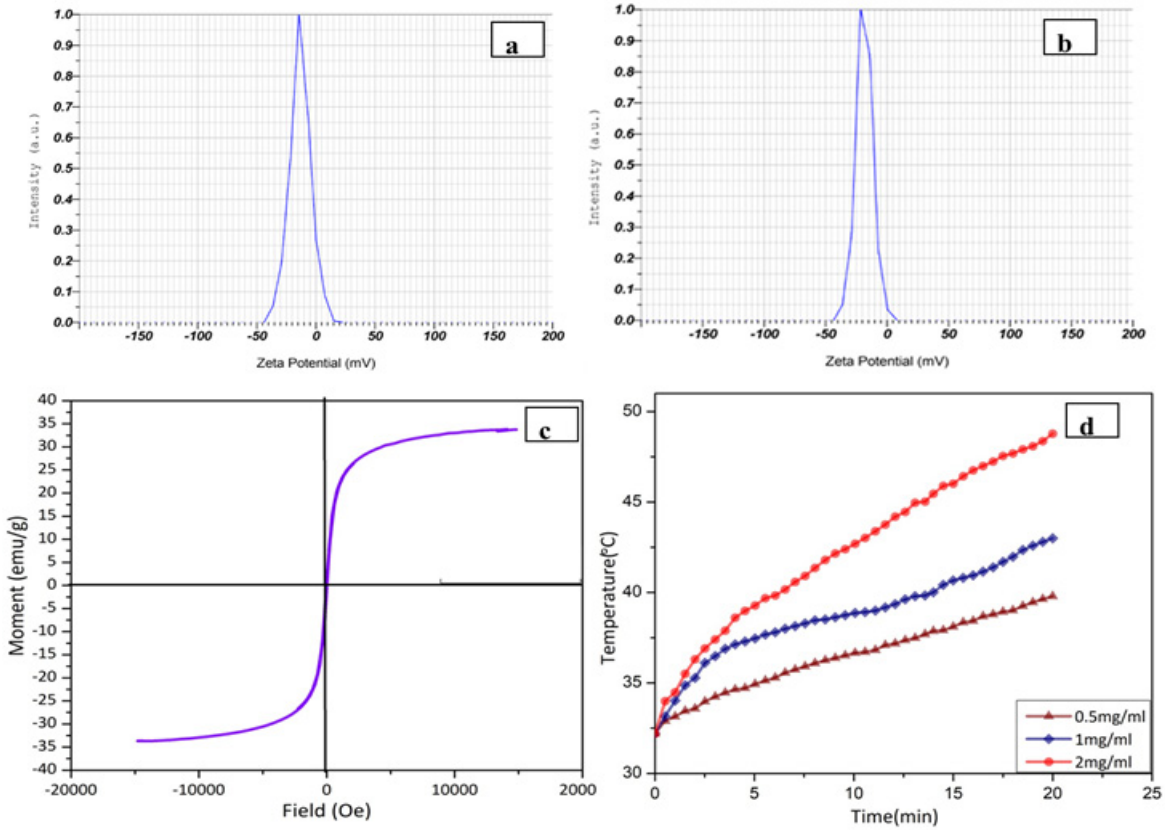

Figure 3: Zeta potential of (a) un-crosslinked and (b) cross-linked of G-IONPs sample; Magnetization plot (VSM) of (c) G-IONPs-DOX; (d) heating profile of G-IONPs sample with various concentrations (0.5,1,2 mg/ml) under alternating magnetic field (d)

nanoparticles (IONPs) (60-80emu/g) [32,38]. The reduced M_s value observed due to the gelatin coated IONPs owes to the existence of the diamagnetic shell around the outer covering ultimately quenching the magnetic moment of the magnetic nanoparticles [53]. The phenomenon of superparamagnetism is said to be exhibited by single domain particles at a state of uniform magnetization without interaction to the other domains within the particle in a colloidally stable solution [54-56].Moreover, as the M_s value also depends on the pH value maintained during the synthesis reaction, reports have shown that at pH more than 10

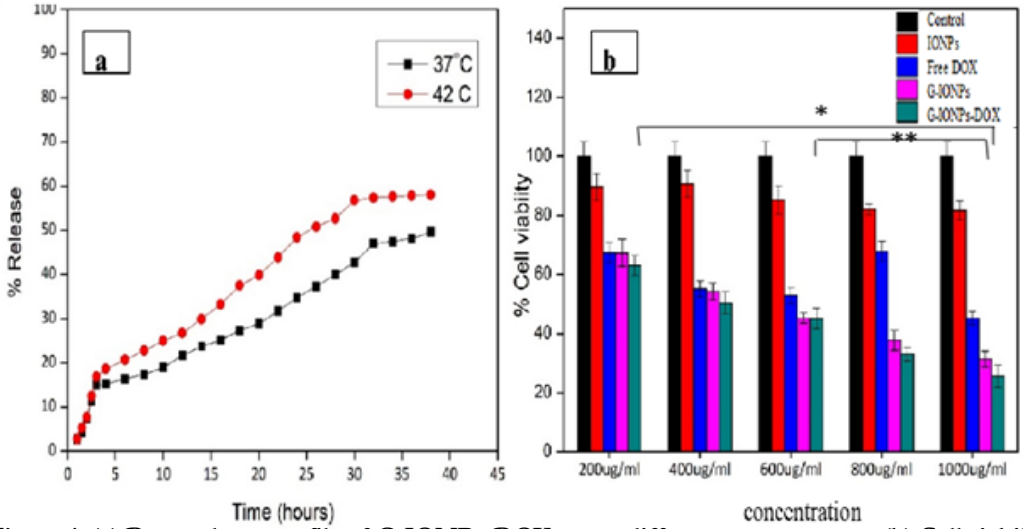

Figure 4: (a) Drug release profile of G-IONPs-DOX at two different temperatures; (b) Cell viability assay carried out on MG-63 (osteosarcoma) cell line for 24 hours using different concentrations of sample (P-value <0.05 (*), P-value <0.01 (**))

there is an increase in the M_s value due to smaller particle size effect [62]. The super paramagnetic behaviour is depicted by the (G-IONPs) nanoparticles with zero remanance and coercivity having a relatively lower saturation magnetization value which is associated to effects like oxidation [58] or the agglomeration due polymer coating yet interestingly it is still effective for biomedical application and cancer therapy which is further pointed in the calorimetric hyperthermia studies. The M_s (saturation magnetization) influences the rate of heating of the magnetic particles under an alternating magnetic field (AMF). As shown in figure 3(d), the heating profile of the nanocarriers taken at three different concentrations(0.5,1 and 2 mg/ml) and subjected to an external AC magnetic field of 30kA/m strength and 316kHz of frequency shows an appreciable increase in the temperature from 38-47ºC which is the desired condition for hyperthermia to occur in cancerous cells. Higher concentration showed a more efficient heating efficiency to reach the desired temperature at a faster rate, due to which these nanocarriers can serve as a delivery system where there is a subsequent drug release due to the heat generated through oscillation of magnetic moments and simultaneously for therapeutic purposes like magnetic-field induced thermotherapy. Energy of heat broadly interrupts cellular pathways specifically effecting protein structure and function making cells susceptible to heat damage [59,60]. Inhibition of DNA repair due to heat enables cells to overcome effects of ionizing radiation with more effective treatment [61]. Hyperthermia results in heat induced shock response in cells, causing cell death through a sequence of biochemical changes based on thermal dose, known as 'time-at-temperature' [62,63].

Sustained drug release is the one vital property needed for drug delivery systems, where the amount of drug released is controlled by an external factor. The release of DOX has been observed at two different temperatures; physiological temperature (37ºC) and a slightly high temperature used in hyperthermia (42ºC) have been shown in figure 4(a). The release of the drug observed at a higher temperature can be related to the point that the gelatin network of chains expands and undergo a quicker reduction attributing to the kinetic energy produced due to the heat generated in the thermo-sensitive polymer causing enhanced diffusion process resulting in efficient drug release. When these drug-loaded nanocarriers are brought in contact with the human body they encounter blood, body fluid and other tissues which may result in host response along with side effects due to their interaction with the biological system. Thus, the biocompatibility, cytotoxicity and degradation of these nanocarriers have to be immensely focused since it is a major prerequisite which highly influences their usage in therapeutics. Cancer tissues are reported to have a lower pH range when compared to normal tissues, which pave the way for pH-based drug delivery systems [64].

In order to evaluate the cell viability and toxic effect of G-IONPs crosslinked with genipin, compared to the control cells without the effect of drug loaded magnetic nanoparticles, MTT assay method was carried out on the osteosarcoma cells (MG-63) at an increasing concentration of 200, 400, 600, 800 and 1000 µg/ml for 24 hours to check the cytotoxicity percentage after the assay. The results in figure 4(b) have shown that as the concentration of the particles increased, the cytotoxicity levels also increased and cell viability was reduced comparing to the percentage of viable cells in the control to 60%, 55%, 50%, 35% and 24% for the concentrations of 200, 400, 600, 800 and 1000µg/ml respectively (for each, $P < 0.05$).

The studies also reports that the G-IOPs crosslinked with genipin show less viability of cells stating that the amount of drug loaded after cross-linking seems to be more effective to inhibit the growth of cells in 24 hours of cell culture. This could also relate to the amount of drug attached to the GIOPs after drug loading as with the activity of genipin, as they show more cytotoxicity levels (as seen in figure 4a)They indicate retention of anticancer activity by effective inhibition of osteosarcoma cells (MG-63) and also seem to promote death in cells due to the drug release in a dose-dependent way due to the degradation of particles within the cells following subsequent release of drug which is in accordance with previous studies [65,66]. This validates that the cross-linking of genipin with doxorubicin has been effective to reduce the growth and proliferation of cancer cells which is very essential to control the cancer activity and also prevent from relapsing [38].

Conclusion

The gelatin coated magnetic nanocarriers for drug delivery were synthesized using the co-precipitation method and cross-linked using genipin with the model drug doxorubicin. The results from this study exhibit the successful coating of gelatin on the surface of IONPs followed by the effective conjugation of the drug. The spherical morphology of the fabricated nanoparticles has been observed in the SEM images while the X-ray diffraction analysis confirms the magnetite phase formation nanoparticles. The highly crystalline purity of the samples has been confirmed by characteristic peaks of the absorption bands of gelatin and genipin. Moreover, the high colloidal stability as shown in the zeta potential values resulted in stable loading and temperature sensitive release profile of the drug, DOX. Interestingly, the saturation magnetization of the gelatin coated nanoparticles was around 37emu/g and hyperthermic studies revealed that it reaches a temperature of 40ºC in less than 15 minutes which is very feasible condition for hyperthermia based cancer treatment strategy. The cell viability assay was carried out for control (2D-cell culture), and the DOX loaded genipin - cross-linked particles were observed with the lower percentage of cell viability thus increased cytotoxicity effect displayed when compared to the gelatin coated nanoparticles. This validates that the cross-linking of genipin with doxorubicin has been effective to reduce the growth and proliferation of cancer cells. This study can be further extended and done by tagging of a protein for specified site binding demonstrating a more efficient targeted drug delivery system also including the bio distribution profile and *in vivo* monitoring within a system.

Acknowledgements

Authors wish to acknowledge VIT, Vellore for providing the seed grant for transdisciplinary research as financial support.

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