Shape induced acid responsive heat triggered highly facilitated drug release by cube shaped magnetite nanoparticles

Debarati De¹ and Madhuri Mandal Goswami^{2,a)}

¹Centre for Research in Nano Science and Nano technology, Block-JD-2, Sector-III, Salt Lake, Kolkata 700106, India

²S.N. Bose National Centre for Basic Sciences, Block-JD, Sector-III, Salt Lake, Kolkata 700106, India

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This paper reports a very simple yet better method for synthesis of cube shaped magnetite nanoparticles (MNPs) and their application in the drug delivery system (DDS). Structural analysis was done by XRD measurements to confirm the phase of the material, and morphological information was obtained through TEM analysis to confirm the shape and size of the particles. It has been shown that these particles can be decomposed in acid medium. These acid-decomposable magnetite nano-particles have been used for heat triggered, remote-controlled, on demand delivery and release of a cancer drug doxorubicin for research and therapeutic purposes. Here, we have shown that the pH stimulated and heat-triggered release of drug from our MNPs significantly enhances the release efficiency. In this case, we observe that pH induced release is far better in comparison to heat-triggered release. From these inspiring results, it may be expected that this methodology may become a significant step towards the development of a pH-sensitive heat triggered drug delivery system minimizing drug toxicity. *Published by AIP Publishing.* [<http://dx.doi.org/10.1063/1.4971439>]

INTRODUCTION

Nano-biotechnology is playing an important role in modern research.¹⁻³ Being at the interface of nano-science and biotechnology, it bears huge potential to cater the both.^{4,5} Problems of biology can be tackled in this domain of knowledge with insights from the nano-world that can bring new solutions from a completely different perspective. Obviously, scientists are trying to use this new weapon to fight cancer, which is still an open battlefield.^{6,7} Conventional cancer treatments contain many undesirable side effects, and scientists are constantly in search of new techniques with minimum health hazards. This search has many directions. One important direction is to minimize the intake of medicine for treatment because cancer medicines are themselves very harmful. These drugs not only attack cancer cells, they destroy the normal healthy cells too. If such harmful drug can be transported more precisely to the cancer affected region only, other parts of the body will not suffer from the poisonous effect of those chemicals.

There is another positive reason to channelize our research in this direction. The entire protocol is minimally non-invasive in nature, which is a growing trend in the medical community. So the entire problem has finally been reduced to manufacture and engineer a new system that can perform these jobs. Researchers have discovered many new avenues for it, such as nuclear therapy, electromagnetic therapy, hyperthermia therapy, and radiation therapy, and synthesis of suitable materials for use in such therapies is also becoming a challenge.⁸⁻¹³ Among them, recently, research on therapy of cancer treatment using thermal energy in a direct or indirect manner is an upcoming important track. Thermal energy can be used directly to kill cancer

^{a)}Email: madhuri@bose.res.in. Tel.: 91-33-2335-5705/6/7/8. Fax: +91-33-2335 3477/1364/2733/9176.

cells and to increase the efficiency of drugs too.^{14,15} For example, gold nanoparticles can successfully convert photons into thermal energy in drug release systems and in photothermal cancer therapy.^{16,17} By utilizing this thermotherapy concept, treatment of diseases with hyperthermia and the release of drugs from mesoporous nanoparticles and thermosensitive polymer-coated carriers are being investigated.¹⁸ Misra *et al.*^{19–21} have described controlled drug release using a magnetic drug carrier consisting of magnetic nanoparticles encapsulated with smart polymer and drug is encapsulated within the thermo-sensitive polymer. Undoubtedly, these works have their own potentiality but the process is lengthy and not so easy. In contrary, our method is very simple and easy to implement. On the other hand, we have proposed how alternating current (AC) magnetic field will trigger the drug release from outside the body and shown the combined effect of pH and heat in drug release.

The conversion of electromagnetic energy into thermal energy by magnetic nanoparticles has the potentiality to be used for therapeutic applications.^{22–24} Hence, research on heat induction by magnetic nanoparticles is attracting considerable interest for their use in this field. It is known that when an external alternating current (AC) magnetic field is applied, the magnetic particles are heated up and the response can be obtained from the specific loss power (SLP) measurement.²⁵ In addition to that, it can be monitored in a non-invasive way from outside the body by directing and controlling the magnetic field required. Nowadays with the help of magnetic nanoparticles induced hyperthermia, people are trying to find a new path for cancer therapy through proper selective drug delivery and stimulated drug release to increase the efficacy of drugs as well as decrease the side effects.^{26–28} Therefore, selective delivery and controlled release of cancer drugs in desired location of body is very important research. In this regard, the development of stimuli-responsive nanomaterials (NPs) for cancer treatment has been receiving extensive attention in recent years and now has become a principal field in cancer research. Different stimuli such as light, electric field, near infrared (NIR), pH, and enzymes are used to release the drug in a controlled way.^{29,30} Many nano carriers including liposome, polymer, micelles, DNA based nano carriers, and plasmonic nano-particles have been developed.^{31–34} These tailor made nano-carriers are being more and more customized for improvement of delivery and release of drugs in controlled ways to increase drug efficiency and reduce side effects. These nano-carriers are functionalized by proper receptor molecules (e.g., antibody) so that they can be targeted selectively to a specific region of interest or tagged with different stimuli dependent systems to bring a control over drug release.³⁵

All of these are nice ideas and have their own potentials. But there are some difficulties also. These organic systems are not adequately stable from chemical as well as mechanical viewpoint and are very costly too. Additionally, they are easily susceptible to microbiological attack; therefore, it is difficult to achieve control over the drug release rate, which is very much essential. Polymer nanoparticles (NPs) also suffer from the problem of the large size effect. Scientists have tried to mimic the drug delivery using some dye or DNA instead of drug molecules to avoid many real problems.^{36,37}

We have moved forward in a different way. We have concentrated on inorganic magnetic nanoparticles as they have the following advantages: They

- (i) can be prepared with very broad size ranges with very low dispersity.
- (ii) can be visualized by magnetic resonance imaging.
- (iii) are able to be guided by means of an external magnetic field.
- (iv) are capable to generate heat under AC magnetic field to trigger drug release as well as simultaneously produce hyperthermia triggered ablation of cancer tissue.

As these types of particles can be heated with an external AC-magnetic field, this thermal energy can be used to combat cancer cells because they cannot survive near 45 °C.^{38,39} If we can generate that amount of thermal energy, we can wipe out them. This procedure is known as hyperthermia in technical terms and many works have been directed to explore it. But here also lies the same problem. We have to generate heat in the close proximity of cancer cells so that normal cells do not get affected in this heating procedure and remain intact. These two different rewards can be achieved with magnetic nanoparticles, one from hyperthermia and the other

from targeted and selective drug delivery to the affected region. We have devoted our research to manufacture them with some desired and preferred properties.^{40–44}

Our goal is to find some paths for cancer therapy employing magnetic properties of magnetic particles. We already have discussed that hyperthermia treatment is a proposed cancer therapy and how it will work for magnetic nanoparticles. There are many parameters to be checked before the use of particles clinically for hyperthermia therapy. But if this hyperthermia technique can be utilized for known cancer drugs to be released with a controlled manner that will also be a great achievement in this field of research. But before clinical practice, it is necessary to study the topic extensively with change of different parameters to optimize the conditions.

In some previous studies, it has been observed that the shape of particles is a crucial factor for the application of these particles as drug delivery agents.^{45,46} Some of the previous studies also have shown that particles other than spherical shape are inhibited to be recognizable by the immune system. Moreover, cellular interaction and circulation of the drug carrier can be enhanced with a proper particle shape.⁴⁷ In one paper, it has been shown that the aspect ratio determines the quantity of mesoporous silica nanoparticle uptake by a small GTPase-dependent macropinocytosis mechanism.⁴⁸ In some other paper for instance, blood circulation time was shown to be extended for PEGylated tobacco mosaic virus with a rod or disc shape in contrast to samples having a spherical shape.⁴⁹ Therefore, particles with shape other than spherical are of importance for the use of drug delivery and drug release as these kinds of particles will be body compatible and will have many other differences in properties (like magnetic, heating, flow rate in blood, stability, etc.), which will broaden the scope to modify the drug delivery and release in the body.

In this paper, we have reported synthesis of cube shaped ferro-magnetic magnetite nanoparticles in a very simple method and shown that these particles will serve as a very good nanocarrier drug delivery system (DDS) with different stimuli dependent drug release properties. These particles have the properties which can be utilized to deliver this type of magnetic system to a proper region directing by magnetic field externally and which in turn again will help to release the drug too in the region of interest inside the body. Here, we have shown that the combined effect of heat and pH simultaneously causes to achieve better efficiency over the release of anti cancer drug. Facilities of this technique are that the process is non-invasive as magnetic field can be applied from outside the body to deliver the DDS in the proper region.

EXPERIMENTAL

Materials and method

All the reagents required for preparation of the particles, such as iron chloride, ethylene glycol, urea, Tx-100, and oleylamine, are purchased from Sigma-Aldrich.

Synthesis of materials

For synthesis of magnetite nanoparticles (MNPs), 4.05 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ is dissolved in 70 ml ethylene glycol. 10 g of Urea is added to the solution and stirred vigorously until a homogenous solution is obtained. 2 ml of Tx-100 is added to the solution and the stirring continues for the next 6 h along with continuous heating. After about an hour, the solution turns dark yellowish, when 2 ml of oleylamine is added. After 6 h, the solutions are allowed to cool. The volume of the solution is doubled by adding ethanol and kept overnight undisturbed. The solutions are centrifuged and thereafter the particles obtained are washed with de-ionized water, ethanol, and acetone, 2 to 3 times in each case. After drying, the particles they are ground well in a mortar and annealed at 300 °C for 1 h under nitrogen atmosphere before further characterization is done.

Loading of drugs to MNPs

The drug doxorubicin (DOX) is loaded in the cube shaped MNPs, and drug loading efficiency and release efficiency are checked with the help of UV-Vis absorption spectroscopy.

40 mg of prepared magnetite nano-crystals is taken in 1 ml of deionized water; then, 40 μ l of ammonium hydroxide solution is added to this and stirred for 30 min; and then, 100 μ l of aqueous 1.4×10^{-3} M DOX solution is added under stirring condition, and stirring is continued for another 30 min to allow the drug to be loaded in the nanoparticles. Then, the drug loaded particles are dried. The dried particles are then gently washed with de-ionized water to remove non-bound drug.

***In vitro* experiment on heating effect of drug loaded MNPs**

For *in vitro* study, human lung carcinoma cell line (A549) of about 10^6 cells were seeded in 6-well plate and incubated for 24 h. Next day, these cells were exposed to 150 μ g/ml of drug loaded MNPs for 2 h under thermal environment of 43 °C keeping in a incubator. After 2 h of incubation at this temperature, the plates were shifted back to 37 °C again. Another control set was kept for 24 h at 37 °C in a CO₂ incubator without treating with particles.

We treated the HeLa cell line with DOX loaded magnetite particles too and incubated for 4 h maintaining the temperature at 43 °C. For cell culture HeLa cell line was maintained in RPMI 1640 media with 10% fetal calf serum (FCS) in 5 cm culture dishes with 100 000 U/L of penicillin and 100 mg/l of streptomycin in a humidified atmosphere of 5% CO₂ in the incubator. 200 μ g/ml of DOX loaded particles were added to this cell line and then incubated for 4 h maintaining the temperature at 43 °C. After completion of 4 h, an aliquot of cell line solution was treated with trypan blue dye and 20 μ l from this solution was put on glass slide and covered with the cover slip, and the image was taken with a high resolution optical microscope.

Characterization techniques

The phase and crystallinity of as-synthesized MNPs are characterized by X-ray diffraction (XRD), and the morphology of the particles is investigated by transmission electron microscopy (TEM). All the XRD patterns were measured in a Rigaku Miniflex II desktop X-ray diffractometer using Cu K α ($\lambda = 1.5418$ Å) radiation whereas the TEM analyses were performed in TECHNAI G² TF20 at 200 kV. For TEM study, the samples were prepared by drop-casting the well dispersed alcohol solution of the as-prepared sample on a 300-mesh carbon-coated copper (Cu) grid followed by drying it in a vacuum desiccator. To investigate the drug release part by these particles, UV-Vis absorbance spectra of aqueous solution of drug in different conditions have been recorded by a Shimadzu model UV-2600 spectrophotometer using a quartz cuvette of 1 cm path length.

RESULTS AND DISCUSSIONS

Structural characterization of magnetite nanoparticles

Figure 1 shows the XRD patterns for as-synthesized MNPs. All the diffraction peaks were analyzed and observed that all the peaks exactly match with the magnetite, which indicates a cubic inverse spinel structure with a face-centered cubic (fcc) unit cell (JCPDS 19-629) composed of 32 Oxygen anions, 16 Fe(III) cations, and 8 Fe(II) cations. Half of the Fe(III) cations are tetrahedrally coordinated, while the other half and all of the Fe(II) ions are octahedrally coordinated.^{50,51} To confirm the magnetite phase, lattice parameters were calculated considering the XRD peaks 511, 440, and 533, which occur at 56.93, 62.57, and 74.1078, respectively, corresponding to 2θ values. The high angle peaks are considered here to minimize the error. The calculated lattice parameters obtained are 8.401, 8.397, and 8.398 Å for XRD peaks 511, 440, and 533, respectively. Taking the average of these lattice parameters, we get 8.398 Å, which matches with the lattice parameter for magnetite. Hence, it can be concluded that the particles are of pure phase magnetite. From the broadening of the peak, the crystallite size is calculated using Debye Scherrer's equation and seen to be of about 18 nm.

The TEM images are shown in Figure 2. From this figure, we clearly see that particles are of cube like shape with size 18 ± 1.5 nm. Some of the particles here look like hexagonal. This again proves the cubic shape of particles rejecting the possibility of being flat tiles like. From

FIG. 1. X-ray powder diffraction pattern for MNPs.

some viewing angles, these cube shaped particles look like hexagonal in shape. We have seen this type of change in shape by tilting the image in different angles during TEM image analysis. As the TEM image gives two dimensional views, so cube shaped particles if observed from other angles may look like hexagonal, rectangular, square, etc., which are clearly shown in left side top of Figures 2(a), 2(b), 2(c), and 2(d). Hence, it can be concluded that our particles are cube shaped, not flat square tiles like shape. From the high resolution TEM image (Figure 2(f)), the inter-planar distance between the lattice fringes is calculated and observed to be about 0.252 nm, which corresponds to the distance between (311) planes of the magnetite crystal lattice. In the in-set of figure (Figure 2(e)) on the right side, the electron diffraction pattern is shown. Analysis of this pattern again confirms that the particles are of pure phase magnetite.

AC magnetic characterization of magnetite nanoparticles

We are interested to use the particles for drug release by the AC magnetic field triggered hyperthermia technique. Therefore, we have performed AC magnetic measurements on these cube shaped particles in our lab made set-up where we can produce about maximum 90 kA/m AC magnetic field at nearly 700 Hz frequency. The AC hysteresis loops for the cube shaped particles are measured changing the AC field frequency from 50 Hz to 600 Hz, few of the representative curves for hysteresis loop measurements are shown in Figure 3(a). In this case, we see that the hysteresis

FIG. 2. TEM image for MNPs: on left side at the corner, (a), (b), (c), and (d) show the pictures of the particles from different viewing angles; on the right side corner picture, (e) shows the electron diffraction pattern and (f) shows the HRTEM image of MNPs.

FIG. 3. AC magnetic field dependent measurements: (a) is the measure of Hysteresis loops at different AC field frequencies and (b) is a plot of SLP Vs AC field frequency.

loop area of the cube shaped particles increases with increasing frequency of AC field. It is known that magnetic nanoparticles can either behave superparamagnetically or ferromagnetically depending on frequency. In our case, particles are behaving ferromagnetically. Hergt *et al.* have demonstrated nicely how the magnetic nanoparticles when exposed to a magnetic AC field may show remarkable heating effects related to losses during the magnetization reversal process of the particles, which is quantitatively defined by specific loss power (SLP).⁵² The temperature enhancement occurs in magnetic nanoparticles under the influence of an external high frequency magnetic field. In our case, the variation of SLP (proportional to the product of hysteresis loop area and frequency) with frequency is shown in Figure 3(b). From the SLP plot, we can have an idea about the heat dissipation by the particles. This figure reflects that the power loss of the particles increases with increasing frequency of applied AC field, which is consistent with our previous published results.⁵³ We have measured the loss up to 600 Hz AC field in our set up because after that the impedance of the circuit becomes so high that the reduced field is not enough to saturate the hysteresis loops. But in spite of this limitation, we have measured the temperatures of the solutions with dispersed magnetic particles at different applied AC frequencies. From these results, we observed that when 10 mg of particles is dissolved in 3 ml of solution and at applied AC frequency 600 Hz, the temperature of the solution becomes 43 °C.

Studies on drug (doxorubicin hydrochloride) release of MNPs triggering with different stimuli

The amount of DOX loaded in this system is calculated from the absorbance value obtained from UV-visible absorption spectra at ~500 nm of initial DOX solution and solution collected after washing the particles. We see that the loading efficiency in this case is 72%, which is much better compared to a previous published result.⁵⁴ We presume that as particles are functionalized with OH⁻ by treating them with NH₄OH and drug is acidic in nature, which in combination facilitate the loading efficiency of drug following simple acid-base reaction. On the other hand, it is known that surface area for cubic particles is higher compared to spherical particles of the same volume. Hence, cubic particles would have better drug loading capacity compared to spherical particles. Therefore, the shape parameter may be another reason for higher loading efficiency in our case. These drug loaded particles are employed for drug release experiment triggering them by two different stimuli such as temperature and pH. For these studies in each case, 10 mg of the above mentioned dried drug loaded particles are taken in 3 ml of phosphate buffer solution (PBS) maintaining proper pH and temperature, and the absorbance of the solution is measured in recorded interval of time for 4 h. The experiments are performed at two different pH levels (5 and 7.4) and at two temperatures (37 °C and 43 °C). The drug release activity of our MNPs at two different temperatures at 37 °C and 43 °C keeping the pH=7.4 and another similar experimental results on drug release activity at higher temperature (43 °C) at lower pH=5 are shown in Figure 4. Here, we observe that at lower pH and higher temperature drug is released with a very high rate. To investigate high sensitivity at lower pH, we

FIG. 4. Drug release activity by MNPs at different temperatures and pH levels.

planned another experiment. At first, 100 ml buffer solution of pH ~ 5 was prepared by the proper method and a small amount of magnetite was taken in 2 ml of this buffer solution. Then, the UV-Vis absorption spectrum of the above sample containing in buffer solution was recorded at zero time. The sample was then stirred for 10 min and took several absorption spectra measured each in 10 min intervals. On the other side, a small amount of Fe(III) ion was added to 2 ml of above mentioned buffer solution. The Fe(III) is obtained by dissolving the same amount of sample in 2 ml of HCl solution. The absorption spectra were taken for this Fe(III) ions solution dissolving it in buffer solution. All the UV spectra are shown in Figure 5. From these curves, it has been observed that there is a peak at about 312 nm ($\lambda_{\max}$) for all the curves. This indicates that our samples are dissolved and Fe(III) ions are formed at lower pH. On the other hand, we observe here that intensity of UV spectra for the particles taken in lower pH buffer, measured in 10 min interval of time, increases with progress of time, which again give the evidence of dissolution of particles with very slow rate though. Hence, this result supports the information that the drug molecules bind with oxygen site of the magnetite particles during loading through the reaction as shown in the schematic diagram (Figure 6). At lower pH, the surface of the particles is dissolved and drugs are detached from the particles.⁵⁵ The environment around cancer cells is more acidic than normal cells and it falls in the range of 5 to 5.5

FIG. 5. All the UV-visible absorption spectra for magnetite sample in low pH buffer solution measured at 10 min interval of time under stirring condition and for Fe(III) ions taken in the same buffer solution.

FIG. 6. Schematic diagram on the mechanism of drug loading and release by MNPs.

pH level.^{56,57} Hence, the release of drugs by our particles is much more significant and shows a high potentiality in the use of particles for such purpose. On the other hand, when temperature is concerned, it is observed that the percentage release of the drug is higher at 43°C than in the case of 37°C . At higher temperature due to thermal agitation, vibration of drug loaded particles becomes higher, which breaks the bonding between drug and particles and hence drug release becomes easier. So we observe that by this method triggered by both heat and pH, the drug release property can be enhanced significantly compared to others.^{58–60}

We have shown that under AC magnetic field, magnetic particles are heated up and this heating can be controlled by changing the value of frequency and field of AC magnetic field. Hence, by changing the applied AC magnetic field and frequency of AC field, the release of drug can be controlled noninvasively and pulsed release is also possible according to requirement by utilizing this field triggered method.

***In-vitro* study on heating effect of DOX loaded MNPs**

To see the heating effect of drug loaded particles on cancer cell line, a comparative study was made treating the cells with particles and without particles, and the transmitted images were taken in a EVOS-FL microscope (Invitrogen, USA), which are shown in Figures 7(a) and 7(b). Here, we see that after treatment with DOX loaded particles at 43°C (Figure 7(a)), most of the cells are floating and those are dead but in the case of control almost all cells remain alive.

The death rate of cancer cells was checked with HeLa cell line also as this is a cell type in an immortal cell line used in scientific research. We treated the HeLa cell line with DOX loaded magnetite particles under the temperature 43°C following the method as mentioned in the Experimental section. To check the cell death, an aliquot of cell line solution was treated with trypan blue dye, and the image was taken in a high resolution optical microscope. The image is shown in Figure 7(c). From this figure, it is evident that most of the cells are killed by this method. From these experiments, we can see that by this method, the rate of cancer cell death is very high, which is the most desirable goal in the cancer research community. By using

FIG. 7. Transmitted images of cancer cells after treatment with the drug loaded sample of dose $150 \mu\text{g/ml}$ at 43°C are shown in (a), for control one (at 37°C) is shown in (b) and optical microscopy image after treatment with drug loaded particles at 43°C is shown in (c).

our method, we are able to kill the cancer cells in high rate that may give a new direction to manage the problem in cancer research in a better way as cancer is a disease involving abnormal cell growth with the probability to invade or spread to other parts of the body.

It is known that DOX is a cytotoxic drug, which is usually administered to cancer patients by the intravenous and whenever appropriate, intravesical and intra-arterial routes. It is a drug used in cancer chemotherapy and it works by intercalating DNA with the most serious adverse side effects like damaging heart, liver, etc.^{61,62} It is generally used in the treatment of a wide range of cancers, including hematological malignancies (blood cancers, like leukaemia and lymphoma), malignant tumours, and soft tissue sarcomas. Therefore, controlled release of this drug in a selected region without spreading it all over body is very desirable to the clinical as well as scientific community, which can be achieved by our method by applying the AC magnetic field of suitable amplitude and frequency from outside the body. On the other hand, efficiency of drug release by our proposed method can be enhanced simultaneously by triggering with AC magnetic field as well as pH and due to the improved efficiency, it is possible to decrease the administered dose significantly as compared to other drug delivery systems.^{31,37}

CONCLUSION

We have synthesized cube shaped particles of magnetite. Structural characterization of these particles shows the pure phase of magnetite. Magnetic measurements offer promising assurance regarding their suitability for the application of drug release by heat triggered way. On the other hand, pH responsive drug release is also possible by this particle. pH and temperature dependent drug release studies give us new information about better treatment for cancer and other diseases by controlled release in a non-invasive way. At 43°C , release takes place with higher rate so by this method by applying AC magnetic field from outside the body; the controlled release of drug can be achieved. Pulsed release of drug is also possible according to the requirement of the patient by on off application of AC magnetic field. Loading efficiency is also higher for these particles compared to other published results. These magnetic nanoparticles offer a new horizon in the area of drug delivery as well as release of drugs. This study provides us a promising tool as the drug delivery system when the problems of toxicity, terrible side effects, localized cancer treatment, and cost are concerned.

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