Plasmonic/Magnetic Multifunctional nanoplatform for Cancer Theranostics

M. Ravichandran¹, Goldie Oza², S. Velumani³, Jose Tapia Ramirez³, Francisco Garcia-Sierra⁴, Norma Barragan Andrade⁴, A. Vera⁵, L. Leija⁵ & Marco A. Garza-Navarro⁶

A multifunctional magneto-plasmonic CoFe₂O₄@Au core-shell nanoparticle was developed by iterative-seeding based method. This nanocargo consists of a cobalt ferrite kernel as a core (Nk) and multiple layers of gold as a functionalizable active stratum, (named as Nk@A after fifth iteration). Nk@A helps in augmenting the physiological stability and enhancing surface plasmon resonance (SPR) property. The targeted delivery of Doxorubicin using Nk@A as a nanopayload is demonstrated in this report. The drug release profile followed first order rate kinetics optimally at pH 5.4, which is considered as an endosomal pH of cells. The cellular MR imaging showed that Nk@A is an efficient T₂ contrast agent for both L6 (r₂-118.08 mM⁻¹s⁻¹) and Hep2 (r₂-217.24 mM⁻¹s⁻¹) cells. Microwave based magnetic hyperthermia studies exhibited an augmentation in the temperature due to the transformation of radiation energy into heat at 2.45 GHz. There was an enhancement in cancer cell cytoxicity when hyperthermia combined with chemotherapy. Hence, this single nanoplatform can deliver 3-pronged theranostic applications viz., targeted drug-delivery, T₂ MR imaging and hyperthermia.

Cancer is the second leading disease which causes major mortality and morbidity worldwide¹. In cancer therapy, it is crucial to increase the drug specificity and drug efficacy to minimise or completely eradicate significant side-effects on patients². Cancer nanotherapeutics overcome many serious drawbacks of chemotherapy such as non-specific targeting, lower efficacy, insolubility of drug moieties in water and oral bioavailability³. Accordingly, Superparamagnetic Iron Oxide Nanoparticles (SPIONs) are exploited as an important nanomaterial for cancer detection as well as therapeutics⁴. Such magnetic nanoparticles (NPs) gained its momentum because of their single-domain ordering along with their large surface to volume ratio (providing large surface area for attachment of biological entities). Hence, this property makes them a suitable candidate as a contrast agent, drug-carrying cargo and hyperthermal agent⁵. The doping of SPIONs with cobalt ions further enhances their magnetic property, thus forming CoFe₂O₄ nanokernels (Nks). These spinel ferrite Nks possess ca. 20–30 times higher magneto-crystalline anisotropy as compared to SPIONs; this increases the performance of materials for biomedical applications⁶–⁸. Specifically, these Nks are mostly used in biomedicine than any other spinel structure because of their enhanced magnetic property and large anisotropy⁹. The increased superparamagnetism makes them an efficient system for theranostics¹⁰–¹².

Such superparamagnetic Nks are reactive and toxic to cells; hence, gold NPs are used for creating a shell on the magnetic core. This architecture is biocompatible and chemically inert in the physiological system¹³. The core-shell nanoparticles (CSNPs) possess unique optical and magnetic properties, thus creating an efficient platform for nanomedicine¹⁴. The significant benefit of the gold nanoshell is to provide complete protection to the inner magnetic core from a plethora of environmental factors¹⁵. This coat also acts as an excellent platform for surface modifications¹⁶,¹⁷, real-time imaging and drug carrying cargos¹⁸,¹⁹.

¹Program on Nanoscience and Nanotechnology, Av. 2508 National Polytechnic Institute, Gustavo A. Madero, San Pedro Zacatenco, 07360 Mexico City, Mexico. ²Department of Genetics and Molecular Biology, Av. 2508 National Polytechnic Institute, Gustavo A. Madero, San Pedro Zacatenco, 07360 Mexico City, Mexico. ³Department of Electrical Engineering, Av. 2508 National Polytechnic Institute, Gustavo A. Madero, San Pedro Zacatenco, 07360 Mexico City, Mexico. ⁴Department of Cell Biology, Av. 2508 National Polytechnic Institute, Gustavo A. Madero, San Pedro Zacatenco, 07360 Mexico City, Mexico. ⁵Department of Mechanical and Electrical Engineering, Universidad Autonoma de Nuevo Leon, San Nicolás de Los Garza, Nuevo León, 66451 Mexico City, Mexico. Correspondence and requests for materials should be addressed to S.V. (email: velu@cinvestav.mx) or J.T.R. (email: jtapia@cinvestav.mx).
The major hurdle in synthesising CSNPs is that there is no uniform coating of gold shell on the surface of the iron oxide core, even though the ratio between iron and gold is 1:720,21. Therefore, gold iteration is a method that improves the formation of CSNPs and controls precisely the thickness of Au shell22,23 on the magnetic core. Even though iron oxide and gold CSNPs have been explored extensively24,25 for more than two decades, there are very few reports about CoFe2O4@Au nanoparticles21,26.

Doxorubicin (Dox) is one of the potential and most widely used anti-cancer agents for various types of cancers. This drug has shown inimical side effects on healthy cells such as cardiotoxicity, mucositis and myelosuppression27–29. These adverse effects are minimised by targeted drug delivery which uses specific molecules such as folic acid (FA) since cancerous cells overexpress folate receptors on their surface30. Apart from synaphic delivery of drugs, the most crucial parameter is its actual release for killing the cancerous cells. The effective release is dependent on different types of stimuli such as internal (alterations in pH, temperature, redox condition as well as the enzyme activities) or external (such as a magnetic field, radiations and ultrasound)31.

Hyperthermia induced by external magnetic field is the most celebrated mechanism that enhances drug release efficiency of the system and are easiest to be used32. The synergistic action of hyperthermia and chemotherapy induces apoptosis as well as necrosis in the cancer cells followed by an enhanced immune response. There is a huge impact of hyperthermia-combined chemotherapy on the immune system of hosts since they induce both adaptive as well as innate immunity. Hence, thermo-chemosenitization is considered as the future of clinical research33.

This work reports multiple iterative gold seed coated cobalt iron oxide nanokernels (Nk@A) as a tri-pronged cancer theranostic agent (Fig. 1). The attachment of FA on the surface of Nk@A was used for tethering folate receptors present on cancerous cells30. Furthermore, Dox moieties orchestrated on FA attached Nk@A were responsible for their anti-cancer activity. Hence, these nanocargos act as proficient drug delivery missiles that targets cancer cells. The drug release profiles were studied using release kinetic models. Moreover, these Nk@A also served as a T2 contrast agent for MR imaging. Finally, such a complex nanocargo was exploited for microwave based localized hyperthermia of cancer cells.

Results and Discussions

Characterization of Plasmonic/Magnetic NPs. XRD analysis was carried out to detect the purity and phase crystallinity of the synthesized Nk and Nk@A (Fig. 2). XRD pattern of Nk cubic spinel phase exhibited well-defined diffraction peaks that match with the CoFe2O4 structures (JCPDS card no. 22-1086)34. XRD pattern of Nk@A showed reflections that correlated well with the FCC ordering of Au (JCPDS card no. 04-0784), thus confirming the formation of a nanoshell onto Nk. In this case, only Au diffraction peaks were observed due to the heavy metal atom effect of Au35,36. The average particle sizes (Dxrd) of Nk and Nk@A were calculated by considering the most intense peaks [Nk (220), (311), (440) and Nk@A (111)]. According to Scherrer equation, the average crystalline size calculated for Nk was 9.68 nm and for Nk@A, 16.69 nm. The particle sizes obtained were well corroborated with corresponding TEM images.
Figure 2. XRD spectrum representing the formation of Nk and Nk@A.

Figure 3a–d† shows TEM, HR-TEM, HAADF-STEM images and line scan of Nk and Nk@A (1st, 3rd & 5th iterations). TEM image of Nks were spherical in shape with the size range of 11–14 nm exhibiting high crystallinity with aggregation which is illustrated in Fig. 3a(a†). The crystal lattice structure of Nks were distorted on the surface due to the curvature effect. Hence, the gold shell could grow epitaxially on the surface of Nks due to a large lattice mismatch. This leads to the formation of Nk as a core and gold as a shell. As iteration increased, the size of the core-shell is also increased five times from 1st to 5th iteration with Au nanoshell. This increment was due to the continued conjugation of nanogold onto the surface of the core Nk, which lead to the formation of Nk@A. HAADF-STEM analysis clearly demonstrated the discrimination between the core and the shell. This is because this contrast is directly proportional to atomic number (Z). In the 1st iterative step, the Nk was coated with Au nanoshell of around 1–1.5 nm (Fig. 3b(b†)) without any aggregation. This proves that the Au iterations not only forms a shell but also stabilizes the nanoparticles. However, in order to enhance the SPR property, the iteration was continued; thus leading to the formation of a nanoflower, that constituted of the collective core made of Nk encapsulated by a thick Au shell. The nanocluster showed jagged-like morphology due to non-homogeneously aggregated Nk, which leads to highly asymmetric coating of Au layer (Fig. 3c(c†))35. The thick gold nanoshell formation after multiple iterations in the Nk@A solution could be inferred from the colour change (Figure S1a in the †ESI). Figure 3c† illustrates the line scan analysis of 3rd iterated nanoflowers showing the elemental distribution of Co, Fe and Au in a single nanoparticle. The line scan confirms that Au signal is seen on the surface of Nks and Co, Fe signals are enriched in the inner core of the Nks. But as the iterations (5th iterations) continued, the Au seeds started to fill in the empty space of the knobby structures32. This resulted in the formation of separated spherical Nk@A and consecutively the shell size increased to 5–6 nm (Fig. 3d(d†)). Similar kind of NPs were obtained for Au@Fe3O4, which had a thin shell of Au36. Figure 3d† represents the corresponding line scan, which clearly shows the Co K, Fe K edges in the core and Au L edges in the shell similar to that of 3rd iterated particles. Additionally to the line scan intensities illustrated in Fig. 3c†,d†, it is interesting to define the stoichiometry represented by the Au L0s, whose ratio is higher than the core elements such as Co and Fe K0. This proves that the Au signal is exhibited strongly than the signal from the core. Therefore, these results suggest that the formation of CSNPs expressing typical elemental composition of high Au content than the core elements, is well corroborated from XRD spectra. The colour mapping shown in Fig. 3(a,a†,b,b†,c,c†,d,d†) proves that the nanostructures are made up of two different metals depending on the electron density of the atoms. This colour mapping was carried out using the Digital Micrograph 3.7.0 by Gatan software. EDS spectra was carried out to determine the composition of CSNPs for the 1st, 3rd and 5th iterations (Figure S1b,c,d in the †ESI) showing the signal of Au, Co & Fe37. The spectra clearly distinguishes the different Au iterations from 1 to 5 just by increasing order of Au signal intensity.

The magnetic property of Nk@A is imperative to have an effective penetration in the cancer cell38. SQUID analysis (Fig. 4) shows a decrement in magnetic saturation (MS) from 74 to 45 emu/g, along with the diminishing hysteretic features. As temperature increased from 5 to 312 K, coercivity (HC) and remanence decreased from 146 to 320 E and from 8 to 2 emu/g, respectively (Table S1 in the †ESI).

This magnetic behaviour was attributed to the thermal relaxation of the magnetic moments of the Nk@A. This behaviour was also ascribed to the re-orientation of the magnetic moments of NPs caused by the thermal energy once it surpassed the magnetic energy imposed by the applied field. Thus, the magnetic characteristics of the Nk@A can be ascribed to those expected from a soft ferromagnetic material, even at 312 K. This kind of ferromagnetic character can be understood from MR imaging which showed high relaxivity values without the interference of Au nanoshell. Moreover, in the case of hyperthermia, the Nk@A showed increased heat dissipation under microwave irradiation in a short span of time.

XPS measurements determined the binding energies and composition of Nk@A. The elements viz., Au, Co, Fe, C and O existed within the range from 0 to ≈1300 eV. Core level spectra were recorded and represented in Figure S3a in the †ESI. Fe2p3/2 and 2p1/2 peaks from Fig. 5a situated at around 711.7 and 725 eV respectively,
were broadened due to the presence of Fe$^{3+}$ ions in tetrahedral sites$^{39,40}$. There is also a satellite peak of Fe$^{3+}$ at 719.6 eV, which confirms the presence of Fe$^{3+}$. The orbitals of Co (Fig. 5b) showed that Co2p3/2 electrons exhibited binding energies at 781.2 eV, which corresponds to ions. This may be due to the substitution of Fe$^{3+}$ ions with Co$^{2+}$ in the tetrahedral site. There is also the existence of a peak at 786.6 eV, which again confirms the presence of Co$^{2+}$. This is in accordance with the XRD data, which proves that there are no mixed phases of CoO or Fe$_2$O$_3$ in the Nk samples$^{41}$. Figure 5c shows Au binding energies with doublet peaks at 83.8 and 87.4 eV thus denoting the Au state of Au4f7/2 and Au4f5/2, respectively. This shows that gold ions are completely converted into metallic Au$^0$, leading to the formation of CSNPs.

Interestingly, the spectrum did not show any detectable Cl2p signal$^{42}$ which further proved the complete reduction of Au onto the surface of Nk (Fig. 5d). The O1s peak showed in Fig. 5e confirmed the presence of oxygen atoms$^{43}$. The C1s signature markers of carbon atom binding energy at 284.7 eV were taken as a reference (Fig. 5f$^{43}$). Therefore, XPS pattern was in good agreement with XRD data, TEM-EDS and line scanning results.

**Tethering folic acid linker and Doxorubicin molecules on Nk@A.** UV-Visible absorption spectra of different iterations of Nk@A were performed as seen in Fig. 6a. As the number of gold iterations on Nk increased from one to three, there was a bathochromic shift of surface plasmon resonance peak (SPR) from 541 to 546 nm. In addition, as the iteration increased from three to five, this SPR peak further shifted from 546 to 551 nm. This bathochromic shift in the SPR peak was related to the increment in the thickness of gold shells on the surface of the magnetic core$^{44}$. Moreover, as iterations increased, the charge density and the amplitude of the free electron oscillation inside the particles also increased. This may be due to the increased surface coating of CTAB, thus causing enhanced plasmonic absorption$^{44}$. The spectra of Nk and Au seeds are also shown in Figure S2 in the †ESI. UV-Visible spectra of FA attached Nk@A (Fig. 6b) shows distinct peaks at 280.8 and 375.2 nm, which are signature markers of FA. Accordingly, there was a bathochromic shift in the SPR peak after FA attachment which showed a peak at 562.4 nm; this shift confirmed that FA formed a complex with Nk@A$^{45}$. Dox attachment was
confirmed from the peak (inset) at 490.7 nm with minor red-shift of Nk@A peak (538.9 nm) and slight blue-shift of the FA attachment (268.7 & 375.2 nm). These shifts ensured the formation of the Dox-FA-Nk@A complex, which is shown in Fig. 6c.

FTIR spectra of functional organic markers on the surface of Nk@A is shown in Fig. 7a. Spectra A represented only CTAB peaks (Table S2 in the †ESI)46, which proved that there was an excess amount of CTAB in the solution. However, spectra B which was analyzed after repeated centrifugation and washing the Nk@A solution, showed a minor peak of CTAB which stabilizes the Au shell (Table S2 in the †ESI). This was also demonstrated from its cytocompatibility towards L6 cells, which was evident from MTT assay and confocal microscopy studies.

Figure 7b shows FTIR spectra of FA-Nk@A and Dox-FA-Nk@A. Spectra A represents both activated FA, which show bands at 1640.7 cm$^{-1}$ and 1718 cm$^{-1}$ expressing –CH stretch and –NH stretch, respectively. FA conjugation to Nk@A was confirmed from –NH and –CH stretch; while the asymmetric stretching of primary amines –NH and bending vibrations of –CO confirmed the formation of amide linkage between FA and Nk@A at 1585.2 cm$^{-1}$47. Spectra B represents the attachment of Dox moieties onto FA-Nk@A. The interaction between these molecules was via amide linkage, which involved –NH amino group of FA and –COOH carboxylic group of Dox. Bands representing these attachments were 1436.8 cm$^{-1}$ that represents anhydride –CO stretch and 1651.4 cm$^{-1}$ denotes amide stretch of –CO. The peak at 2915 cm$^{-1}$ was a classic peak of secondary –NH$_2$ bending and peak at 3000.68 cm$^{-1}$ corresponded to primary –NH$_2$ bending47.

TGA analysis was carried out for all samples in the temperature range of 30–900 °C under N$_2$ flow atmosphere and any change in % weight loss was recorded. TGA graph (Figure S3b in the †ESI) shows Nk, Nk@A, FA-Nk@A, Dox-FA-Nk@A, activated FA and Dox. Initially, activated FA and Dox showed a gradual weight loss from 30 to 100 °C. The degradation of these moieties was rapid because they are completely organic in nature. Nk showed a rapid degradation at 122 °C, which was due to the complete evaporation of water molecules. Then,
there was a solid plot up to 555 °C and a drop at 728 °C followed by a slow degradation of bound chlorides and hydroxides. In the case of Nk@A, the initial weight loss from 30 to 288 °C was due to the complete desorption of water and CTAB molecules from the surface. The second degradation, from 289 to 595 °C, was a result of the covalent interaction of CTA⁺ ions; the final degradation at a higher temperature, from 596 to 900 °C, was most likely due to the electrostatic interaction of the ammonium group from CTAB attached to the Au NPs surface.

In FA-Nk@A complex, the weight loss in the range of 30–100 °C was due to the desorption of intercrystalline water molecules. The second degradation, in the range of 101–750 °C, was due to FA covalent attachment, which was seemingly induced by the disintegration of groups like hydroxyl, carboxyl and amino groups present in FA.

Dox-FA-Nk@A complex initially showed degradation of water moieties and weak surface interactions of hydrogen with the Nk@A. The second decomposition from 96 to 740 °C, was due to the decomposition of FA and Dox from the NPs complex and the decomposition from 741 to 900 °C is due to slow degradation of Nk@A.

Intracellular localization of Dox & Dox-FA-Nk@A. CryoTEM analysis confirmed Nk@A internalization and endocytic cavities that contained NPs (Fig. 8a,b) with aggregation, as the pH was acidic. This observation gave an insight towards the mechanism of receptor-mediated endocytosis of Nk@A, which can be attributed to FA and folate receptor interactions. Moreover, the cellular uptake of NPs is also dependent on the surface charge. Zeta potential (ζ) values for Nk, Nk@A, FA-Nk@A and Dox-FA-Nk@A complex were in the range of +11.9 to +12.1 mV, +33.4 to +37.7 mV, −35.9 to −38.4 mV, and +16.8 to +19.4 mV, respectively (Figure S3c in the †ESI). This charge dependency is due to the stability of the complex, which conciliates the harsh physiological milieu, such as in the bloodstream or inside the cell. The mechanism of cellular uptake for positively charged nanoparticles is interaction of the positive moieties with the negatively charged cell surface. Rotello and coworkers studied the effect of surface charge on the stability of amine functionalized gold nanoparticles. It was found that net positive charge caused more displacement of ligands in extracellular and intracellular environments.
in normal tissues at neutral pH, while it gains its activity in cancerous tissue at lower pH. Hence, Dox-FA-Nk@A was highly water-soluble as well as hydrophilic at lower pH as compared to neutral pH. Hence, Dox-FA-Nk@A was internalized in PBS with agar gel. These agar phantoms were used to evaluate the T1 and T2-weighted images shown in Fig. 9, which was attributed to the high regression coefficient value ($R^2 = 0.9865$). This confirms that the drug release is pH as well as concentration dependent mechanism.

Magnetic Resonance Imaging in normal and cancerous cells. Nk@A was employed as an efficient MR imaging contrast agent. It was cultured with both L6 and Hep2 cells. The cells were harvested and then suspended in PBS with agar gel. These agar phantoms were used to evaluate the $T_1$ and $T_2$-weighted images shown in Fig. 13a (only $T_2$-weighted images). As the concentration increased from 0.08–0.64 mM of Nk@A, there was an increment in $T_2$-weighted image, which became darker with both cells. The linear relationship was calculated in order to obtain the longitudinal relaxivity $r_1$ and transverse relaxivity $r_2$ of both L6 and Hep2 cells incubated with Nk@A (Fig. 13b,c). The $r_1/r_2$ and $r_2/r_1$ values were calculated and are shown in Table S3 in the †ESI. We found that the $r_2/r_1$ value of L6 was 83.15 and for Hep2 it was 120.68. This was much higher than $r_1/r_2$ of both, showing more than 80% cell death at high concentrations.
which confirmed that Nk@A were efficient T2 contrast agent as compared to T1. But in the previous report for CoFe2O4@Au, r2/r1 value was around 3321. So, we concluded that the increment in the r2/r1 value was mainly due to Au iterations.

The r2 value of Nk@A with L6 was 118.08 mM−1s−1 and Hep2 was 217.24 mM−1s−1. This is highly comparable to the clinically used MRI contrast agent such as Feridex 105 mM−1s−1 65, which shows noticeable changes after injecting iron oxide NPs66. Therefore this confirmed that the Au nanoshell around Nk did not play a role in Nk core spin67.

But the r2 value with Hep2 cells showed significant increment when compared to L6 cells because of gold iterations. The other reason might be the uptake of gold coated magnetic NPs by cancerous cells was higher than that of normal cells68 because the electrostatic interactions of surface charge from gold coating cause more interaction with the cell69. Finally, we demonstrated that the Nk@A can be effectively used as an efficient T2 contrast agent.

Microwave based Hyperthermia therapy. Hyperthermia therapy involves increase in the temperature of tissues or cells, so that they become more susceptible to anti-cancer drugs. Nk@A nanoparticles were tested as a hyperthermal agent under microwave (MW) irradiation by using a microcoaxial double slot antenna as an applicator. The increment of temperature as a function of time was measured under ISM (Industrial, Scientific and Medical) approved frequency of 2.45 GHz in order to induce localized hyperthermia. The applicator was inserted in the phosphate buffered saline (PBS) containing Nk@A of various concentrations (10–125 μg/ml) and the increment of temperature was measured by using noninterfering fiber optic probes. The MW was irradiated using a home-made setup70 for 150 sec at 6 W. Interestingly, the temperature increment was very rapid and reached 45°C in around 75 sec, which was enough to kill cancer cells; the temperature raised up to 50–60°C within 150 sec for Nk@A (Fig. 14a).

The PBS was used as a control which showed maximum rise in temperature as water has the highest absorption of MW71. This temperature increment was purely based on the Au iterations on Nks. Au nanoshell as well as...
Figure 11. Confocal microscopy study representing the morphology of Hep2 cells treated with nanoparticles for 24 h (a) Nk, (b) Nk@A, (c) FA-Nk@A which shows negligible cell death but (d) Dox-FA-Nk@A clearly shows the increased cell death at higher concentration of nanoparticles (Scale bar-20μm).

Figure 12. (a) Cumulative release of Dox at 3 different pH, (b) Various plots representing different fitting in kinetic models of drug release.
superparamagnetic core led to energy increment which may be due to magnetic anisotropy as compared to Nks in xylene at 2.45 GHz. The temperature increment was much higher when compared with Au@-Fe₃O₅, which was around 38°C at 2.45 GHz in water, for 10 mins at a power of 120 W.

Figure 13. (a) T₂ contrast image of Nk@A as the concentration increases the darkening effect also increases depending upon the type of cells, (b) r₁, (c) r₂ relaxivity values of Nk@A incubated with L6 and Hep2 cells.

Figure 14. (a) Plot showing increment of temperature as a function of time with the increased concentrations of Nk@A as a potential hyperthermal agent, MTT assay of Hep2 cells (24 h) subjected to hyperthermia (50°C for 50 sec): (b) in-vitro hyperthermia where cells treated with Nk@A which represents the killing of cells as the concentration increases by producing enough heat, (c) in-vitro chemohyperthermia where cells treated with Dox-FA-Nk@A which represents the killing as the concentration increases by producing heat and also release of Dox.
**In-vitro Hyperthermia and Chemo-Hyperthermia therapy.** Chemo-hyperthermal effect or thermo-chemosensitization is quantified by the interaction of anti-cancer drugs with cells at elevated temperature. The thermal enhancement ratio (TER) for Dox is 1 at two different temperatures (41.5 °C versus 43.5 °C, respectively)75. There is a relationship between drug-heat interactions on cell cytotoxicity. This pharmacodynamics is responsible for enhanced killing of cells. The mechanism of thermal enhancement for drug cytotoxicity includes enhanced drug uptake as well as DNA damage and retardation of DNA repair31. There are previous studies, which reported the thermal enhancement of cellular cytotoxicity when drug interaction with cells takes place at higher temperatures76. In this report, an increase in Nk@A concentration shows an increased cell mortality in addition to MW radiation exposure for 50 sec. The cells interacting with increased Nk@A concentration, but without any exposure to MW irradiation (Fig. 14b), showed comparatively lesser mortality than the exposed ones. Both Au shell and superparamagnetic core absorb MW, due to which hyperthermia is induced in the cells, thus consequently leading to cell death. The viability of Hep2 cells was suppressed more by Dox-FA-Nk@A when exposed to microwave as compared to unexposed ones (Fig. 14c). The Dox concentration was considered based on its half-maximal inhibitory concentration of 12.5 μg. Hence, here the Dox tethered Nk@A concentration was in the range of 2–14 μg/ml. The IC50 value of Dox-FA-Nk@A-MW was 8 μg as compared to the IC50 value of Dox-FA-Nk@A, which is 12 μg. This clearly shows thermal enhancement of Dox cytotoxicity at lower IC50 values in comparison with Dox alone. Hence, Dox orchestrated Nk@A efficiently inhibited the cell viability at elevated temperatures and very low concentrations of Dox, thus improving the therapeutic proficiency along with minimum side-effects. Concurrently, this combined therapy shows efficient synergism, thus exhibiting mininal effect on Hep2 cells. In summary, we have developed SPR enhanced Nk@A by multiple iterative method, which proved to be a promising nanomaterial with multifunctional properties specifically in the field of cancer nanotheranostics. These multiple iterations provided a new platform for surface functionalization. This helped in the efficient delivery of drugs, following first order rate kinetics. This Nk@A was also used as a competent MRI contrast agent and proved to be an effective T2 agent with high relaxivity values in the presence of both L6 and Hep2 cells. Finally, Nk@A was used as a hyperthermal agent. In an in-vitro study using Hep2 cells, both Nk@A and Dox-FA-Nk@A on exposure to microwave irradiation using 2.45 GHz for 50 sec showed cell mortality. It was found that there is an enhancement of cell mortality when MW based hyperthermia collates with Chemotherapy. With this efficiency, Nk@A can be used for potential applications as a single nanomaterial for 3 different uses, from tracking, diagnosing to therapeutics.

**Materials and Methods**

**Materials.** Ferric (III) chloride (FeCl₃·6H₂O, 97%), Cobalt (II) nitrate hexahydrate (CoNO₃·6H₂O, 99.999%), sodium hydroxide (NaOH, >98%), Gold (III) chloride trihydrate (HAuCl₃·3H₂O, ≥99.9%), L-Ascorbic acid (AA) (C₆H₈O₆, ≥99%), Dimethyl sulfoxide (DMSO) (CH₃SOCH₃, ≥99.9%), Sodium chloride (NaCl, ≥99.5%), Hexadecyltrimethylammonium bromide (CTAB) (CH₃(CH₂)₁₅N(Br)(CH₃)₃, ≥99.0%), Folic acid (FA) (C₁₉H₁₉N₇O₈, ≥97%), N-Hydroxysuccinimide (NHS) (C₅H₄NO₆, ≥98%), N,N'-Dicyclohexylcarbodiimide (DCC) (C₁₂H₁₀N₂, ≥99%), Triethylamine (TEA) ((C₂H₅)₃N, ≥99%), Doxorubicin hydrochloride (Dox) (C₂₇H₂₉NO₁₁•HCl), MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide), Hoechst Stain solution, Phalloidin–Tetramethylrhodamine B isothiocyanate, and ethanol (CH₃CH₂OH, ≥98.9%) were purchased from Sigma-Aldrich (Mexico). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and streptomycin were obtained from Gibco, Life Technologies. Agarose (UltraPure, Agarose) was purchased from Invitrogen, Thermo scientific. Materials.

**Synthesis of Cobalt ferrite (Nk) and gold-coated Nk (Nk@A) NPs.**

**Nk preparation.** Magnetic Nk were synthesized using co-precipitation method. The precursors 0.5 M of ferric (III) chloride and 0.25 M of cobalt (II) nitrate hexahydrate were taken in the ratio of 1:0.5 and in order to avoid the precipitation of the salts, they were initially dissolved separately in 10 ml of nitrogen (N₂) degassed DI water and mixed with 1.5 M solution of 40 ml NaOH, which was used as a reducing agent. The entire synthesis process was carried out under N₂ atmosphere for 1.5 h at 80 °C (pH 12). The black resultant precipitate was separated using a strong magnet and it was washed 3 times with DI water. The pure Nk were further used for Nk@A formation.

**Nk seed preparation.** 200 μl of synthesized Nk was centrifuged and dispersed in 1 ml of DI water (pH adjusted to 7). Then, 400 μl of DMSO was added and the mixture was stirred continuously under N₂ atmosphere at 75 °C for 3 h to reduce the aggregation of NPs.

**Preparation of gold seed solution.** Au coating was carried out for 5 iterations, 5 aliquots of gold seed solution were prepared freshly by mixing 0.5 ml (1 M) of CTAB, 1 ml (50 mM) of ascorbic acid, and 100 μl (1M) of HAuCl₄ solution. This solution complex mixture was sonicated for 15 mins. The golden yellow color immediately changed to a milky orange color and then to a milky white color (Figure S1b, (b*) in the †ESI).

**Synthesis of Nk@A CSNPs.** The seed solution of Nk and gold were used to synthesize Nk@A. In this case, the ratio of 1:5 was taken. This was because we had already optimized the ratio with 1:5, 1:7, and 1:9 and found that 1:5 was appropriate for the Nk@A. Initially, 1 part of milky white colored gold seed solution was added drop-wise to the faint brown-colored Nk seed solution. This solution mixture was stirred for 2 h continuously until the brown colored solution turned into purple. Then, the gold iteration was continued for 5 times for every 2 h. Finally, a dark purple color Nk@A solution was obtained. Then, these Nk@A were magnetically separated (after each iteration) by magnetic separation technique and they were washed twice with a mixture of hexane and ethanol to obtain high purity NPs excluding excess gold NPs. The washed NPs were centrifuged again twice to remove excess CTAB from the solution.
FA Activation and attachment to Nk@A. Activated FA was used for the attachment onto the surface of Nk@A. The reason behind the activation of FA was to activate the carboxylate group. This was carried out by dissolving FA (0.25 g) into 20 ml of DMSO which was subjected for 1 h sonication to ensure complete dispersion. Later, carboxylate group present in FA was activated by mixing 0.125 gm of DCC and 0.225 gm of NHS. The complete reaction was carried out under N₂ atmosphere at 30 °C for 12 h (FA/DCC/NHS molar ratio: 2:1:2). The resultant product was filtered using Whatman filter paper; then it was further used for attach onto Nk@A.

Activated FA was used to attach onto the Nk@A. This attachment was carried out by mixing 1 ml of activated FA and 10 ml of Nk@A under N₂ atmosphere and stirring the mixture continuously for 5 h. Then, N₂ atmosphere was detached and the reaction was continuously stirred for 24 h. Finally, this reaction mixture was filtered using Whatman filter paper. Then the process of dialysis was carried out to eliminate unreacted FA using a 3000 kDa dialysis membrane in PBS (pH 7.4). After centrifugation, the pellet was again dialyzed in DI water for a period of 24 h. The activated FA binding onto the surface of Nk@A was studied using UV-Vis spectroscopy analysis.

Synthesis of Dox-FA-Nk@A CSNPs complex. Anthracycline antibiotic Dox was used to kill cancerous cells using FA-Nk@A. Activated FA functionalization on Nk@A acted as an anchor for the binding of Dox. To bind Dox, 5 ml FA-Nk@A was mixed with 1 ml of TEA and ml of DMSO as a solvent; finally, 400 μl of 2.36 mM Dox solution were added. This mixture was purged using N₂ gas under continuous stirring at 60 °C for 5 h. The final complex solution was dialyzed to remove the unbound or excess of Dox. The Dox binding was studied by characterization techniques, like FTIR and UV-Vis spectroscopy.

Dox loading efficiency and in-vitro Dox release. Before initiating the drug release studies, it was very significant to determine the Dox loading efficiency because the exact amount of Dox bound onto FA-Nk@A complex was so decisive to calculate the proper drug delivery study (in the †ESI). Dox release studies were carried out by dialysis process. Drug release study was determined at 3 different PBS at 37 °C in an incubator of pH 5.3, 6.8 and 7.4 with continuous stirring to simulate the intercellular, intracellular and external environment of cancer cells. To carry out this study, 2 ml of dialyzed Dox-FA-Nk@A complex was used. Then, 1 ml of sample was withdrawn from the system every 40 mins to determine the drug content. To compensate the PBS solution as soon as 1 ml was drawn, it was replaced with the same equivalent volume. The amount of Dox released was determined using UV-Vis spectrophotometer at 485 nm which was the signature absorbance of Dox. All the experiments were repeated thrice for all the samples. This drug release study was also explained with different drug kinetics models in order to explain the release mechanism.

Characterization. UV-Vis spectroscopy. UV-Vis spectra was obtained using Shimadzu Corporation UV-2401PC UV-Vis spectrometer.

X-Ray diffraction (XRD) measurement. The crystallinity of the Nk and Nk@A were recorded by using XPert PRO XRD spectrometer (PANalytical B.V., Holland) from 10 to 80° (2θ value) using Cu K-α radiation (0.15418 nm).

Fourier transform infrared (FT-IR) spectroscopy. FT-IR spectra were obtained on a Nicolet iS50 FT-IR Spectrometer (Thermo Scientific).

Thermogravimetric analysis (TGA). Thermal analysis was carried out for liquid samples using a TGA Q50 (TA Instruments) from 30 to 900 °C under nitrogen flow with a heating rate of 10 °C/min.

High Resolution Transmission electron microscopy (HRTEM). TEM images were taken on a HRTEM (JEOL, JEM-ARM200F) equipped with HAADF-STEM (high-angle annular dark-field scanning transmission electron microscopy) detector and Oxford XMax 80 Energy Dispersive X-Ray Spectrometer (EDS). The sample was prepared in such a way that aggregation in the grid was avoided. Then, 10 μl of sample were dispersed in 100 μl of isopropanol, which was sonicated for 30 mins; then a drop of sonicated nanoparticle dispersion was placed onto the amorphous carbon-coated 200 mesh copper grid (Ted Pella, Inc.). Finally, the sample was allowed to dry at ambient temperature before it was loaded into the microscope.

CryoTEM. The internalization of NPs inside the cells was confirmed by cryoTEM analysis. 5 μl of Nk@A nanoparticles suspensions were made into a thin liquid film which was prepared on lacy carbon grid (Pelco, USA) and then quenched into liquid ethane to freeze the sample using a Leica EM-CPC chamber. Tecnai F20 (FEI) operated at 200 kV to obtain the images which were recorded with a USCI1000 slow scan CCD camera (Gatan) at 50000x.

X-Ray photoelectron spectroscopy (XPS) Analysis. XPS analysis were performed using a K-Alpha X ray Photoelectron Spectrometer (XPS) System (Surface Analysis, Thermo Scientific). Monochromated, Micro-focused Al Kα was used as an X-ray source type. The binding energy of C1s carbon at 284.7 eV was used for calibration.

Magnetic measurements. Nk and Nk@A magnetic properties were measured using a superconducting quantum interference device (SQUID), (Quantum Design, MPMS3). The magnetization hysteresis of the samples was obtained by changing H between +70 to −70 kOe at 3 different temperatures, like 5 K, 300 K and 312 K. The hysteresis of the samples was also obtained at 100 kOe using a temperature interval from 1.8 to 312 K.
Zeta potential measurements. The zeta potential values were determined by using a Zetasizer Nano ZS90 (Malvern instruments) at 25 °C at a wavelength of He-Ne laser 633 nm, Max 4 mW at a scattering angle of 90° using a Universal ‘dip’ cell kit. Data were obtained using a monomodal acquisition according to the Smoluchowski theory. The measurements were repeated 3 times. Before the analysis the samples were well sonicated for 1 h to avoid aggregation.

Magnetic Resonance Imaging (MRI) experiments. MR imaging was performed with a 7T clinical Signa HDxt scanner (Varian). T1-weighted images were acquired using the following parameters: T1, Repetition time TR = 2000 ms, fast spin echo, FOV = 3 × 3 cm, Echo time TE = 15–250 ms, slice thickness = 4 mm and resolution 256 × 256 points. For T1 measurements, coronal spin-echo sequences with fixed echo time (TE) = 24 ms and varying repetition time (TR) (25 ms to 4 s) were used. Nk@A suspensions were taken at varying concentrations.

Microwave (MW) experiment setup. The setup used to apply the MW electromagnetic field to perform the experiments consisted of a generator (SML 03, Rhode & Schwarz, Germany) set to a frequency of 2.45 GHz. This signal was then amplified using an RF & MW module power amplifier (1164-BBMQ6AHM, Empower, USA). The output power was then monitored through the use of a dual direct coupler (DC7154M, Amplifier Research, USA) and a power meter (PM2002, Amplifier Research, USA) to ensure an output power of 6W and to monitor the reflected power of the system. To adjust the standing wave ratio (SWR) to an optimal value, a coaxial stub tuner (1878C, Maury Microwave Corp., USA) was used along a network analyzer (E5071B, Agilent Technologies, USA) to measure and reduce the SWR to a minimum prior to each experiment.

Temperature Measurements. Non electromagnetic interfering optical fiber probes temperature sensors (M3300, Luxtron, USA) were used to record temperature increment. The temperature was measured inside the PBS. Each test lasted 200 sec in order to study the temperature response as a function of time. The temperatures were recorded every second during the experiments using True Temp software (Luxtron, USA).

Confocal Imaging. Z-series confocal images were collected using Zeiss LSM 700 confocal microscope fitted with a 40X oil-immersion lens. Images were processed using Zen 2012 software.

All the above analysis and characterization techniques used liquid sample (FTIR, TGA, UV-Vis spec, HRTEM, Zeta potential, Flow cytometry analysis), powder form (SQUID), and thin film (XRD).

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**Author Contributions**

M.R. and G.O. conceived and designed the experiments, M.R. performed the experiments, analysed and wrote the manuscript text, F.G.-S. evaluated the results for cytotoxicity experiments, N.B.A. performed all the cellular experiments, A.V. and L.L. interpreted hyperthermia results, M.A.G.-N. carried out SQUID analysis and interpreted them, G.O., S.V. and J.T.R. reviewed the manuscript.

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