5.1. Synthesis of FePt Nanoparticles

The FePt nanoparticles (FePt NP) were synthesized under inert condition using previously described method with slight modifications [1]. Briefly, platinum acetylacetonate (0.5 mmol) was mixed to the solution of dioctylether (20 mL) and 1,2-hexadecanediol (1.5 mmol) and heated to 100 ^{0}C for 5 min to facilitate complete solubilization of platinum acetylacetonate. Oleic acid (0.5 mmol), oleylamine (0.5 mmol) and iron acetylacetonate (1 mmol) were added to the mixture and heated to reflux (265 ^{0}C). The refluxing was done for 30 min after which the heat source was removed and the reaction mixture was allowed to cool at room temperature. The inert environment created using nitrogen was removed and synthesized FePt nanoparticles were obtained as black precipitate by addition of ethanol (40 mL). The obtained nanoparticles were separated using centrifugation at 16000 rpm for 30 mins. The black precipitate was separated by decanting the yellow brown supernatant. The black precipitate was dispersed in hexane in presence of oleic acid (0.5 mmol) and oleylamine (0.5 mmol) and precipitated using ethanol. The precipitated nanoparticles were finally obtained by centrifugation and redispersed in hexane.

5.2. Phase transfer of oleic acid coated FePt NP

The phase transfer of FePt NP from hydrophobic phase to aqueous phase was carried out using base-bath-assisted procedure with slight modification [2]. Briefly, 10 mL of oleic acid coated FePt NP dispersion in hexane (50 mg/mL) was added to 20 mL of washing solution containing water (10 mL), 2-Propoanol (10 mL) and potassium hydroxide (1 g). The resultant mixture was ultra-sonicated for 30 mins with occasional vortexing. The mixture was kept under stirring at room temperature for 18 h after which the FePt NPs were separated by centrifugation at 15000 rpm for 20 min. The supernatant was discarded and obtained nanoparticles were dispersed in water.

5.3. Surface modification of FePt Nanoparticles

5.3.1. Grafting of amine groups on FePt NP Using APTES (NH_2-FePt)

Surface modification of phase transferred FePt NP was done using APTES (3-Aminopropyl)triethoxysilane) according to previously described method with some modifications [3]. Accurately weighed quantity of vacuum dried FePt nanoparticles (500 mg)

were dispersed in dry toluene using ultra-sonication for 15 min under ambient conditions followed by dropwise addition of APTES (2.1 mmol). The resultant reaction mixture was refluxed at 80^0 C for 12 h under magnetic stirring. The amine modified FePt nanoparticles were separated using NdFeB permanent magnet and washed repetitively with acetone to remove traces of unreacted silanes and dried under vacuum for 24 h.

5.3.2. Grafting of amine groups on FePt NP Using PEI (NH_2-FePt)

Surface modification of phase transferred FePt NP was done using PEI according to previously described method with some modifications [4]. Aqueous solution (10mL) of bPEl-25kDa (20%) was directly poured into the FePt dispersion (10mL, 5mg/mL) and heated to 90 °C and kept at that temperature for 1 hour under magnetic stirring. The amine modified FePt nanoparticles were separated using NdFeB permanent magnet and washed repetitively with water to remove traces of unreacted PEI and dried under vacuum for 24 h.

5.3.3. Grafting of carboxyl groups on FePt NP using PAA (COOH-FePt)

COOH-FePt nanoparticles were synthesized by the thermal decomposition route [5]. Briefly, platinum acetylacetonate (0.5 mmol) was mixed to the solution of dioctylether (20 mL) and 1,2-hexadecanediol (1.5 mmol) heated to 100 ^oC for 5 min to facilitate complete solubilization of platinum acetylacetonate. Oleic acid (0.5 mmol), oleylamine (0.5 mmol) and iron acetylacetonate (1 mmol) were added to the mixture. PAA (0.4 mmol) was added to a round-bottomed flask (equipped with a stirrer and a condenser) containing 20 mL of 2-pyrrolidone (boiling point 245 °C). Nitrogen was purged into this mixture for 30 minutes to remove any dissolved oxygen. This mixture was subsequently held at 210 °C for 30 min. Furthermore, refluxing at 265 ^0C was carried out for 60 min under vigorous mechanical stirring. The resultant dispersion was then cooled to room temperature and a 5:1 volume ratio of diethyl ether/acetone was added to separate out the particles. To remove any unreacted PAA, particles were washed three times with Milli-Q water followed by diethylether and acetone (5:1) for removal of unreacted and surface adsorbed reactants. The washed particles were dispersed in water.

5.3.4. Grafting of thiol groups on FePt NP Using (SH-FePt)

SH-FePt nanoparticles were synthesized by the thermal decomposition route [6]. Briefly, platinum acetylacetonate (0.5 mmol) along with 1,2-hexadecanediol (1.5 mmol)was mixed into dioctylether (20 mL) and heated to 100 ^{0}C for 5 min to facilitate complete solubilization of platinum acetylacetonate. Oleic acid (0.5 mmol), oleylamine (0.5 mmol) and iron acetylacetonate (1 mmol) were added to the mixture and heated to reflux (265 ^{0}C). The refluxing was done for 30 min after which the heat source was removed and the reaction mixture was allowed to cool at room temperature. The inert environment created using nitrogen was removed and synthesized FePt nanoparticles were obtained as black precipitate by addition of ethanol (40 mL). Dried FePt nanoparticles were then redispersed in a mixture of Dimethylsulfoxide (DMSO) (5mL) and 3-mercaptopropionic acid (100µL). The nanoparticles were kept on magnetic stirring for 12 h after which they were separated by centrifugation, and the precipitate was dispersed in water.

5.3.5. Post functionalization of NH_2-FePt NP with Carboxylic acid groups (B-FePt)

Succinic anhydride (0.3 mmol) dissolved in 5 mL anhydrous DMF was added dropwise to the dispersion of NH_2-FePt NP (200mg) in 50 mL DMF under nitrogen blanket. The resulting reaction mixture was left for 24 h on magnetic stirring at room temperature. After 24 h, stirring was removed and bi-functional NH_2-FePt-COOH NP were separated using NdFeB permanent magnet and washed 5 times with acetone to remove traces of DMF and unreacted succinic anhydride and kept under vacuum at room temperature to obtain dried nanoparticles [7].

5.4. Synthesis of Fe_2O_3@FePt Nanoparticles

Synthesis of Fe_2O_3@FePtnanoparticles was done according to previously reported procedure by Li and Sun et al, with slight modification [8]. Based on a colloidal template, FePt nanoparticles were synthesized by mixing iron acetylacetonate (0.5 mmol) and platinum acetylacetonate (0.125 mmol), precursors for Fe and Pt, respectively. Platinum acetylacetonate (0.125 mmol) along with 1,2-hexadecanediol (1.5 mmol) was mixed to the solution of dioctylether (20 mL) and heated to 100 ^{0}Cfor 5 min to facilitate complete solubilization of platinum acetylacetonate followed by the injection of oleic acid (0.08 ml, 0.6 mmol) and oleylamine (0.08 ml, 0.6 mmol). The precursor solution was then placed into a 10 mL Teflon-lined stainless steel autoclave and gradually heated

to 250 ^{0}C for 1 hr. The synthesized Fe_2O_3@FePtnanoparticles were obtained as black precipitate by addition of ethanol (40 mL). The obtained nanoparticles were separated using centrifugation at 16000 rpm for 30 mins. The black precipitate was separated by decanting the yellow brown supernatant. The black precipitate was dispersed in hexane in presence of oleic acid (0.5 mmol) and oleylamine (0.5 mmol) and precipitated using ethanol. The precipitated nanoparticles were finally obtained by centrifugation and redispersed in hexane.

5.4.1. Synthesis of Fe_2O_3@FePt-SH

Fe_2O_3@FePt-SH nanoparticles were synthesized by the thermal decomposition route [6,8]. Dried Fe_2O_3@FePt nanoparticles were then redispersed in a mixture of DMSO (5mL) and 3-mercaptopropionic acid (100µL). The nanoparticles were kept on magnetic stirring for 12 h after which they were separated by centrifugation, and the precipitate was dispersed in water.

5.4.2. Synthesis of Fe_2O_3@FePt-COOH

Fe_2O_3@FePt-COOH nanoparticles were synthesized by the thermal decomposition route [8]. Based on a colloidal template, FePt nanoparticles were synthesized by mixing iron acetylacetonate (0.5 mmol) and platinum acetylacetonate (0.125 mmol), precursors for Fe and Pt, respectively. Platinum acetylacetonate (0.125 mmol) along with 1,2-hexadecanediol (0.75 mmol) was mixed to the solution of dioctylether (20 mL) and heated to 100 °C for 5 min to facilitate complete solubilization of platinum acetylacetonate followed by the injection of oleic acid (0.08 ml, 0.6 mmol) and oleylamine (0.08 ml, 0.6 mmol). PAA (0.4 mmol) was added to a round-bottomed flask (equipped with a stirrer and a condenser) containing 20 mL of 2-pyrrolidone (boiling point 245 °C). Nitrogen was purged into this mixture for 30 minutes to remove dissolved oxygen. This mixture was subsequently held at 210 °C for 30 min. Furthermore, refluxing at 265 ^{0}C was carried out for 60 min under vigorous mechanical stirring. The colloidal precursor solution was then placed into a 10 mL Teflon-lined stainless steel autoclave and gradually heated to 250 ^{0}C for 1 hr. The synthesized Fe_2O_3@FePtnanoparticles were obtained as black precipitate by addition of ethanol (40 mL). The obtained nanoparticles were separated using centrifugation at 16000 rpm for 30 mins. The black precipitate was separated by decanting the yellow brown supernatant. The black precipitate was dispersed in hexane in presence of oleic acid (0.5 mmol) and oleylamine (0.5 mmol) and precipitated using ethanol. The resultant dispersion was then cooled to room temperature and a 5:1 volume ratio of diethyl ether/acetone

was added to separate out the particles. To remove any unreacted PAA, particles were washed three times with Milli-Q water and diethylether: acetone mixture (5:1). The washed particles were dispersed in water.

5.4.3. Synthesis of Fe_2O_3@FePt-NH_2Using PEI and PLL

For synthesis of Fe_2O_3@FePt-NH_2, aqueous solution (10mL) of bPEl-25kDa (20%) was directly poured into the Fe_2O_3@FePt dispersion (10mL, 5mg/mL) and heated to 90 °C and kept at that temperature for 1 hour under magnetic stirring. The amine modified Fe_2O_3@FePt nanoparticles were separated using NdFeB permanent magnet and washed repetitively with water to remove traces of unreacted PEI and dried under vacuum for 24 h. Synthesis of Fe_2O_3@FePt-NH_2 was also done alternatively using Poly-l-lysine (PLL). The synthesized Fe_2O_3@FePt (5mL, 5mg/mL) nanoparticles were dispersed in PLL (0.3 mmol) solution and stirred for 24h. The resultant dispersion was dialyzed against distilled water with dialysis membrane (MWCO 10000) for 48 h to remove unreacted PLL.

5.4.4. Synthesis of Fe_2O_3@FePt-NH_2-HA

Synthesis of Fe_2O_3@FePt-NH_2-HA was done using EDC/NHS as reported earlier [9]. Briefly, hyaluronic acid (HA) (50 mg) was first dissolved in 5 mL distilled water and heated at 70^0 C for 15 min and was allowed to cool at room temperature. Fe_2O_3@FePt-NH_2 nanoparticles(5mg/mL) were mixed in DMSO (5mL) along with EDC (7 mg) and NHS (3 mg). The mixture was kept on stirring for 3 h and then added drop wise to HA solution. The mixture was kept on constant stirring for 12h. The nanoparticles were separated using NdFeB permanent magnet. The obtained nanoparticles were washed with water thrice to remove unbound HA followed by separation of unreacted EDC/NHS following dialysis (dialysis membrane, cutoff MW 5000) against distilled water for 24h.

5.4.5. Synthesis of Fe_2O_3@FePt-NH_2-FITC

Synthesis of Fe_2O_3@FePt-NH_2-HA was done as reported previously using EDC/NHS. Briefly, Fe_2O_3@FePt-NH_2 nanoparticles (5mg/mL) were dispersed in DMSO (5mL) along with EDC (7mg) and NHS (3mg) and kept on stirring for 3h. After 3h, FITC solution (FITC dissolved in ethanol (1mg/mL)) was added drop wise to nanoparticles dispersion and kept on stirring for 12h in dark. The FITC conjugated Fe_2O_3@FePt-NH_2nanoparticles were separated using NdFeB

permanent magnet and purified by dialysis (dialysis membrane, cutoff MW 5000) against mixture of ethanol and distilled water (30:70) for 24h.

5.4.7. Synthesis of Fe_2O_3@FePt-NH_2/COOH

Synthesis of Fe_2O_3@FePt-NH_2/COOH was done using two different methods [10, 11]. In the first method, succinic anhydride (0.3 mmol) was dissolved in 5 mL anhydrous DMF and added dropwise to the dispersion of Fe_2O_3@FePt-NH_2 (200 mg)in 50 mL DMF under nitrogen blanket. The resulting reaction mixture was left for 24 h on magnetic stirring at room temperature. After 24 h, stirring was stopped to obtain Fe_2O_3@FePt-NH_2/COOH by separating them using NdFeB permanent magnet. They were washed 5 times with acetone (20mL) to remove traces of DMF and unreacted succinic anhydride and kept under vacuum at room temperature to obtain dried nanoparticles Fe_2O_3@FePt-NH_2/COOH.

In the second method, polyaspartic acid (PASA) was used to synthesize Fe_2O_3@FePt-NH_2/COOH. Briefly, Fe_2O_3@FePt (20 mg) nanoparticles were dispersed in 5 ml hexane and added onto 5 mL PASA (25 mg/ml) solution in water to form a two phase mixture. The mixture was sonicated for 15 min and then centrifuged. The aqueous phase was washed three times with hexane (20mL) and the passed through a PD-10 column for purification and solvent exchange. Briefly, nanoparticle dispersion (10 mL) was loaded onto a column. Borate buffer (3.5 mL) (50 mM, pH 8.5) was added to the column and deepest colored fraction at the bottom of column was collected.

5.4.8. Synthesis of Fe_2O_3@FePt-NH_2-TPP

Synthesis of Fe_2O_3@FePt-NH_2-TPP was done using (3-carboxypropyl) triphenylphosphonium bromide (CTPB) as per previously reported literature [12]. For conjugation of TPP, CTPB and EDC were dissolved in 0.1M 4-morpholineethanesulfonic (MES) at concentration of 20 mmol L^{-1}. To 5 mL dispersion Fe_2O_3@FePt-NH_2 (10µg mL^{-1}), 300µL of CTPB was added followed by 300µL of EDC. The reaction mixture was left for stirring at room temperature for 4h. The formed Fe_2O_3@FePt-NH_2-TPP was separated using NdFeB permanent magnet and purified by dialysis (dialysis membrane, MW cutoff 5000) against distilled water for 24h to remove unreacted reagents.

5.4.9. Synthesis of Fe_2O_3@FePt-NH_2-TPP-COOH

For conjugation of TPP, CTPB and EDC were dissolved in 0.1M MES at concentration of 20 mmol L^{-1}. To 5 mL dispersion Fe_2O_3@FePt-NH_2/COOH (10µg mL^{-1}), 300µL of CTPB was added followed by 300µL of EDC. The reaction mixture was left for stirring at room temperature for 4h. The formed Fe_2O_3@FePt-NH_2/COOH-TPP was separated using NdFeB permanent magnet and purified by dialysis (dialysis membrane, MW cutoff 5000) against distilled water for 24h to remove unreacted reagents.

5.4.10. Synthesis of Fe_2O_3@FePt-NH_2-TPP-COOH-Ctx

The Fe_2O_3@FePt-NH_2/COOH-TPP nanoparticles were separated by centrifuging purified dispersion at 16000 rpm for 30 min. To the Fe_2O_3@FePt-NH_2/COOH-TPP suspension in PBS (10 mL, 0.01 M), a solution of NHS (2.3 mM), EDC (0.28 M) and NHS-PEG-MAL was added under magnetic stirring for 15 min followed by addition of 50 µg of *chlorotoxin* (Ctx) dissolved in 1 mL water and was left to incubate for 24h at room temperature [13]. The Fe_2O_3@FePt-NH_2/COOH-TPP-Ctx nanoparticles were obtained by dialysis (dialysis membrane, MW cutoff 5000) against distilled water for 24h and centrifugation at 16000 rpm for 30 min.

5.4.11. Synthesis of Fe_2O_3@FePt-NH_2/COOH-Drug Conjugate

A solution of EDC (35 mg) and NHS (25 mg) was prepared by dissolving them in 5 mL of distilled water. Lenalidomide (Ld) (10 mg) was dissolved in 5 mL DMSO while Fe_2O_3@FePt-NH_2/COOH (100 mg) were dispersed in 5 mL DMSO using ultrasonication [14]. The solution of EDC/NHS was added to Fe_2O_3@FePt-NH_2/COOH dispersion and kept at room temperature for 3 h after which drug solution was added to the above reaction mixture dropwise and kept for stirring for 12 h at room temperature. The Fe_2O_3@FePt-NH_2/COOH-Drug Conjugates were separated using NdFeB permanent magnet, washed with acetone/water (30mL, 40:50) to remove traces of DMSO and unconjugated drug. The obtained Fe_2O_3@FePt-NH_2/COOH-Drug conjugates were freeze dried (as per protocol given in section 4.6) and stored for further use.

5.4.12. Synthesis of Fe_2O_3@FePt-NH_2-TPP-COOH-Drug Conjugate

For synthesis of Fe_2O_3@FePt-NH_2/COOH-TPP-drug conjugate, same procedure was followed as described above except, Fe_2O_3@FePt-NH_2/COOH-TPP nanoparticles were used in place ofFe_2O_3@FePt-NH_2/COOH nanoparticles.

5.4.13. Synthesis of Fe_2O_3@FePt-COOH-DOTA-NH_2

For conjugation of DOTA-NH_2 to Fe_2O_3@FePt-COOH, DOTA-NH_2(25mg) was activated using EDC and NHS at pH 5.5 (MES buffer, 10mL) for 30 min with DOTA: EDC: NHS molar ratio of 10:5:4 [15]. The activated mixture was added to Fe_2O_3@FePt-COOH (10mL, 5mg/mL) and incubated overnight at 4 ^{0}C. The unreacted EDC and NHS were removed through PD-10 column and dialysis membrane (MWCO 10000). The obtained Fe_2O_3@FePt-COOH-DOTA-NH_2 nanoparticles were freeze dried (as per protocol given in section 4.6) and stored for further use.

5.4.14. Synthesis of Fe_2O_3@FePt-NH_2-DOTA-NCS

For conjugation of DOTA-NCS to Fe_2O_3@FePt-NH_2, DOTA-NH_2 (25mg) was activated using EDC and NHS at pH 5.5 (MES buffer, 10mL) for 30 min with DOTA: EDC: NHS molar ratio of 10:5:4 [15]. The activated mixture was added to Fe_2O_3@FePt-NH_2nanoparticles (10mL, 5mg/mL) and incubated overnight at 4 ^{0}C. The unreacted EDC and NHS were removed through PD-10 column and dialysis membrane (MWCO 10,000).The obtained Fe_2O_3@FePt-COOH-DOTA-NCSnanoparticles were freeze dried (as per protocol given in section 4.6) and stored for further use.

5.4.15. Synthesis of Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx

Synthesis of Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx nanoparticles was done in three steps. In the first step, Fe_2O_3@FePt-NH_2/COOH-TPP nanoparticles were synthesized as described before (section 5.4.9). The nanoparticles were purified using dialysis (MWCO 10,000) and separated using magnet. In the second step, the conjugation of LND to Fe_2O_3@FePt-NH_2/COOH-TPP was done using EDC/NHS [16]. Briefly, LND (10 mg) was dissolved in distilled water (4 mL) and cooled on ice. For conjugation of LND to Fe_2O_3@FePt-NH_2/COOH-TPP, LND (10mg), EDC (7 mg), and NHS (3 mg) were dissolved in distilled water (3 mL) and stirred at room temperature for 4 h. The LND solution was then mixed with Fe_2O_3@FePt-NH_2/COOH-TPP (2 mL of 20 mg/mL in distilled water), and was stirred continuously for 16 h at room temperature. After the reaction, the dispersion was purified by passage through Sephadex G-25. In the third and final step, HA (10mg) was mixed in to the suspension of obtained Fe_2O_3@FePt-NH_2/COOH-TPP-drug in PBS (10 mL, 0.01 M), a solution (5mL) of NHS (2.3 mM), EDC (0.28 mM) and NHS-PEG-MAL (5mg) was added under magnetic stirring for 15 min followed by addition of 50 µg of *chlorotoxin* dissolved in 1 mL water and was left to incubate

for 24h at room temperature. The Fe_2O_3@FePt-NH_2/COOH-TPP-drug-HA-Ctx nanoparticles were obtained by dialysis against distilled water for 24h and centrifugation at 16000 rpm for 30 min.

5.4.16. Synthesis of Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx (pH responsive HA-Drug Conjugate)

Synthesis of Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx nanoparticles was done using two different approaches. The first approach involved three steps. In the first step, Fe_2O_3@FePt-NH_2/COOH-TPP nanoparticles were synthesized as reported previously (section 5.4.9). The synthesized nanoparticles were purified using dialysis and separated using magnet. In the second step, the conjugation of LND to Fe_2O_3@FePt-NH_2/COOH-TPP was done using a *cis*-acontityl bond [17]. Briefly, LND (10 mg) was dissolved in distilled water (4 mL) and cooled on ice. *Cis*-acontityl anhydride (5 mg) dissolved in 1,4-dioxane (200 mL) was slowly added to the LND solution (4mL) with continuous stirring. The pH of the reaction mixture was adjusted to 9.0 using NaOH (0.5 M) keeping the reaction mixture in ice. After 30 min, the pH of reaction mixture was adjusted to 7.0 using 1M HCl and the mixture was stirred for another 30 min followed by addition of HCl (1 M) till precipitate of *cis*-aconitic anhydride-LND conjugate was formed. After 15 min on ice, the precipitate was recovered by centrifugation (8000 rpm, 10 min). For conjugation of aconitic anhydride-LND conjugate to Fe_2O_3@FePt-NH_2/COOH-TPP, aconitic anhydride-LND conjugate (10mg), EDC (7 mg), and NHS (3 mg) were dissolved in distilled water (3 mL) and stirred at room temperature for 4 h. The solution was then mixed with Fe_2O_3@FePt-NH_2/COOH-TPP (2 mL of 20 mg/mL in distilled water), and was stirred continuously for 16 h at room temperature. After the reaction, the dispersion was purified by passage through Sephadex G-25. In the third and final step, HA (10mg) was dissolved in distilled water (5mL) and heated at 70^0 C for 15 min and was allowed to cool at room temperature. It was then added to the suspension of obtained Fe_2O_3@FePt-NH_2/COOH-TPP-drug in PBS (10 mL, 0.01 M), a solution of NHS (2.3 mM), EDC (0.28 M) and NHS-PEG-MAL (Maleimide) (5mg) was added under magnetic stirring for 15 min followed by addition of 50 µg of *chlorotoxin* dissolved in 1 mL water and was left to incubate for 24h at room temperature. The Fe_2O_3@FePt-NH_2/COOH-TPP-drug-HA-Ctx nanoparticles were obtained by dialysis against distilled water for 24h and centrifugation at 16000 rpm for 30 min.

In the second approach, pH sensitive HA-LND conjugate was synthesized. Firstly, carboxymethyl HA (CMHA) was synthesized according to previously described method [18]. For this synthesis, 500mg Hyaluronic acid was dissolved in 45% NaOH(5ml) and stirred using magnetic stirrer at room temperature for 2 hours to deprotonate the HA. The deprotonated HA was then reacted with chloroacetic acid (500mg) in Isopropanol (12.5 ml) for 2.5 hours, followed by precipitation with methanol for 30 minutes. pH was adjusted to 7.0 using methanol. The precipitates were separated by filtration and the obtained CMHA was dissolved in deionized water (20ml). After this, it was purified against deionized water by dialysis (dialysis tubing) for 24 hrs and lyophilized to obtain CMHA (Carboxymethylated Hyaluronic Acid). For lyophilisation, first step included deep freezing of content of dialysis bag at -70° C for 24 hours in deep freezer to form dry ice cake. The formed dry ice cake containing vials were then transferred to the Freeze Dryer (AdVantage 2.0 BenchTop Freeze Dryer / Lyophilizer, SP Scientific, USA.) and lyophilized at -70° C for 24 hrs. [19]. In the second step, accurately weighed amount of CMHA(100mg) and Hydrazine hydrate (3.0ml) were subsequently dissolved in 4ml deionized water. The mixture was stirred using magnetic stirrer until absolutely dissolved solution was formed. After that 60 mg EDC.HCL was added to the above solution and stirred at room temperature for 2 hours. Finally, CMHA-LND conjugate was synthesized by dropwise addition of different amount of drug (40 mg, 60 mg and 80 mg in 5ml of DMSO) to the above polymer solution and it was reacted at room temperature for next 16hrs.The resultant solution was then precipitated using methanol (30ml) and the precipitates were collected by centrifugation at 3500 rpm for 15 minutes. After this, precipitates were washed thrice with methanol (15ml) to remove unreacted LND. The obtained precipitates were subjected to vacuum drying and the supernatant was used to calculate the amount of unconjugated drug. In the third step,Fe_2O_3@FePt-NH_2/COOH-TPP nanoparticles (50mg) were dispersed in5 ml of distilled water and 5 ml of DMSO in a beaker. Different quantities of CMHA-drug conjugates (100mg, 120mg, 140mg) were separately dispersed in 5 ml distilled water in another beaker. Both the dispersions were mixed and incubated with stirring on magnetic stirrer for 16 hours. Prepared final nanoparticles were separated using magnet. They were dispersed in distilled water and lyophilized to obtain Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA. To the suspension of obtained Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA in PBS (10 mL, 0.01 M), a solution of NHS (2.3 mM), EDC (0.28 M) and NHS-PEG-MAL (Maleimide) (5mg) was added under magnetic stirring for

15 min followed by addition of 50 µg of *chlorotoxin* dissolved in 1 mL water and was left to incubate for 24h at room temperature [20]. The Fe_2O_3@FePt-NH_2/COOH-TPP-drug-HA-Ctx nanoparticles were obtained by dialysis against distilled water for 24h and centrifugation at 16000 rpm for 30 min.

5.5. Optimization of FePt core using Quality by Design Approach (QbD)

Risk analysis on the synthesized FePt nanoparticles with respect to particle size, and elemental composition was done to identify potential high risk factors for further optimization studies. Risk assessment study was done using fishbone diagram to select critical variables which will provide valuable information related to critical formulation and process variables which should be controlled to obtain quality product.

5.5.1. Experimental design

5.5.1.1. Risk analysis (Identification of critical variables)

In order to identify the critical processing variables, Ishikawa diagrams were used [21]. Ishikawa diagrams were constructed to establish relation between the different process/formulation variables and the characteristics affected by them, for identifying factors with potential risk of affecting final product quality attribute. Two final product quality attributes were selected for study (particle size and elemental composition of alloy nanoparticles).

5.5.1.2. Preliminary investigation of critical variables

After identifying the variables which might affect the product quality attribute, preliminary investigation of variables based on previous research and risk priority was done. Effect of selected variables was studied on particle size and elemental composition for calculating optimal lower and upper value for screening study [21].

5.5.1.3. Optimization using Response surface methodology

Box-Behnken design (BBD) is a response surface methodology (RSM) involving a group of statistical and mathematical methods useful for the modeling and analyzing problems associated with method or experimental work. It serves as a tool to optimize the response that is influenced by various parameters/factors either related to process or formulation [22]. It also quantifies the relationship between the independent parameters/factors and the obtained response surfaces.

BBD is rotatable or nearly rotatable second-order design based on three-level incomplete factorial design. In the present investigation, a three variable BB-RSM was used to study the effect of selected factors on desired response. BBD was generated by SAS JMP software, Version16, which gave a total of 15 experimental runs.

5.5.1.4.Establishment of design space

Overlay plot was plotted for establishing design space. Overlay plot gives a range within which, variation in the value of CPP will not affect the final response (in present case is the final product quality attribute). An overlay plot was plotted between the significant critical variables affecting product quality attributes, identified after screening phase [23]. The design space was analyzed for repeatability.

5.5.1.5.Analysis of design space robustness

Analysis of established design space is very necessary from the scale up point of view. It is very important to check and analyze if obtained design space holds correct and the area upper and lower to establish design space yields response which is undesired [24]. With respect to above consideration, analysis of design space was done by plotting overlay plot using JMP (SAS) 16 with response higher and lower to the established design space. The software gave values for variables in and around established design space along with expected value of the desired responses

5.5.1.6. Statistical analysis

All results were analyzed using statistical software package Minitab 16 and JMP (SAS). The experimental data obtained were validated by ANOVA combined with the F-test. The determination coefficient (R^2, agreement between the experimental results and predicted values obtained from the model) and the model F-value (Fisher variation ratio, the ratio of mean square for regression to mean square for residual) were applied for statistical evaluation.

5.6. Characterization of Synthesized Nanoparticles

The various synthesized FePt and Fe_2O_3@FePt nanoparticles were characterized for particle size and surface potential using Malvern Zetasizer (USA).

5.6.1. Particle size and Zeta Potential

For particle size analysis, each sample was diluted ten times with filtered double distilled water to avoid multi-scattering phenomena and placed in disposable sizing cuvette. Polydispersity index was noted to determine the narrowness of particle size distribution. Analysis was performed in triplicate and the results were expressed as mean ± SD. For zeta potential, each sample was suitably diluted 10 times with filtered double distilled water and placed in a disposable zeta cell. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system [25].

5.6.2. Spectroscopic Analysis:

FTIR analysis was performed for Fe_2O_3@FePt, Fe_2O_3@FePt-NH_2-HA, Fe_2O_3@FePt-NH_2/COOH, Fe_2O_3@FePt-NH_2-TPP, Fe_2O_3@FePt-NH_2-TPP-COOH, Fe_2O_3@FePt-NH_2-TPP-COOH-Ctx, Fe_2O_3@FePt-NH_2/COOH-Drug,Fe_2O_3@FePt-NH_2-TPP-COOH-Drug, Fe_2O_3@FePt-COOH-DOTA-NH_2, and Fe2O3@FePt-NH_2-DOTA-NCS and Fe_2O_3@FePt-NH_2/COOH-TPP-drug-HA-Ctx. 100 scans in the region from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} were accumulated to obtain FT-IR spectra (FTIR spectrometer, IR Affinity -1S (Shimadzu, Japan). FTIR-spectrum of prepared nanoparticle was measured in the solid state by preparing a Potassium Bromide (KBr) pellet. The sample was previously ground and mixed thoroughly with KBr, an infrared transparent matrix at 1:100 (sample KBr) ratio. The KBr pellets were prepared by applying 10-12 metric ton of pressure in a motorized pellet press (Kimaya engineers, India). The pellets were then scanned over a wavelength range of 4000-400 cm^{-1} and a spectrum was obtained by using a FTIR spectrometer, IR Affinity -1S (Shimadzu, Japan) [26].

5.6.3. X-Ray diffraction and Thermometric Analysis

PXRD data was obtained using an X-ray diffractometer (BrukerAxs, D8 Advance; Germany). Patterns were obtained using the X-ray diffractometer (Bruker D8 advance) with Cu source of radiation. Measurements were performed at a voltage of 40kV and 25 mA [27]. Thermo gravimetric analysis (TGA) was carried out using a TA instrument Q600 thermal balance. Typically 5–10 mg of nanoparticles were heated to 600 ^oC with 10 ^0C min^{-1} in air and kept isothermal at 600 ^oC for 30 min to determine the amount of organic content on the nanoparticles surface carried out between RT and 700 ˚C at a heating rate of 10 ˚C /min in air atmosphere using the α-Al_2O_3 standard [28].

5.6.4. Electron Microscopy, Elemental Analysis and Magnetic Susceptibility

Elemental analysis of FePt and Fe_2O_3@FePt nanoparticles was also performed by energy dispersive spectroscopy using Philips LEO 1530-2 FESEM/EDS, with 15 kV accelerating voltages. The morphology of nanoparticles was observed using Transmission Electron Microscopy (TEM). XPS was done to study the chemical states of Fe and Pt in FePt nanoparticles using PHI 5000 Versa Probe II, FEI Inc. Atomic force microscopy was done using Atomic Force Microscope (NT-MDT-INTEGRA, INTEGRA, USA). For this, the samples were dispersed in water and directly used for analysis. Surface topology of a sample in x, y as well as z direction was checked using sharp tip at the end of cantilever that moves over the sample.AFM measurements were performed in contact mode. Magnetic susceptibility of nanoparticles was studied by measuring the magnetic susceptibility as a function of the applied magnetic field H using a vibrating sample magnetometer (Lakeshore VSM 7410) with a maximum applied magnetic field of 20 kOe. The hysteresis of the magnetization was obtained by varying H between +20000 and −20000 Oe at 300 K. Raman spectroscopy was performed using Horiba JobinVyon, Model LabRam HR [29].

5.6.5. NMR Spectroscopy

The NMR spectra were recorded on a Bruker Advance II 400 MHz NMR spectrometer equipped with a 5 mm ^{1}H cryo probe head with a z-gradient (Bruker BioSpin, GmBH, Germany)in $CDCl_3$ at 25 °C. The data was acquired and processed using the TopSpin 3.1 software (Bruker). The pulse sequences from the Bruker pulse sequence library were used for ^{1}H NMR. A line broadening factor of 0.3 Hz was applied and base line correction was performed prior to integration of the ^{1}H NMR signals. Sample quantities of lyophilized CMHA and (typically 50 mg), was used for recording NMR spectrum [30].

5.6.6. DSC Analysis

DSC analysis was carried out using a Differential Scanning Calorimeter (Shimadzu, Japan) at a heating rate of 10˚C per minute in the range of 30 ˚C to 200 ˚C under inert nitrogen atmosphere at a flow rate of 40ml/min. Sample (LND, HA, CMHA, CMHA-LND conjugate and *cis*-aconitic anhydride-LND conjugate was taken (~4-8 mg) in an aluminium pan, crimped and sealed. An empty aluminium pan was used as reference.

5.6.7. Electron Spin Resonance Spectrometry (ESR)

ESR spectra were measured at room temperature with JES-FA200, JEOL spectrometer. The conditions for ESR were as follows: X-band, 100 kHz modulation with 3.2-G amplitude; microwave power 0.99500 mW, central magnetic field 3385 G with scan 400 G, scan time 200 s. The mean height of the three peaks in each signal is taken as the relative intensity of the NO and ROS signal. 5mg of sample was used for analysis [31].

5.6.8. Raman Spectroscopy

Raman spectrum was recorded on a LabRam HR, Horiba JobinYvon, Japan spectrometer. The excitation sources were 325 nm (HeCd laser), 442 nm (HeCd laser), 514.5 nm (Ar ion laser), and 632.8 nm (HeNe laser). The scattered light was detected with a Peltier-cooled CCD detector (Renishaw) with spectral resolution ~2 cm^{-1}.Raman spectrum was calibrated using the 520 cm^{-1} silicon band. The spectrum was recorded in wave numbers. Spectral manipulations such as baseline adjustment, smoothing, and normalization were performed with the WiRE 3.3 software [32].

5.7. Determination of carboxyl modification

To determine the degree of carboxyl modification of CMHA, a modified titration assay was used with slight modifications. Dry CMHA (12.5 mg) was dissolved in 1.0 ml of 0.1N NaOH and diluted with 1.5ml of DI water. This solution was then titrated with 0.05N HCl, using phenolphthalein as an indicator [33]. Blank titrations contained no CMHA. The degree of substitution was calculated using the equation 5.1 & 5.2.

$n_{COOH} = (V_b - V) * C_{HCl}$ (Equation 4.1)

Where, n_{COOH}= moles of carboxyl groups

V_b= volume of HCl needed to titrate blank

V = volume of HCl needed to titrate sample

C_{HCl} = conc. of HCl

$$DS = \frac{W_{DSU} * n_{COOH}}{m_{dry} - MW_1 * n_{COOH}}$$ (Equation 5.2)

Where, DS = degree of substitution

MW_{DSU} =MW of an unsubstituted disaccharide unit

m_{dry} = mass of dry CMHA

MW_1 = increase in MW due to carboxyl group substitution

The percent carboxyl modification was then calculated DS × 100%.

5.8. Estimation and optimization of Conjugation Efficiency of CMHA-Lenalidomide conjugates

The extent of LND conjugation on CMHA was determined by measuring amount of unconjugated LND using ultraviolet (UV) spectrophotometric analysis. After every centrifugation cycle of the conjugates, the supernatant was collected and stored in a stoppered vial. The analysis was performed by measuring the absorbance of the supernatant in methanol at 220nm after diluting it with sufficient quantity of methanol. This will give the amount of unconjugated LND. From the amount of unconjugated LND, conjugated amount of LND was calculated and % conjugation efficiency was finally obtained using following equation;

% Conjugation Efficiency = (Conjugated amount of LND) / (Total amount of LND added) * 100

5.9. Estimation of drug loading

The quantity of lenalidomide (LND) conjugated on Fe_2O_3@FePt-NH_2/COOH-TPP-drug was determined indirectly by measuring the quantity of lenalidomide remaining in the supernatant based on the absorbance of the samples at 250 nm. The standard curve was obtained (as mentioned in analytical method development section) and the sample absorbance was measured using UV–visible spectrophotometer (Shimadzu UV-1700). The percentage entrapment efficiency (%EE) and conjugation efficiency (%CE) was calculated using the formula:

$$\% EE = \frac{Dt - Df}{Dt} * 100$$

$$\%CE = \frac{Wt - Wc}{Wt} * 100$$

Where, Dt= total amount of drug added to Fe_2O_3@FePt-NH_2/COOH-TPP; Df=amount of free drug in solution; Wt=total weight of lyophilized Fe_2O_3@FePt-NH_2/COOH-TPP-drugnanoparticles; Wc=weight of lyophilizedFe_2O_3@FePt-NH_2/COOH-TPP nanoparticles.

5.10. Redispersibility of lyophilized Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx nanoparticles.

Manual shaking method was employed to check the redispersibility of Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx nanoparticles [34]. For this, lyophilized Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx nanoparticles (10mg) were taken in eppendorf containing 3 ml of double distilled water. It was then shaked manually and time required for complete redispersibility of Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx nanoparticles was noted. For measurement of particle size and zeta potential, weighed quantity of lyophilized Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx nanoparticles (100 mg) were taken in a test tube containing 5 ml of double distilled water and sonicated for 3 minutes using a bath sonicator. The resulting dispersion was subjected to particle size and zeta potential measurement using Malvern Zetasizer.

5.11. *In vitro* Magnetic Hyperthermia

The heating ability of FePt and Fe_2O_3@FePt nanoparticles was evaluated by time dependent calorimetric measurements using an induction heating unit (Note: heating ability of surface functionalized nanoparticles was not assessed as the functional groups attached to synthesized nanoparticles have no effect on magnetic hyperthermia). Induction heating of FePt and Fe_2O_3@FePt nanoparticles (2.5, 5, 10 and 20 mg/mL) for hyperthermia application was performed in eppendorf tube (2 mL) with an induction heating unit (Easy Heat 8310, Ambrell; UK) having a 6 cm diameter (4 turns) heating coil. The FePt and Fe_2O_3@FePt nanoparticles were dispersed in 1 mL of distilled water in Eppendorf tube with ultrasonication for 30 min for uniform and efficient dispersion of nanoparticles. The tube was then placed in the centre of the coil with applied frequency of 265 kHz [35]. Heating of nanoparticle samples was carried out for 10 min with current between 200-400A. The calculation of developed magnetic field by current of 200, 300 and 400A was calculated according to the given formula (Eq. 4.3):

$$H = \frac{1.257ni}{L} inOe \qquad \text{Eq. 4.3}$$

Where “n”= number of turns; “i”=applied current and “L” denote the diameter of the turn in centimetre.

5.12. *In vitro* NIR triggered Hyperthermia (Photothermal) and Dual Magnetophotothermal Measurement

Laser triggered hyperthermia was induced by a near infrared continuous laser at 808 and 1024 nm with external adjustable power supply as per previously reported literature [36]. The aqueous dispersion of FePt and Fe_2O_3@FePt nanoparticles contained in a 0.5 mL Eppendorf tubes were irradiated by laser source alone and in combination with magnetic hyperthermia by placing Eppendorf tubes in same sample holder used for only magnetic hyperthermia (the distance between Eppendorf tubes and laser source was 2-3 cm and the laser spot was 1 cm^2. The laser power range employed was 0.8 and 2.5 W/cm^2).

5.13. LND Release in Tumor Mimicking Environment

The *in vitro* pH triggered drug release studies were carried out in a water bath at 37 °C under mild shaking motion (50 rpm) [37]. Free LND and the drug loaded FePt and drug loaded Fe_2O_3@FePt nanoparticles were taken into dialysis bags (MWCO = 6000), and were immerged into tubes containing 20 ml phosphate buffer saline (PBS, pH 7.4, pH 6.5, pH 5.5 and 4.5) as the release media. The tubes were incubated in a shaking water bath at 37 °C during the test. Sampling was done at a predetermined period by removing 2.0 ml of the buffer solution from the tubes and 2.0 ml fresh buffer solution was added into the tubes to maintain the total solution volume. The amount of LND released was determined using UV spectrophotometer at the absorbance wavelength of 250 nm.

5.14. Release of Iron (Fe) from SPANs

A portion of the aqueous suspension of drug loaded FePt and drug loaded Fe_2O_3@FePt nanoparticles containing 1 mg of Fe equivalent was dispersed in dialysis tubing (MWCO) 1000, Spectrum Laboratories, Inc.), and was further immersed into a 30 mL PBS (pH 7.4 and 4.8) maintained at 37 °C [38]. Samples were withdrawn (1 mL) after pre-determined time intervals from the PBS solution and the concentration of Iron was analyzed using ICP-OES (as described in material and protocol section 4.3.10).

5.15. Mucus penetration Study

For studying mucus penetrating ability of SPANs (Fe_2O_3@FePt alloy nanoconjugates (Fe_2O_3@FePt-NH_2/COOH-Drug-HA-Ctx)) and M-SPANs (Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx) nanoparticles, mucin (350 mg) was equilibrated in a constant temperature culture vibrator at 37°C for 15 minutes at a speed of 100 rpm to ensure homogeneous dispersion of mucin. It was then placed in the donor chamber of a modified Franz diffusion cell with a polycarbonate membrane (pore size 2 μm) located between the donor and receptor chambers to support the mucus [39]. Then 50 μL of FITC conjugated SPANs and M-SPANs nanoparticles (5mg/mL) were added onto the surface of the mucin and equilibrated for another 15 minutes in the vibrator under the same conditions. The receptor chamber was filled with 6.4 Phosphate Buffered Saline (PBS). At fixed time intervals, 1 mL samples from the receptor chamber were withdrawn and replaced with the same volume of pre-warmed fresh phosphate-buffered saline. The concentration of FITC was determined using a microplate reader (Fluoroskan Ascent CF (Labsystems, USA).

5.16. Interactions

The interaction of synthesized nanoparticles (Fe_2O_3@FePt-NH_2-HA, Fe_2O_3@FePt-NH_2-FITC, Fe_2O_3@FePt-NH_2/COOH, Fe_2O_3@FePt-NH_2-TPP, Fe_2O_3@FePt-NH_2-TPP-COOH, Fe_2O_3@FePt-NH_2-TPP-COOH-Ctx, Fe_2O_3@FePt-NH_2/COOH-Drug, Fe_2O_3@FePt-NH_2-TPP-COOH-Drug, Fe_2O_3@FePt-COOH-DOTA-NH_2, Fe_2O_3@FePt-NH_2-DOTA-NCS andFe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx) with mucin, plasma and extracellular matrix protein [40, 41] was studied. The effect on surface charge due to the interaction between nanoparticles and mucin was studied according to previously reported method with slight modifications. 1% w/w dispersion of nanoparticles and mucin in PBS (pH 6.4) were prepared separately. The dispersions were sonicated on a bath sonicator for 30 min followed by centrifugation at 50,000 rpm for 15 min. The supernatants were separated and dispersion of obtained pellet of nanoparticles and mucin were suspended in PBS (pH 6.4). The two dispersions were mixed and incubated for 1 h followed by particle size and zeta potential measurement.

For studying the interaction of nanoparticles with extracellular matrix proteins, brain extracellular matrix (ECM) proteins were isolated from freshly collected mouse brain as

previously described. Nanoparticles were dispersed in PBS (pH 7.4) by sonication for 15 min and then added to ECM protein solution and stirred for 1 h at 37^0 C. After 1 h, the mixture was centrifuged at 15000 rpm and obtained pellet was redispersed in double distilled water. The nanoparticle dispersion was analyzed for change in particle size and zeta potential.

Hemolysis study was performed to study the interaction of prepared nanoparticles with erythrocytes [42]. In brief, blood was collected from rat by retro-orbital plexus puncture into EDTA solution containing tubes and was centrifuged at 2000 rpm for 10 min. Obtain pellet of erythrocytes were washed thrice with PBS (7.4) and then dispersed in PBS. The test samples were incubated at 37 ^{0}C for 2 h. NaCl (0.1M) was used as negative control (as it is isotonic with the intracellular solute concentration). After incubation for 2 h, absorbance of the sample solution was measured at 540 nm by UV–Vis spectrophotometer (UV–Vis, Shimadzu UV-1601). The percentage hemolysis was calculated using formula given below:

$$\% \, Hemolysis = \frac{At - An}{Ac - An} * 100$$

Where: At=absorbance of test samples at 540 nm; An= absorbance of negative control at 540 nm and Ac= absorbance of positive control at 540 nm.

The hemolytic index was also calculated according to ASTM F756-00 standards, according to which, 0–2 % is non-hemolytic; 2–5 % is mildly hemolytic and ≥5 % is hemolytic [43].

5.17. Stability Studies

Stability of synthesized nanoparticles (Fe_2O_3@FePt-NH_2-HA, Fe_2O_3@FePt-NH_2-FITC, Fe_2O_3@FePt-NH_2/COOH, Fe_2O_3@FePt-NH_2-TPP, Fe_2O_3@FePt-NH_2-TPP-COOH, Fe_2O_3@FePt-NH_2-TPP-COOH-Ctx, Fe_2O_3@FePt-NH_2/COOH-Drug, Fe_2O_3@FePt-NH_2-TPP-COOH-Drug, Fe_2O_3@FePt-COOH-DOTA-NH_2, Fe_2O_3@FePt-NH_2-DOTA-NCS andFe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx) was assessed in simulated nasal fluid (SNF) and DMEM. Stability was assessed with respect to change in particle size (aggregation) and zeta potential. Simulated nasal fluid was prepared according to previously reported literature [44, 45]. The synthesized nanoparticles (2mL, 5mg/mL; Fe_2O_3@FePt-NH_2-HA, Fe_2O_3@FePt-NH_2-FITC, Fe_2O_3@FePt-NH_2/COOH, Fe_2O_3@FePt-NH_2-TPP, Fe_2O_3@FePt-NH_2-TPP-COOH, Fe_2O_3@FePt-NH_2-TPP-COOH-Ctx, Fe_2O_3@FePt-NH_2/COOH-Drug, Fe_2O_3@FePt-NH_2-TPP-COOH-Drug,

Fe_2O_3@FePt-COOH-DOTA-NH_2, Fe_2O_3@FePt-NH_2-DOTA-NCS and Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx) were added to SNF and DMEM (5 mL) and incubated for 2 hr. After incubation, the nanoparticles were separated and were analyzed by Zetasizer (Nano ZS 90, Malvern Instruments Ltd., Malvern, UK).

5.18. Nanoparticles and Protein Corona

The interaction between nanoparticles and proteins present in biological medium is inevitable. Following *in vivo* administration, nanoparticles get covered by a protein corona as they interact with proteins present in biological media which influences its behavior at cellular level [46]. To assess the interaction of SPANs (Fe_2O_3@FePt alloy nanoconjugates (Fe_2O_3@FePt-NH_2/COOH-Drug-HA-Ctx)) and M-SPANs (Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx) with serum proteins, nanoparticles (2 mL, 5 mg/mL) were incubated with 50 % (v/v) human plasma (5 mL) at physiological body temperature followed by size and surface potential measurement at predetermined time intervals up to 48 h.

References

1. H. Jang, I.-S. Kang, J. Kim, J. Kim, Y. J. Cha, D. K. Yoon, et al. Nanofluidic chip for liquid TEM cell fabricated by parylene and silicon nitride direct bonding. Nanotechnology. 2017, 28(37), 375301-375309.
2. B. Sutens, T. Swusten, K. Zhong, J. K. Jochum, M. J. Van Bael, E. V. Van der Eycken, et al. Tunability of Size and Magnetic Moment of Iron Oxide Nanoparticles Synthesized by Forced Hydrolysis. Materials. 2016, 9(7), 554-564.
3. V. Paredes, E. Salvagni, E. Rodríguez-Castellon, F. Gil, J. Manero. Study on the use of 3-aminopropyltriethoxysilane and 3-chloropropyltriethoxysilane to surface biochemical modification of a novel low elastic modulus Ti–Nb–Hf alloy. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2015, 103(3), 495-502.
4. P. Y. Keng, I. Shim, B. D. Korth, J. F. Douglas, J. Pyun. Synthesis and self-assembly of polymer-coated ferromagnetic nanoparticles. ACS Nano. 2007, 1(4), 279-92.
5. Y. Shi, M. Lin, X. Jiang, S. Liang. Recent advances in FePt nanoparticles for biomedicine. Journal of Nanomaterials. 2015, 2015, 2.
6. Ö. Metin, S. F. Ho, C. Alp, H. Can, M. N. Mankin, M. S. Gültekin, et al. Ni/Pd core/shell nanoparticles supported on graphene as a highly active and reusable catalyst for Suzuki-Miyaura cross-coupling reaction. Nano Research. 2013, 6(1), 10-17.
7. C. I. Olariu, H. H. Yiu, L. Bouffier, T. Nedjadi, E. Costello, S. R. Williams, et al. Multifunctional Fe_3O_4 nanoparticles for targeted bi-modal imaging of pancreatic cancer. Journal of Materials Chemistry. 2011, 21(34), 12650-12659.
8. C. Xu, Z. Yuan, N. Kohler, J. Kim, M. A. Chung, S. Sun. FePt nanoparticles as an Fe reservoir for controlled Fe release and tumor inhibition. Journal of the American Chemical Society. 2009, 131(42), 15346-51.
9. N. T. Thanh, L. A. Green. Functionalisation of nanoparticles for biomedical applications. Nano Today. 2010, 5(3), 213-30.
10. D. T. Nguyen, K.-S. Kim, Functionalization of magnetic nanoparticles for biomedical applications. Korean Journal of Chemical Engineering. 2014, 31, 1289-1305

11. R. A. Sperling, W. J. Parak. Surface modification and functionalization of colloidal inorganic nanoparticles. Philosophical Transactions of Royal Society A. 2010, 368, 1333–1383.
12. X.-H. Wang, H.-S. Peng, L. Yang, F.-T. You, F. Teng, A.-W. Tang, et al. Poly-L-lysine assisted synthesis of core–shell nanoparticles and conjugation with triphenylphosphonium to target mitochondria. Journal of Materials Chemistry B. 2013, 1(38), 5143-5152.
13. K. E. Sapsford, W. R. Algar, L. Berti, K. B. Gemmill, B. J. Casey, E. Oh, et al. Functionalizing nanoparticles with biological molecules: developing chemistries that facilitate nanotechnology. Chemical Reviews. 2013, 113(3), 1904-2074.
14. E.-K. Lim, T. Kim, S. Paik, S. Haam, Y.-M. Huh, K. Lee. Nanomaterials for theranostics: recent advances and future challenges. Chemical Reviews. 2014, 115(1), 327-94.
15. L. Esser et al., Gadolinium-functionalized nanoparticles for application as magnetic resonance imaging contrast agents via polymerization-induced self-assembly, Polymer Chemistry. 2016, 7, 7325-7337.
16. Q. Qu, X. Maa, Y. Zhao, Targeted delivery of doxorubicin to mitochondria using mesoporous silica nanoparticle nanocarriers, Nanoscale, 2015, 7, 16677-16686
17. B. Wang, P. Liu, B. Shi, J. Gao, P. Gong, Preparation of pH-Sensitive Dextran Nanoparticle for Doxorubicin Delivery, Journal of Nanoscience and Nanotechnology. 2015, 15 (4), 2613-2618
18. G. Yang, G. D. Prestwich, B. K. Mann, Thiolated Carboxymethyl-Hyaluronic-Acid-Based Biomaterials Enhance Wound Healing in Rats, Dogs, and Horses, ISRN Veterinary Science, 2011, (2011), 1-7.
19. W. Abdelwahed, G. Degobert, S. Stainmesse, H. Fessi, Freeze-drying of nanoparticles: Formulation, process and storage considerations, Advanced Drug Delivery Reviews, 2006, 58, 1688-1713
20. W. Gu, G. Song, S. Li, C. Shao, C. Yan, L. Ye, Chlorotoxin-conjugated, PEGylated Gd2O3 nanoparticles as a glioma-specific magnetic resonance imaging contrast agent, RSC Advances., 2014, 4, 50254-50260.
21. K. Pramod, M. A. Tahir, N. A. Charoo, S. H. Ansari, J. Ali. Pharmaceutical product development: A quality by design approach. International Journal of Pharmaceutical Investigation. 2016, 6(3), 129-138.

22. R. V. Lenth. Response-Surface Methods in R, using rsm. Journal of Statistical Software. 2009, 32(7), 1-17.
23. X. Y. Lawrence. Pharmaceutical quality by design: product and process development, understanding, and control. Pharmaceutical research. 2008, 25(4), 781-91.
24. N. Cressie. Statistics for spatial data: John Wiley & Sons. 2015, 2nd edition, DOI: 10.1002/9781119115151
25. R. J. Hunter. Zeta potential in colloid science: principles and applications: Academic press. 2013, 2nd edition, 1-373.
26. J. Schmitt, H.-C. Flemming. FTIR-spectroscopy in microbial and material analysis. International Biodeterioration& Biodegradation. 1998, 41(1), 1-11.
27. S. A. Speakman. Basics of X-ray powder diffraction. Massachusetts-USA, 2011a Disponívelem:< http://prism mitedu/xray/Basics% 20of% 20X-Ray% 20Powder% 20Diffraction pdf. 2011.
28. R. B. Prime, H. E. Bair, S. Vyazovkin, P. K. Gallagher, A. Riga. Thermogravimetric analysis (TGA). Thermal analysis of polymers: Fundamentals and applications. A John Wiley & Sons, Inc., Publication. 2009, 1st edition, 241-317.
29. N. Colthup. Introduction to infrared and Raman spectroscopy: Elsevier. 2012, 3rd edition, 1-547.
30. H. Friebolin, J. K. Becconsall. Basic one-and two-dimensional NMR spectroscopy: VCH Weinheim. 1993, 5th edition, 1-442.
31. M. H. Levitt. Spin dynamics: basics of nuclear magnetic resonance: John Wiley & Sons. 2001, 2nd edition, 1-740.
32. N. Colthup. Introduction to infrared and Raman spectroscopy: Elsevier; 2012, 3rd edition, 109-337.
33. R. J. Wendling, A. M. Christensen, A. D. Quast, S. K. Atzet, B. K. Mann. Effect of Carboxymethylation on the Rheological Properties of Hyaluronan. PLOS ONE. 2016, 11(9), e0162849, 1-15.
34. Y.-P. Sun, X.-q. Li, J. Cao, W.-x. Zhang, H. P. Wang. Characterization of zero-valent iron nanoparticles. Advances in Colloid and Interface Science. 2006, 120(1), 47-56.
35. F. Sonvico, S. Mornet, S. Vasseur, C. Dubernet, D. Jaillard, J. Degrouard, et al. Folate-conjugated iron oxide nanoparticles for solid tumor targeting as potential specific

magnetic hyperthermia mediators: synthesis, physicochemical characterization, and in vitro experiments. Bioconjugate Chemistry. 2005, 16(5), 1181-1188.

36. A. Espinosa, R. Di Corato, J. Kolosnjaj-Tabi, P. Flaud, T. Pellegrino, C. Wilhelm. Duality of iron oxide nanoparticles in cancer therapy: amplification of heating efficiency by magnetic hyperthermia and photothermal bimodal treatment. ACS NANO. 2016, 10(2), 2436-46.
37. J. L. Markman, A. Rekechenetskiy, E. Holler, J. Y. Ljubimova. Nanomedicine therapeutic approaches to overcome cancer drug resistance. Advanced Drug Delivery Reviews. 2013, 65(13), 1866-79.
38. C. Xu, Z. Yuan, N. Kohler, J. Kim, M. A. Chung, S. Sun. FePt nanoparticles as an Fe reservoir for controlled Fe release and tumor inhibition. Journal of the American Chemical Society. 2009, 131(42), 15346-51.
39. A. Jachak, S. K. Lai, K. Hida, J. S. Suk, N. Markovic, S. Biswal, et al. Transport of metal oxide nanoparticles and single-walled carbon nanotubes in human mucus. Nanotoxicology. 2012, 6(6), 614-22
40. X.-H. Peng, X. Qian, H. Mao, A. Y. Wang. Targeted magnetic iron oxide nanoparticles for tumor imaging and therapy. International Journal of Nanomedicine. 2008, (3), 311-321.
41. O. Veiseh, J. W. Gunn, M. Zhang. Design and fabrication of magnetic nanoparticles for targeted drug delivery and imaging. Advanced Drug Delivery Reviews. 2010, 62(3), 284-304.
42. S. K. Loeb, S. Valiyaveettil. Investigating the toxicity of iron (III) oxide nanoparticles, zinc (II) oxide nanorods and multi-walled carbon nanotubes on red blood cells. http://www.nus.edu.sg/nurop/2010/Proceedings/FoS/Chemistry/Stephanie%20Katharine%20Loeb_NT081367E.pdf
43. M. VafaHomann, D. Johansson, H. Wallen, J. Sanchez. Improved ex vivo blood compatibility of central venous catheter with noble metal alloy coating. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2016, 104(7), 1359-65.
44. I.-L. Hsiao, Y.-J. Huang. Effects of serum on cytotoxicity of nano-and micro-sized ZnO particles. Journal of Nanoparticle Research. 2013, 15(9), 1829-1845.

45. Y. Zhao, Z. Zhang, W. Feng. Toxicology of Nanomaterials: John Wiley & Sons. 2016, 1st edition, 1-432.
46. M. Rahman, S. Laurent, N. Tawil, L. H. Yahia, M. Mahmoudi. Nanoparticle and protein corona. Protein-nanoparticle interactions: Springer. 2013, 1st edition, 21-44.