4.1.Sources of Chemicals

Polyethylenimine 2, 25 kDa (PEI2 and PEI25, respectively), Poly(acrylic scid) (PAA), Poly(aspartic acid) (PASA) and Poly (l-lysine) (PLL)were purchased from Sigma-Aldrich (St Louis, USA), Hank‘s balanced salt solution (HBSS), trypsin/EDTA methylthiazolyldiphenyl tetrazolium bromide (MTT), spectrophometric - grade dimethyl sulfoxide (DMSO), heparin sulfate, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N,N'-dicyclohexylcarbodiimide (DCC) were purchased from Sigma–Aldrich (St. Louis, USA). Anhydrous chloroform ($CHCl_3$), tetrahydrofuran (THF), dimethyl formamide (DMF) and diethyl ether were purchased from Fisher Scientific (Rankem, India). Sodium chloride was purchased from Fisher Scientific (Mumbai, India). 30% Acrylamide/Bis Solution 37.5:1 was obtained from Sigma-Aldrich (St Louis, USA). Platinum acetylacetonate (Pt(acac)2) (97%), iron (III) acetylacetonate (Fe(acac)3), 1,2-hexadecanediol (Technical grade 90%), dioctylether (99%), oleylamine (technical grade 70%), oleic acid (99%), anhydrous hexane (95%), acetone (≥99.5%), EDC, N-hydroxysuccinimide, 3-Aminopropyltriethoxysilane (APTES), pyridinesulfonate, ferrozine, neocuproine, ammonium acetate, mucin type II porcine and tributylamine were purchased from Sigma-Aldrich, India. (3-carboxypropyl)triphenylphosphonium bromide (CTPB), 4-morpholinoethanesulfonic acid (MES), FITC, Poly-l-lysine and tributylamine were purchased from Sigma-Aldrich, India. Potassium hydroxide, ascorbic acid and succinic anhydride were purchased from Himedia, India. Hyaluronic acid was obtained as gift sample from Mil Laboratory Private Limited, Vadodara, India. Chlorotoxin was obtained as gift sample from Alomone labs, Egypt. DOTA was purchased from Macrocyclics, Inc, USA. All other chemical and reagents used were of analytical grade.

Dulbecco‘s Modified Eagle Medium (DMEM) cell culture media, Minimum Essential Medium Eagle (MEM), ultrapure nuclease-free water, penicillin/streptomycin (10,000 U/mL/10,000 mg/mL) and bovine serum (FBS) were purchased from Invitrogen (Grand Island, USA) and HiMedia Labs (Mumbai, India). The sterile tissue culture plates (24, 48 and 96 well) and sterile tissue culture flasks (25 and 75 cm2) were obtained from Thermo Scientific and BD Falcon, respectively. The Millex GS syringe filter and Stericup-GP (150 ml, 0.22 µ) were purchased from Millipore, India.

All the reagents and solvents required for syntheses were purified by general laboratory techniques before use. Melting points were determined using silicon oil bath type melting point apparatus. The completion of the reaction was monitored by thin layer chromatography (TLC) on silica gel pre-coated plates (60F254, Merck, 0.25 mm thickness), visualizing in ultraviolet light (254nm) or iodine vapors.

4.2. Instruments Used

Sonicator: Modern Industrial Corporation (Mumbai, India)
Ultracentrifuge: Sigma 3k 30 (Sigma Centrifuge, USA)
Fluorescence Microscope: FSX100 (Olympus, USA)
Particle Size Analzyer: Malvern Zetasizer (Malvern Instruments, USA)
Zeta Potential Measurement: Malvern Zetasizer (Malvern Instruments, USA)
Flow Cytometer: FACSCalibur flow cytometer (BD Biosciences, USA)
Transmission Electron Microscope: TEM Philips, FEI-Morgagni (Philips, Japan)
UV-Visible Spectrophotometer: UV 1800 (Shimadzu, Japan)
Fluorescence Microplate Reader: Fluoroskan Ascent CF (Labsystems, USA)
Lyophilizer: (Advantage 2.0 Bench Top Freeze Dryer/ Lyophilizer, SP Scientific, USA)
Confocal Microscope: Zeiss LSM 510 confocal microscope (Germany)
ELISA Plate reader: Bio-Rad, Hercules, CA, USA

4.3. CHARACTERIZATION TECHNIQUES

Different techniques were used to characterize the properties of the particles. Dynamic light scattering studies were performed for measuring size of nanoparticles. Transmission electron microscopy (TEM) was used to characterize the size and crystal structure of the nanoparticles through electron diffraction. Scanning electron microscopy (SEM) was used to determine the surface morphology of the particles. Structural studies were carried out by X-ray diffraction (XRD) measurements. Vibrating sample magnetometer was employed for the magnetic characterization of the nanoparticles. X-ray diffraction (XRD) was used for structural characterization. Inductively coupled plasma-optical emission spectroscopy (ICP-OES) and energy dispersive X-ray (EDX) was used for compositional characterization of nanoparticles.

Spectroscopic characterization includes Raman spectroscopy and X-ray photoelectron spectroscopy (XPS).

4.3.1. Dynamic light scattering (DLS) studies

DLS also known as Quasi-elastic light scattering (QLES) is based on change in average intensity of light [1]. When a particle is placed in the path of monochromatic beam of light, there is temporal fluctuation of scattered light due to the Brownian motion of the particles. Zetasizer Nano instrument (Worcestershire UK) was used to determine the size of the nanoparticles. To analyze size, the nanoparticles were suspended in double distilled water in a concentration less than 5mg/mL and sonicated prior to measurement. The measurements were carried out in automatic mode and the values were presented as average value of 20 runs.

4.3.2. Zeta Potential

Zeta potential measurement is done to assess the surface charge of the particles [2]. It represents the effective charge on the particles; it is, therefore, related to the net electrostatic repulsion between particles. The surface charge is further important to determine surface phenomenon such as biological interaction which includes, blood protein, cell surface proteoglycans and other bio-molecules. To analyze size, the nanoparticles were suspended in double distilled water in a concentration less than 5mg/mL and sonicated prior to measurement. The measurements were carried out in automatic mode and the values were presented as average value of 20 runs. Smoluchowski approximation was used to calculate zeta potential from the electrophoretic mobility.

4.3.3. Electron microscopy

Electron microscopy is a very important technique to analyze the morphology, size and structure of nanoparticles. Electron microscopes generate images with much higher resolution than light microscopes due to short wavelengths of the accelerated electrons [3]. The resolution of transmission electron microscopes can be below angstrom if lens aberrations can be minimized. Two main different types of electron microscope, TEM and SEM, are commonly used. SEM is mostly used to characterize the surface and subsurface of nanostructure systems. TEM is used for imaging the internal microstructure of ultrathin specimens and nanoparticles.

4.3.4. Transmission Electron Microscope (TEM)

A 300-mesh copper grid with a carbon film deposited on a formvar backing, purchased from Ted Pella, were used for preparing TEM samples. A drop of the nanoparticle dispersion solution in octane was put on the formvar side of the TEM grid. The solvent was allowed to evaporate in air and assemble on the grid. Images were recorded on the negative films by the camera installed at the bottom portion of the TEM. Electron Microscopy (PHILIPS Model) operated at voltages of 20-200kv with a resolution of 2.4 Ao. The sample was allowed to dry on the slide and the slide was introduced into the instrument and scanned under the microscope for viewing of particles

4.3.5. Scanning electron microscopy (SEM)

A scanning electron microscope uses a tiny electron beam to scan across the sample. In SEM, the signals are observed on the same specimen site as the incoming electron beam. Morphology and size of the developed nanoparticles was investigated using Scanning Electron Microscopy (Zeiss Model) operated at voltages of 20-200kv with a resolution of 2.4 Ao). The samples were mounted on an Aluminum sample stub or Silica substrates using double-sided carbon stick tabs. The sample was made conductive by plating it with gold for 1 min. The anodized sample was placed in the cavity of the instrument and scanned under the microscope for determination of particle morphology.

4.3.6. Atomic Force Microscopy (AFM)

AFM is used to study the surface of the nanoparticles in atomic resolution and measure the force at nano Newton scale [4]. In this, an atomically sharp tip is scanned over the surface with a feedback mechanism that enables piezoelectric scanner to maintain the tip at constant force and height above the sample surface. As the tip scans the surface of nanoparticles, moving up and down with the contour of the surface, the laser beam is deflected off the attached cantilever into a dual element photodiode. The primary purpose of this technique is to quantitatively measure surface roughness with a nominal 5 nm lateral and 0.01nm vertical resolution. To analyze sample, approximately 1mg nanoparticles were dispersed in double distilled water and sonicated. 2 μL of this dispersion was deposited on clean mica plates and allowed to dry overnight at room temperature. The plates were analyzed using Atomic Force Microscope (NT-MDT-INTEGRA, USA) and height differences on the surface were indicated by color code where lighter indicates higher height.

4.3.7. Energy Dispersive X-ray Spectroscopy (EDS)

One important issue during the characterization of the specimen is the element composition [5]. The energy dispersive X-ray spectroscopy (EDS) is the commonly used technique to determine the element composition of the phase. Grisham's EDX (Energy Dispersive X-ray) detector connected to the SEM was used for studying the elemental composition of samples. The samples were mounted on an Aluminum sample stub or Silica substrates using double-sided carbon stick tabs. The sample was made conductive by plating it with gold for 1 min. The anodized sample was placed in the cavity of the instrument (Philips LEO 1530-2 FESEM/EDS) and scanned under the microscope for selection of area to be analyzed.

4.3.8. X-ray diffraction (XRD)

Experimentally, if we know the wavelength of the X-ray λ and measuring θ we can know the atom plane spacing d. By knowing d, the crystal structure and phase can be identified. Another application of XRD is to estimate the particle size by using Sherrer's formula: $t = 0.9\lambda/\beta \cos\theta$

Where, β is the half maximum width of the peak in radians, λ is the wavelength of the X-ray and θ is the corresponding constructive interference angle.

Philips expert pro MPD diffractometer (PAN analytical Inc. Germany) an automated diffractometer equipped with Bragg-Brentano geometry with resolution of 0.001 A0 was used to perform powder X-Ray diffraction study. About 10 mg of powdered sample was sprinkled over vacuum grease coated glass slide at room temperature to form a sample layer of approximately 0.5 mm thickness. The samples were targeted using, Cu Kα radiation (λ = 0.15418 nm) as target filter having a voltage/current of 40 KV/30 mA and scanned at speed of 40/min, radiation diffraction angle (2θ) ranged from 5° to 70° i.e. $3 \leq 2\theta \leq 400$. Step width was 0.040C and step counting time was 0.5 second and acquisition time was 1 hr.

4.3.9. Vibrating sample magnetometer (VSM)

The vibrating sample magnetometer (VSM) is based on Faraday's law [7]. It states that a field will be generated in a coil when there is a change in the flux. For a coil with n turns:

$$V = -(na)dB/dt$$

If the coil is in a magnetic field ($B = \mu 0 H$), after we put a sample with a magnetization of M, the field will be:

$$B = \mu 0(H + M)$$

Then the corresponding flux change is:

$$\Delta B = \mu 0 M$$

Combining the above two equations, we get:

$$Vdt = -na\mu M$$

The magnetic properties of nanoparticles were studied by measuring the magnetic susceptibility as a function of the applied magnetic field H. The powdered samples of lyophilized nanoparticles were punched in form of thin tablet and kept inside the magnetization chamber and the curve was obtained 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.

4.3.10. Inductively Coupled Plasma-optical Emission Spectroscopy (ICP-OES)

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis on composition of selected powder samples was processed by digesting them in nitric acid. This technique uses plasma of argon that contains a large amount of cations and electrons [8].Temperatures within the plasma can reach up to 10,000 K, allowing the sample to fully ionize whether injected into the plasma either as an aerosol, thermally generated vapor or fine powder. Recombination with electrons within the plasma generates characteristic x-rays associated with the ions present. A spectrophotometer can then detect the wavelength and intensity of thee mission allowing determination of multiple elements and relative quantities within the sample. The samples were first digested in concentrated Nitric acid followed by injection of digested sample inside the ionization chamber of ICP (Perkin Elmer Optima 5300 DV). The quantitative analysis of samples was done by measuring the intensity at element specific (Iron: 239.5 nm) wavelength.

4.3.11. Raman Spectroscopy

It is the shift in wavelength of the in elastically scattered radiation that provides the chemical and structural information. Raman shifted photons can be of either higher or lower energy, depending upon the vibrational state of the molecule under study [9].It relies on inelastic scattering or

Raman scattering, of monochromatic light, usually from a laser in the visible, near infrared, or near ultraviolet range. The laser light interacts with molecular vibrations, phonons or other excitations in the system, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the vibrational modes in the system. The nanoparticles samples (1mg/mL) were dispersed in solvent (distilled water) and analyzed in Raman spectrophotometer (LabRam HR, Horiba JobinYvon, Japan) at element specific wavelength to assess presence of elements in samples.

4.3.12. X-ray Photoelectron Spectroscopy (XPS)

XPS helps analyze the presence of surface absorbed contamination or change in surface composition of nanoparticles [10]. XPS measurements were performed on a Kratos AXIS Ultra DLD (Kratos, Manchester, UK) instrument in the “hybrid” mode using a monochromatic Al Ka x-ray source and a nominal photoelectron take-off angle of 0^{o}(the take-off angle is defined as the angle between the substrate normal and the axis of the analyzer lens). All samples were run as insulators using a low-energy flood gun for charge neutralization. As a control, similar compositional measurements were acquired for the clean titanium substrates. Measurements were performed on three replicates for each sample. Data analysis was done using the Vision Processing data reduction software.

4.3.13. Differential Scanning Calorimetry (DSC)

Thermal behavior of drug, Poloxamer 188, blank nanoparticles, physical mixture of the drug and polymer and drug loaded nanoparticles were studied usingDSC-60-Shimadzu Corporation, Japan. The instrument was calibrated using indium for melting point and heat of fusion under nitrogen purging was noted. For the analysis, 3-5 mg of the sample was weighed in standard aluminum pans. Each sample was heated under nitrogen purging at rate of 10^{o}C/min, in the temperature range of (0 – 725^{o}C).

4.4. Protocols for Cell Cultures

The U87MG cells were obtained from the cell repository facility of National Center of Cell Sciences, Pune, India. The cell lines were maintained at 16h prior to the experiments as monolayer cultures in Dulbecco‘s Modified Eagle culture Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS) and 1% antibiotic (streptomycin + penicillin).

Cultures were maintained at 37˚C in a humidified 5% CO_2 atmosphere. The protocols for freezing the cells from existing stock, starting the cell culture from frozen stock, expanding the cell culture and counting the cells are based on the protocols and methodology described in Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, 6thEdition, Ed: Freshney R. I., Wiley- Blackwell Publication, 2010 [11].

4.4.1. Freezing Cells from Existing Stock

Solutions:

Dulbecco‘s Modified Eagle Medium with 10% FBS

Penicillin/Streptomycin, Cell Culture Freezing Medium, DMSO, Trypsin

HBSS: Hanks‘Balanced Salt Solution

1) The DMEM medium and HBSS were warmed up in the water bath at 37°C.

2) The cells were taken from incubator and observed for cell growth under the microscope (made sure cells have grown ~80% confluence).

3) DMEM was removed with the help of vacuum connected pipette tip from the culture flask and 5 mL HBSS was added to the flask and allowed to sit.

4) Again HBSS was removed with the help of vacuum connected pipette tip, 1 mL Trypsin solution was added and allowed to stand for 5 min occasionally swirling to dissociate the cells.

5) Then 5 mL DMEM was added to stop the trypsin activity and the cell suspension was transferred into 50 mL tube.

6) The cells were centrifuged at 600 rpm for 6 min.

7) Supernatant was removed with the help of vacuum leaving only the undisturbed pellet.

8) 50 mL Cell Culture Freezing Medium & DMSO was added into the tube.

9) The cell suspension was splitted into small vials (1 mL into each vial).

10) The vials were put into a box and frozen for 1 hour.

11) The box was stored under liquid Nitrogen until further use.

4.4.2. Starting the Cell Culture from Frozen Cells

1) The cell culture vial was taken from liquid N_2 storage and transferred on dry ice (-60°C) to the lab.

2) The cells were thawed in the water bath at 37°C.

3) 5 mL DMEM was taken and added to 50 mL centrifuge tube.

4) The thawed cells were added to 50 mL tube and washed the vial with medium.

5) The cell suspension was centrifuged at 600 rpm for 6 min.

6) The supernatant was removed and 20 mL DMEM was added to the pellet and the cells were re-suspended in DMEM.

7) The cell suspension was equally splitted between two T-25 cm2 flasks (~10 mL into each).

8) The flasks were placed on its side in the incubator. It was made sure that the cap was loose, so as to avoid limiting oxygen supply.

4.4.3. Expanding the Cell Culture

1) DMEM and HBSS were warmed up in the water bath at 37°C.

2) The cells were taken from incubator and observed for cell growth under the microscope (made sure cells have grown ~80% confluence).

3) Medium was removed with the help of vacuum from the T-flask and 5 mL HBSS was added to the flask and allowed to sit (added to non-cell side and allowed to stand on cell side).

4) HBSS was removed with the help of vacuum from the T-flask (away from cells) and added 1mL of trypsin to the flask (on non-cell side) occasionally swirling to dissociate cells. Then 5 mL DMEM was added to the flask.

5) The cell detachment was monitored under the microscope.

6) The Trypsin/medium/cell suspension was collected in a 15 mL test tube and centrifuged for 6 min (at 600 rpm) so that the cells settled at the bottom of the test tube as a pellet.

7) Supernatant was removed with the help of vacuum getting as close as possible to the pellet (without touching the cells).

8) 10 mL of DMEM was added to the test tube and mixed.

9) The cell suspension was splitted equally between 10 T-25 flasks (1 mL in each flask).

10) 9 mL medium was added to each flask.

11) The flask were put (on its side) in the incubator (made sure that the cap is loose).

4.4.4. Cell Counting in Multiwell Plates

1) Medium was removed and cells were washed with 0.5 mL (48 wells) or 1 mL (24 wells) HBSS.
2) HBSS was removed and 50 µL of Trypsin solution was added (48 wells) or 100 µL (24 wells) to each well and incubated for 5 minutes. Cells were observed under the microscope.
3) Cells were pooled from the first well to the other wells of the same kind.
4) Each well was washed with 100 µL tissue culture medium and combined all solutions and measured the volume of cells.
5) The cells were mixed by gently flushing the cell/medium mixture up and down with a pipette.
6) Two independent 1:10 dilutions were made (if necessary) of the cell suspension.
7) Cells were counted with hemocytometer (two counts from each dilution).

*It should be noted that, only those cells were counted which were within two inner lines of the triple line boundary of the four 1 mm3 squares (each of these squares is made of 16 small squares).

The number of cells per mL = Number of cells divided by 4 X 10,000 X dilution factor. For example, if average count is 96 cells in all four squares and cells were undiluted:

Number of cells/mL = 96/4 X 10^4 X 1 = 24,000 cells/mL

4.4.5. Seeding 48-well plates with cells

1) The medium was removed from the T-flask using vacuum and flask was rinsed with 6 mL sterile HBSS.
2) 1 mL trypsin was added and the flask was gently shaken observing cell detachment under the microscope.
3) After the cells were detached, 9 mL of basic medium (with 10% FBS) was added.
4) The cell suspension was pipetted out of the T-flask and transferred to 50 mL centrifuge tube.
5) The cell suspension was centrifuged at 1000 rpm for 7 minutes.
6) The supernatant was removed and small amount of DMEM was added to re-suspend the cells.
7) Sufficient medium was added till enough volume of cell suspension was achieved to seed the plate.

8) 500 µL of cell suspension was added into each well for 48-well plate (1000 µL for 24-well plate) and incubated at 37oC, 5% CO^2 for 24-48 h (depending on the cell concentration).

4.5. Cytotoxicity assay in Multiwell Plates

1) Cells were grown in 96-well plates with 0.5 mL medium (1 mL for 24-well plates).
2) At the time of assay, the medium was removed and cells were washed with HBSS (sterile) once.
3) 0.1 mL of DMEM was added for 96-well plates.
4) Nanoparticles dispersion was added (dispersed in sterile ultrapure water) at desired concentration and incubated the plates for 24 hours.
5) Post 24 h of treatment, the cells were washed twice with HBSS.
6) Just before 24 hours were complete, MTT was dissolved in HBSS (0.55 mg/mL) and filtered using a Millipore sterile 0.22 µm filter and warmed the solution to 37°C.
7) 200 µL of MTT solution was added into each well.
8) The plate was incubated at 37°C for 2 hours followed by removal the supernatant.
9) The plate was dried by dabbing on tissue paper (do not wash) and added DMSO.
10) The formazon crystals were suspended in 100µL of isopropanol containing 0.06 M HCl and 0.5% SDS.
10) The plate was incubated for 5 min in the dark and mixed to dissolve the crystals.
11) Absorbance was read on ELISA plate reader at 570 nm and calculated mean (-/+ SD) of absorbance as a measure of cell activity/cell number.

4.6. Lyophilization

Freeze-drying (lyophilization) involves the removal of water from products in the frozen state at tremendously low pressures. The process is normally used to dry the products that are thermo-labile and would be demolished by heat-drying. The technique has too much potential as a method to solve long-term stability of various nanoparticles. Freeze drying technique was used for the purpose of maintaining the stability of synthesized nanoparticles. Synthesized nanoparticles were freeze-dried using trehalose at 10% concentration. Nanoparticle dispersion was subjected to two stages of freeze drying. First step included deep freezing the ADN dispersion 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. The porous cake thus formed was checked for Redispersibility, Drug Content and Particle Size [12].

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