CHAPTER 2

materials & methods
Chapter 2  47

Introduction

Synthesis of metal nanoparticles using chemical methods and assessment of biocompatibility of synthesized nanoparticles is the main theme of this thesis. Metal nanoparticles prepared by the methods described in the thesis have been characterized by spectroscopic, microscopic and biological techniques. Spectroscopic techniques such as UV-Visible absorption spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), and Microscopic techniques such as Transmission Electron Microscopy (TEM), Atomic Force Microscopy (AFM), and Confocal Laser Scanning Microscopy (CFLSM). Further, the nanoparticles interactions with live cells, their effect on cells and their subsequent cellular internalization has been studied by MTT assay, Trypan Blue dye exclusion test, Enzyme Linked Immuno Sorbent Assay (ELISA), Greiss Reagent test, Fluorescence Activated Cell Sorting (FACS), Phase Contrast microscopy, Confocal Laser Scanning microscopy (CFLSM), and Time lapse Video Microscopy (TLVM). This Chapter describes the materials and methods followed for the experimentation. The basic principles of the techniques used for the physico-chemical and biological characterization have been described in Annexure III.

Materials

2.1 Chemicals and kits

Tissue culture reagents like media, sera and antibiotics were obtained from Gibco. All the salts for buffer preparations and chemicals for SDS PAGE were obtained from USB. Opt’-EIA ELISA kits were obtained from BD biosciences. Paraformaldehyde was obtained from ICN (Aurora, OH). cDNA synthesis kit was obtained from Promega (Madison, USA) and PCR reagents were used from Bangalore Genei (India). All other chemicals and materials were obtained from Sigma and used as received unless otherwise mentioned. Cytochalasin ‘B’ was a kind gift from Dr. S. Ghaskadbi, ARI, Pune and fluorescent latex beads were kind gift from Dr. Sahul Hameed, Vellore, India.

2.2 Plastic wares

Tissue culture grade plastic wares like 25 cm² flasks, 6-well-plates, 24 well plates, 96-well-plates were obtained from Nunclon (DenMark). Other plastic wares like centrifuge tubes were obtained from Tarson and pipette tips, eppendorff tubes etc were obtained from Axygen (CA, USA).
Chapter 2

2.3 Animals

Swiss albino (6 to 8 weeks old) mice were obtained from an inbred colony maintained at the Animal Facility of the National Centre for Cell Science, Pune, India. Animals were housed under controlled conditions of light (12 hour of light and 12 hour of darkness), temperature (24 °C) and humidity (50 %). Mice were maintained on normal chow and water. Experimental protocols were approved by Animal Ethical Committee of the institute (NCCS).

2.4 Cell lines

All cell lines except HUVEC used in the present thesis were procured from American Type Cell Collection (ATCC), Rockville, USA and maintained in respective media (Gibco BRL) supplemented with 10 % heat inactivated Fetal Bovine Serum (FBS) (Gibco BRL). In case of NIH 3T3 cells instead of FBS, 10 % NBCS was used. The cultures were maintained in medium with 2 mM L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin at 37 °C in an atmosphere of 5 % CO₂ in 95 % humidified air. Human umbilical cord vascular endothelial cells (HUVEC) were purchased from Cambrex (East Rutherford, NJ) and were maintained as per the instructions in the medium prescribed by the company. All the cell lines used in the study were tested for mycoplasma by RT PCR analysis and were found mycoplasma free.

Table 2.1 Particulars of cell lines used for the study

<table>
<thead>
<tr>
<th>Cell lines and (Medium)</th>
<th>Type</th>
<th>Source</th>
<th>Remark/Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH 3T3 (DMEM)</td>
<td>Non-cancerous fibroblast</td>
<td>Mouse</td>
<td>Nicely spreading cells, excellent for cytoskeletal studies</td>
</tr>
<tr>
<td>RAW264.7 (DMEM)</td>
<td>Cancerous macrophage</td>
<td>Mouse</td>
<td>Professional phagocytes</td>
</tr>
<tr>
<td>HUVEC (EBM2)</td>
<td>Non-cancerous primary endothelial</td>
<td>Human</td>
<td>Excellent Model for <em>in vitro</em> vasculogenesis studies. Used for wound healing assay</td>
</tr>
<tr>
<td>MCF-7 breast cancer cells (DMEM)</td>
<td>Cancerous epithelial</td>
<td>Human</td>
<td>Model for anti tumor drug screening</td>
</tr>
<tr>
<td>SISK (L-15)</td>
<td>Non cancerous epithelial</td>
<td>Fish</td>
<td>Model for aquatic toxicology</td>
</tr>
</tbody>
</table>
Methods

2.5 Primary Cell culture

2.5.1 Islet Culture

The mouse pancreatic islets were isolated as according to the protocol developed by Bhonde and co-workers [1]. Briefly, male Swiss albino mice were sacrificed by cervical dislocation and their pancreata aseptically removed and were chopped finely using sharp mincing scissors followed by three washes with Hanks Balanced Salt Solution (HBSS). The minced pancreata were then digested with filter sterilized collagenase solution (Sigma, St Louis, MO, USA) 1 mg/ml, 2 mg/ml soybean trypsin inhibitor and 1 % bovine serum albumin (BSA) prepared in DMEM (Dulbecco’s Modified Eagle’s Media). Digestion was carried out at 37°C for 8-10 min on shaker using magnetic stirrer. Digestion was stopped by adding cold DMEM medium supplemented with 10 % FCS. Digest was then washed twice with RPMI 1640 medium and pellet was finally resuspended and cultured in RPMI 1640 media supplemented with 10 % FCS and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) in culture grade Nunclon flasks at 37°C incubator (Forma Scientific, USA), in an atmosphere of 5 % CO₂ and 95 % humidity for 48 hours.

2.5.2 Peritoneal macrophage culture

Murine peritoneal macrophages were isolated according to the method described by Kruisbeek and co-workers [2]. Briefly, 2 ml of 30 g/lit of two month aged fluid thyoglycollate medium was injected intraperitonially into adult male Swiss albino mice for 96 h. This leads to elicitation of resident macrophages into the peritoneal cavity. After four days macrophages were harvested by sacrificing the mice by cervical dislocation. Peritoneal wall was exposed by cutting and pulling back the skin of inguinal area. The peritoneal wall was swabbed with 70 % alcohol followed by 10 ml of injection of sterile PBS into peritoneum using 23 gauge needle and syringe. The PBS along with cells was carefully aspirated back and cells were washed with RPMI 1640 medium. After counting the cells were cultured in RPMI 1640 medium supplemented with FBS and antibiotics for overnight prior to setting up experiments.

2.6 Synthesis protocols for Nanoparticles

2.6.1 Sodium borohydride reduced gold nanoparticles
Chapter 2

In a typical experiment, 100 mL of 2 x 10^{-4} M aqueous solution of chloroauric acid prepared in autoclaved sterile deionized Milli-Q water was reduced by 0.01 g of sodium borohydride at room temperature in a sterile cell culture hood equipped with vertical laminar air flow. The solution resulted in a ruby-red colour indicating the formation of gold nanoparticles. The average size of the nanoparticles was 3.5 ± 0.7 nm [3]. The colloidal gold solution was aged for 24 h to ensure complete reduction of chloroaurate ions followed by 3 times dialysis over a period of 24 h in autoclaved sterilized deionized water using dialysis membrane (molecular weight cut off 12 kDa) to remove excess free borohydride ions and unreduced chloroaurate ions present in the solution, if any. The nanoparticles were concentrated at a factor of 2 by rotavapping at 72 mm and 50 ºC to achieve a final gold nanoparticle concentration of 4 x 10^{-4} mM (assuming complete reduction of chloroaurate ions). The further concentration leads to the agglomeration of particles.

2.6.2 Capping of gold nanoparticles.

The dialyzed gold nanoparticles were capped by lysine (lys) [4] and poly-L-lysine (PLL) by addition of 5 ml of aqueous solutions of 1 mM lys and 0.05 % PLL (Molecular weight ~ 4000) respectively to 20 ml each of 0.1 mM dialyzed gold hydrosols. After addition of lys and PLL to the respective gold nanoparticle solutions and ageing for 12 h, these solutions were again subjected to dialysis to remove uncoordinated lys and PLL molecules. The borohydride reduced colloidal gold and lys and PLL-capped colloidal gold solutions were concentrated by a factor of 2 by rotavapping at 72 mm and 50ºC to achieve a final gold nanoparticle concentration of 0.2 mM (assuming complete reduction of chloroaurate ions).

2.6.3 Fluorescent gold nanoparticles

Surface modification of the gold nanoparticles prepared as detailed above with fluorescein isothiocyanate (FITC) was achieved by addition of aliquots of aqueous solution of FITC to 0.2 mM colloidal gold solutions to achieve a final FITC concentration of 50 μM for emission spectroscopy and confocal microscopy studies. Variously functionalized gold nanoparticles were also capped with FITC to a final FITC concentration of 10 μM for additional emission studies. The solutions were allowed to equilibrate for 12 h under dark at 4 ºC to avoid photo- and thermo-deactivation of FITC molecules. Free FITC molecules in the colloidal gold solutions
were removed by centrifugation at 6000 rpm for 20 min at 4 °C followed by two washings with deionized water and the pellets containing FITC-bound gold nanoparticles were redispersed in deionized water and used for confocal microscopy (CFLSM). However, this low centrifugation speed was chosen to avoid the aggregation of gold nanoparticles at higher speed and thus avoiding the variation in the size of gold nanoparticles for CFLSM studies. The pellet obtained after centrifugation was redispersed in water such that the intensity of the nanogold solution obtained before and after centrifugation was same.

2.6.4 Curcumin reduced gold nanoparticles

In a typical experiment 10 ml of HAuCl₄ was taken into marked test-tubes and different volumes (from 1 µl- 20 µl) of freshly prepared curcumin solution (20 mg/ml in 0.5 M KOH) was added with thorough mixing. The solutions were left overnight at room temperature in dark. Initially, when curcumin is added the solution appears yellow and gradually it turns to muddy purple on leaving in dark which is indicative of nanoparticle synthesis. The nanoparticles were washed and resuspended in Milli-Q water before further analysis and characterization.

2.6.5 Curcumin reduced silver nanoparticles

In a typical experiment 100 ml of 10⁻³ M aqueous silver nitrate solution was reduced by 200µl of freshly prepared curcumin solution in 0.5 M KOH. The solution was kept in dark at room temperature and the synthesis of nanoparticles was monitored by analyzing the aliquots at different time points. To prove that the reducing ability of curcumin occurred only at alkaline pH a control experiment was performed where curcumin solution made in methanol was added to silver nitrate solution. The resultant nanoparticles were centrifuged at 18000 rpm in Sorval RC 2500 refrigerated centrifuge using ss 34 rotor at 10°C for 30 min. In a control experiment silver nitrate solution was kept with KOH alone in the absence of curcumin. Silver nanoparticles were not formed even after prolonged incubation in dark. In another control only curcumin was added. Silver nanoparticles obtained by this method are highly stable in solution without any external stabilizer. In general silver nanoparticles obtained by the reported methods undergo aggregation if there is no stabilizer and in the method described here this problem is overcome by the
capping nature of curcumin constituents on the nanoparticle surface preventing any aggregation.

2.6.6 Tyrosine reduced silver nanoparticles

The tyrosine reduced silver nanoparticles were synthesized as according to the protocols described by Selvakannan et al. [6]. Briefly, 10 mL of 10⁻³ M aqueous silver sulfate solution was taken along with 10 mL of 10⁻³ M aqueous solution of tyrosine and this solution was made into 100 mL with deionized water. To this solution, 1 mL of 10⁻¹ M solution of KOH was added and this solution was allowed to boil. The colorless solution turned yellow during boiling which indicated the formation of silver nanoparticles.

2.7 Physico-chemical characterization of nanoparticles

2.7.1 UV-vis absorption spectroscopy studies

Synthesis of all nanoparticles used in the study and binding of lys and PLL to the gold nanoparticles and their further binding to FITC was monitored by UV-vis spectroscopy on a Jasco dual-beam UV-Vis-NIR spectrophotometer (model V-570) operated at a resolution of 2 nm.

2.7.2 Fluorescence emission spectroscopy studies

Fluorescence spectroscopy is a powerful tool for studying the binding of fluorescent probes like FITC to gold nanoparticles [7]. Quantitative analysis of FITC binding to pure Au, Au·lys and Au·PLL nanoparticles was done using a Perkin-Elmer luminescence spectrophotometer (model LS 50B). Solutions containing FITC molecules were excited at 492 nm (λ_ex = 492 nm) and the emission band was monitored in the range of 500-600 nm. Gold nanoparticle solutions having a final FITC concentration of 0.1 and 0.5 mM were prepared as described previously. The decrease in fluorescence intensity in the supernatants after centrifugation of FITC-bound gold nanoparticles is proportional to the FITC bound to the various gold nanoparticles.

2.7.3 Transmission electron microscopy (TEM) measurements and particle size distribution

Samples were prepared by drop-coating films of the different gold and curcumin reduced silver nanoparticle solutions on carbon-coated copper TEM grids followed by measurements on a JEOL model 1200EX instrument operated...
at an accelerating voltage of 120 kV. For particle size distribution the TEM images were magnified, printed and around 250 spherical nanoparticles were measured manually. The size was compared with standard bar. The different bar size compared nanoparticles were grouped and plotted as size frequency at x axis against particle size at y axis using Microcal origin software.

### 2.7.4 Confocal microscopy (CFLSM) measurements

One drop of sample was mixed with equal volume of antifade mounting medium and was placed over a glass slide followed by mounting with cover glass. The samples were excited by 488 nm argon laser and were acquired and analyzed under confocal microscope.

### 2.7.5 FTIR analysis of curcumin reduced silver nanoparticles

Curcumin reduced silver nanoparticles were synthesized as above and washed twice with ultrapure Milli-Q water using a centrifugation speed of 18000 x g for 20 min at 10 °C. The pellet obtained after washings was mixed with powdered KBr and dried under IR lamp for 10 min. a pinch of curcumin powder was also mixed with KBr, dried under IR lamp and used as reference. The FTIR data was generated from Perkin-Elmer FTIR Spectrum 1 spectrophotometer operated at a resolution of 2 cm⁻¹ with 256 scans.

### 2.7.6 pH and salt dependent stability of nanoparticles

To determine nanoparticle stability with respect to pH, the 100 ml nanoparticles were centrifuged and the pellet was redispersed in 10 mL of ultrapure Milli-Q water. A 50 µL aliquot of the concentrated nanoparticle solution was diluted in 4.5 ml of ultrapure water. pH of curcumin reduced and stabilized AgNP solutions was adjusted by adding dilute HCl and NaOH. In a similar type of experiment the NaCl concentration was also adjusted from 0.15 to 2 M. The stability of the particles was evaluated by taking absorption spectra 1 h after the pH and NaCl concentration of the nanoparticle solution was adjusted.

### 2.8 Biocompatibility of nanoparticles

#### 2.8.1 Viability determination by trypan blue dye exclusion test

The above mentioned cell lines were seeded in triplicates for each concentration to be tested in 60 mm tissue culture plates at a cell density of 1 x 10⁶ cells/plate for 24 h. The cells were treated with increasing concentrations of
nanoparticles for 48 h. Following treatment the cells were dislodged using TPVG. The cells were pelleted and resuspended in medium and an aliquot of cell suspension was mixed with equal volume of 0.4 % Trypan blue dye for 5 min. The cells were subjected to Nebaur’s chamber and counted blindly by two different workers.

2.8.2 Nanoparticle Treatment and Cytotoxicity Determination by MTT Assay

Actively growing RAW264.7 cells were seeded at a density of 1 x 10⁵ cells/well of a 96-well tissue culture plate (Falcon, BD Biosciences, Franklin Lakes, NJ) and incubated overnight. The cells were treated with different concentrations (10, 25, 50, and 100 µM) of borohydride-reduced dialyzed gold nanoparticles for different times (24, 48, and 72 h) in quadruplets. In case of silver nanoparticles, HUVECs were treated with different concentrations (10, 25, 50, and 100 µM) of curcumin reduced dialyzed silver nanoparticles for 48 h in hexaplets. Control cells were used without any nanoparticle treatment. At the end of each exposure, the toxicity level of gold nanoparticles was assessed by 3-(4, 5-dimethylazol-2-yl)-2, 5- diphenyl-tetrazolium bromide (MTT) assay [8]. The MTT assay helps in cell-viability assessment by measuring the enzymatic reduction of yellow tetrazolium MTT to a purple formazan, as measured at 570 nm using Spectra Max 250 UV-Vis micro-plate reader (Molecular Devices, Sunnyvale, CA). All experiments were performed 3 times and the average of all of the experiments has been shown as cell-viability percentage in comparison with the control experiment while untreated controls were considered as 100 % viable.

2.8.3 Reactive Nitric Oxide Species (RNS) Determination.

Nitrite (NO2⁻) content was used as an index of nitric oxide (NO) production. Nitrite levels in supernatants of gold nanoparticle treated cells after 24 and 48 h exposure were determined spectrophotometrically with the Griess reagent. A total of 50 µL of the samples was added to 50 µL of freshly prepared Griess reagent in a 96-well plate and the absorbance was recorded at 540 nm. Dose and time dependent nitrite levels were plotted and compared to nanoparticle untreated control cells.

2.8.4 Reactive oxygen species (ROS) estimation by Flow Cytometry.

Intracellular ROS was detected using flow cytometry as described by Bass and co-workers [9]. Briefly, a stock solution of DCFH-DA (50 mM) was prepared in
methanol and stored at -20 °C in the dark. RAW264.7 macrophage cells were exposed to various concentrations of gold nanoparticles for 24 and 48 h. The green fluorescence of DCF was recorded by exciting the solutions with 488nm argon laser (FL1) using a FACS Vantage system (Becton- Dickinson, San Jose, CA), and 10000 events were counted per sample. FACS experiments were performed 3 times in quadruplets, and the representative FACS data has been shown. Also, the average of the mean fluorescent intensity of all of the experiments has been shown along with the standard deviation bars.

2.8.5 Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RAW264.7 cells were seeded at a density of 1 x 10^6 cells/well of 6-well culture plates and grown overnight. The cells were treated with different concentrations of gold nanoparticles for 3 h. Bacterial lipopolysacchride (LPS) (100 ng/ml) treated cells served as a positive control, and untreated cells served as a standard control for RT-PCR analysis. The total RNA was extracted from controls and gold nanoparticle-treated RAW264.7 cells using the Trizol method (Life Technologies). For cDNA synthesis, 2 µg of total RNA from each sample was incubated with a random primer, 0.1 µM dithiothreitol (DTT), 500 µM dNTPs, 40 units of RNase inhibitor, and 200 units of followed by incubation for 5 min at 95 °C. The transcripts from each sample were amplified from the cDNA using recombinant Taq polymerase (Life Technologies) under the following conditions: 95 °C for 2 min, 94 °C for 1 min, 68 °C for 1 min for TNF and 60 °C for 1 min for IL-1, and 72 °C for 1 min for a total of 30 cycles. β-Actin was used as a loading control. The primers used have been shown in the following table:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>5'-CGG AGG TGG AAC TGG CAG AAG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGT ACA ACC CAT CGG CTG GCA-3'</td>
</tr>
<tr>
<td>IL-1</td>
<td>5'-TCA TGG GAT GAT GAT GAT AAC CTG CT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCC ATA CTT TAG GAA GAC ACG GAT T-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-TGG AAT CCT GTG GCA TCC A-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TAA CAG TCC GCC TAG AAG CA-3'</td>
</tr>
</tbody>
</table>

2.8.6 Enzyme-Linked Immuno-Sorbant Assay (ELISA)

RAW264.7 cells were seeded at a density of 1 x 10^6 cells/well of 6-well culture plates and grown overnight. The cells were treated with different concentrations of
gold and silver nanoparticles for 24 h. Bacterial lipopolysacchride (LPS) (100 ng/ml) treated cells served as a positive control, and untreated cells served as a standard control. The culture supernatants were assayed for the pro inflammatory cytokines TNF for gold and silver nanoparticle and IL-1 for gold nanoparticles, by using Opt-EIA kits (BD Biosciences) according to manufacturer’s instructions.

2.8.7 Effect of Gold Nanoparticles on Total Cell Proteins

Total cell proteins were isolated from actively growing RAW264.7 cells treated with or without 100 µM concentrations of borohydride reduced dialyzed gold nanoparticles for 24 h using RIPA lysis buffer (20 mM Tris at pH 7.5, 150 mM NaCl, 1 % NP-40, 1 % TritonX-100, 0.1 % SDS, 1 % sodium deoxycholate, and the protease inhibitor cocktail). Total protein content was quantified by the Bradford method (BioRad, Hercules, CA), and 25 µg of protein was resolved using 10 % SDS-PAGE [10] and stained using Comassie brilliant blue.

2.8.8 NF-kB studies

The actively growing HUVEC cells were cultured at a density of 5 x 10⁴ cells/per glass cover-slips, and grown for a period of 24 h. The cells were exposed to curcumin reduced silver nanoparticles in a dose and time dependent manner followed by several washings with chilled PBS (pH 7.0). The cells were fixed with freshly prepared 3.7 % chilled paraformaldehyde (ICN, Aurora, OH) for 10 minutes, washed, blocked with 1 % BSA and 1 % FCS in PBS. The cells were permeabilized with 0.1 % Triton-X-100 in PBS and probed with anti-mouse NFkB antibody for 30 min followed by washing several times. The cover-slips were incubated with specific alexafluor-543 conjugated antibody for 45 min, washed thoroughly with PBS and mounted with antifade 1,4-diazobicyclo-2,2,2-octanex (DABCO) in mounting medium. To stain nucleus, DAPI was already added to the mounting medium. The cover-slips were observed and analyzed under confocal laser scanning microscope.

2.8.9 Studies on Islets

The mouse pancreatic islets were isolated from male Swiss albino mice and cultured for 24 h as according to the protocol developed by Bhonde and co-workers [1]. The islets were washed, pooled and counted under dissection microscope. Around 500 islets were taken in each group and treated with indicated concentrations of AuNPs for 24 h. The islets were again washed with Krebs ringer buffer solution
(KRBH) and were incubated at 37 °C and 5 % CO₂ in 300 µL of KRBH buffer with 5 mM glucose for 45 min. The islets were spun and the supernatant was saved at -20 °C. The same islets were again incubated in 300 µL of KRBH with 16.5 mM glucose for 45 min in CO₂ incubator. The supernatant was saved and later on subjected to insulin ELISA. The mouse specific ELISA kits were purchased from Mercodia and insulin secreted by stimulated and unstimulated islets was determined by following the manufacturer’s protocols.

2.8.10 Morphometry analysis

RAW264.7 cells were seeded at a density of 5 x 10^4 cells/ per glass cover slip, Raw 264.7 cells were grown for a period of 24 h. The cells were exposed to either 50 µM or 100 µM gold nanoparticle and 50 µM HAuCl₄ solutions for different time periods in CO₂ incubator. The coverslips were washed with chilled PBS (pH 7.0). The cells were fixed with freshly prepared 3.7 % chilled paraformaldehyde (ICN, Aurora, OH) for 10 minutes, permeabilized with 0.1 % Triton-X-100 and washed with chilled PBS (pH 7.0). The cells were blocked with 0.5 % BSA and probed with phalloidin-TRITC for 30 min followed by several washings to remove unbound reagent. The coverslips mounted with DAPI and antifade 1,4-diazobicyclo-2,2,2-octanex (DABCO) in mounting medium.

HUVEC cells were seeded at a density of 5 x 10^4 cells/ per glass coverslip HUVEC cells were grown till they formed a monolayer. The cells were treated with 25 µM and 50 µM of curcumin-reduced silver nanoparticles and incubated for 48 h and 96 h respectively. The coverslips were washed with chilled PBS (pH 7.0). The cells were fixed with freshly prepared 3.7 % chilled paraformaldehyde (ICN, Aurora, OH) for 10 minutes, permeabilized with 0.1 % Triton-X-100 and washed with chilled PBS (pH 7.0). The cells were blocked with 0.5 % BSA and probed with anti vinculin antibody for 1 h at room temperature. The unbound antibody was removed by several washings and the cells were incubated with FITC conjugated anti-vinculin secondary antibody for 30 min at RT in dark. The cells were thoroughly washed and incubated with phalloidin-TRITC for 30 min. The cells were again washed several times and coverslips were mounted with antifade 1,4-diazobicyclo-2,2,2-octanex (DABCO) in mounting medium. Untreated and unstained cells were taken as controls for experiment.
The mounted coverslips were observed under the confocal laser scanning microscope (Carl Zeiss, Germany) fitted with a CCD-4230 camera, using computer based programmable image analyzer KS300 (Carl Zeiss, Germany).

2.9 Endocytosis of Nanoparticles

2.9.1 Atomic force microscopy (AFM) measurements

Cell imaging was done in the tapping and contact modes by AFM on a VEECO Digital Instruments multimode scanning probe microscope equipped with a NanoScope IV controller. For sample preparation, RAW264.7 macrophage cells were seeded at a density of 5 x 10^4 cells/ml per glass cover-slip and grown for a period of 24 h. The cells were exposed to 50 μM gold nanoparticles for 5 min followed by several washings with chilled PBS (pH 7.0). In another experiment cells were given a pulse of 50 μM gold nanoparticles for 15 min followed by overnight chase in media at 37 °C in humidified atmosphere containing 5 % CO₂ and 95 % air. The cells were fixed with freshly prepared 2 % chilled paraformaldehyde (ICN, Aurora, OH) in PBS for 10 minutes. Paraformaldehyde, unlike glutaraldehyde, preserves the immunogenicity of the cell surface proteins. After fixation cells were washed 5 times with chilled PBS (pH 7.0) and coverslips were attached to metallic packs with conducting double-sided tape. The metallic packs were mounted on a 6399e- piezoscanner (10 μm) of AFM analysis. For tapping mode AFM, 125 μm long etched silicon probes and for contact mode AFM, 100 μm long silicon nitride probes were used. Phase and friction images were collected in the tapping and contact mode respectively at a scanning frequency of 1 Hz.

2.9.2 Confocal laser scanning microscopy (CFLSM) of cells

RAW264.7 cells were seeded at a density of 5 x 10^4 cells/ml per glass coverslip, and grown for a period of 24 h. The cells were exposed to 50 μM FITC-coupled gold nanoparticle solutions for different time periods in CO₂ incubator (or on ice for 1 h) followed by several washings with chilled PBS (pH 7.0). In some experiments, the cells were also treated with lysotracker dye (Molecular Probes, USA) for 30 min. The cells were fixed with freshly prepared 3.7 % chilled paraformaldehyde (ICN, Aurora, OH) for 10 minutes, washed with chilled PBS (pH 7.0) and mounted with antifade 1,4-diazobicyclo-2,2,2-octanex (DABCO) in mounting medium. The coverslips were observed under the confocal laser scanning microscope
(Carl Zeiss, Germany) fitted with a CCD-4230 camera, using computer based programmable image analyzer KS300 (Carl Zeiss, Germany).

2.9.3 TEM studies on cells

RAW264.7 macrophage cells were seeded at a density of $1 \times 10^6$ cells in a 60 mm tissue culture dish and grown overnight. The cells were treated with 50 μM gold nanoparticles for 24 h and washed thoroughly with chilled PBS, pelleted by centrifugation and fixed with 0.1 % glutaraldehyde. Cell cryotomy was performed and images were recorded at 100 kV using a Tecnai 12 Biotwin TEM (FEI, Eindhoven, Netherlands).

2.9.4 FACS studies on cells

RAW264.7 macrophage cells were seeded at a density of $1 \times 10^6$ cells in a 100 mm tissue culture dish and allowed to grow for a period of 24 h. The cells were washed twice with medium and were pre incubated with or without 2 μM Cytochalasin B for 90 min at a final volume of 3 ml per plate. In control as well as treated plates either 2 μl of fluorescent latex beads (diameter 1 μm) or 50 μM Au-Lys-FITC (final concentration) was added. The plates were incubated in dark for another 2 h. The cells were dislodged and washed with ice cold PBS. Cells were transferred to FACS tubes and an equal volume of trypan blue (0.04 %) dye was added to each tube followed by incubation on ice for 10 min. after trypan blue treatment the cells were acquired an analyzed using FACS vantage system (BD).

2.10 Antimicrobial activity

Actively growing *E. coli* culture in its log phase was spread in the form of a lawn on prepared Luria-Agar plate. After spreading small 5 mm diameter paper strips dipped in increasing concentrations of nanoparticles were put on these plates and the plates were incubated at 30 °C in the incubator and were monitored up to 96 hrs to look for any inhibition. No inhibition was seen with the nanoparticles concentrations used as against the antibiotic (Ampicillin 50 μg/ml) that showed a clear zone of inhibition. MIC test was also performed but all the cells in the tubes containing concentrations 0, 0.5, 5, 25, 50, 100 and 200 μM nanoparticles concentrations in a final volume of 5 ml (total) Luria – Broth. Similar experiment was performed in *Saccharomyces cerevisae* (Yeast), where in the procedure followed was similar except the media, which was MGYP in this case.
2.11 In vitro wound healing assay

Cell migration was studied by the in vitro scratch wound healing assay. The HUVEC cells were seeded in 6 well tissue culture plates and allowed to grow in monolayer. When cells reached 100 % confluence, were scratched with a 10 µL sterile pipette tip. The wells were washed thrice with the medium to remove any non-adherent cells and medium was shifted to DMEM with 1 % FCS. The cells were treated with increasing concentrations of curcumin reduced silver nanoparticles. VEGF (50 ng/ml) in medium was used as positive control. The cell migration into wounded area was recorded using time lapse microscope and images were captured at an interval of 20 min.

2.12 Alginate based studies

The hydrogels were prepared by dripping 1.8 % sodium alginate solution through a syringe with 26 gauze needle in 100 mM calcium chloride solution. The resulting 500-800 µM calcium cross linked alginate beads were incubated with 0.1 % poly-l-lysine for 10 min followed by incubation with 100 µM sodium borohydride reduced gold nanoparticle solution. The change in color was an indicative of deposition of gold nanoparticles on to the surface of alginate beads. These beads were co cultured in bacteriological plates with RAW 264.7 cells for 3 h and beads were transferred to fresh tissue culture plates. PLL coated beads were taken as control for co culture experiment. The beads were observed under microscope and adherence of cells over hydrogel was noted.
Chapter 2

2.13 References


