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RESEARCH PAPER

BSA-PLGA-Based Core-Shell Nanoparticles as Carrier System for Water-Soluble Drugs

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ABSTRACT

Purpose Preparation, optimization and *in vitro* evaluation of core-shell nanoparticles comprising of a hydrophilic core of BSA surrounded by a hydrophobic shell of PLGA for loading water-soluble drugs.

Methods A double emulsion method was optimized for preparation of BSA-PLGA based core-shell nanoparticles. Proof of concept for core-shell type structure was established by visual techniques like confocal microscopy and TEM. Characterization was done for particle size, encapsulation efficiency, drug loading and *in vitro* drug release. Cellular uptake was assessed using confocal microscopy, bio-TEM and HPLC assay, and cytotoxic activity was tested by MTT assay in MG-63 osteosarcoma cells.

Results The optimized core-shell nanoparticles showed a particle size of 243 nm (PDI-0.13) and encapsulation efficiency of 40.5% with a drug loading of 8.5% w/w. *In vitro* drug release studies showed a sustained release for 12 h. Cellular uptake studies indicated a rapid and efficient uptake within 2 h. TEM studies indicated that the core-shell nanoparticles were localized in cytoplasm region of the cells. Gemcitabine loaded core-shell nanoparticles showed enhanced cytotoxicity against MG-63 cells as compared to marketed formulation of gemcitabine (GEMCITE®).

Conclusion These results indicate that core-shell nanoparticles can be a good carrier system for delivering hydrophilic drugs like gemcitabine successfully to the cells with enhanced efficacy.

KEY WORDS BSA · core-shell nanoparticles · gemcitabine · water-soluble drugs

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ABBREVIATIONS

BSA	bovine serum albumin
PLGA	poly(lactic acid-co-glycolic acid)

INTRODUCTION

Delivery of hydrophilic small molecules is challenging due to their high water solubility, specifically in the presence of a hydrophobic carrier matrix which poses problems in their encapsulation at desired drug loading and encapsulation efficiency. Hydrophilic macromolecules such as proteins could be encapsulated inside these carriers owing to their large molecule weight which decreases their diffusivity to the external aqueous environment. During the preparation of drug formulations, most carrier systems use aqueous phase as external medium resulting in a rapid outward diffusion of hydrophilic molecules from hydrophobic matrix leading to poor encapsulation. Additionally, low affinity to the polymer matrix and high surface area of nano-carriers makes them more vulnerable to rapid drug release.

To overcome this delivery problem, vesicular systems such as liposomes (1) and polymersomes (2–5) have been tried for this type of drug molecules since they contain the inner aqueous core and are partially successful. Polymeric carrier systems developed using synthetic hydrophobic polymers such as polyester or polyanhydrides are suitable only for hydrophobic molecules (6–9) and partially to hydrophilic macromolecules such as proteins and genes (10,11). In case of small hydrophilic molecules, the same hydrophobic polymers showed either poor pay loads and/or very low encapsulation efficiency (12). Delivery systems for hydrophilic molecule having pH dependent solubility such as doxorubicin hydrochloride (13,14), cloricromene (15), propranolol hydrochloride (16) have also shown partial success in encapsulation in hydrophobic polymers based nano-carriers,

however, this is largely dependent on the decrease in solubility of the drug by varying pH of external phase during encapsulation. It has been reported that doxorubicin is more soluble in acidic form in comparison to its neutral form and thus nanoformulation prepared by neutral form resulted in higher drug loading (0.92%) than the cationic form of the drug (0.65%), still desired encapsulation was not achieved (14). The higher encapsulation was also achieved using ion-pairing or complexation with the polyanions, but was only applicable to charged molecules. Chavanpatil *et al.* have demonstrated (17–19) that aerosol OT-alginate nanoparticles could be used for encapsulating basic, water soluble drugs such as doxorubicin and verapamil while acidic drugs such as fluorescein and diclofenac showed poor drug loadings (20). Vrignaud *et al.* (21) have reviewed the strategies for encapsulating hydrophilic molecules in polymer based nanoparticles with the advantages and drawbacks of each system indicating a clear need to develop a carrier system which could be used for delivering hydrophilic small molecular weight drugs.

In search of new carrier systems, core-shell nanoparticles have shown a great potential to overcome such problems since they provide greater flexibility in designing due to their hybrid nature (22–24). Core-shell nanoparticles either are prepared using inorganic materials which may pose the problem of material accumulation in the body or are prepared for larger biomolecules like DNA which requires very low loading of active molecule (22,25,26). Delivery of small/medium hydrophilic molecules still remains challenging. Arias *et al.* have prepared magnetite/poly(alkylcyanoacrylate) (PACA) core-shell nanoparticles for delivery of 5-fluorouracil (27). Magnetite forms core of the particles which is surrounded by the polymeric shell of PACA containing the drug. The entire drug got released within 3 h from this system. In the present study, core-shell nanoparticles with hydrophilic core using BSA surrounded by hydrophobic shell made of PLGA are prepared as a carrier system for delivering gemcitabine as a potential water-soluble model drug. The hydrophilic core provided suitable environment for encapsulation of hydrophilic low molecular weight drug while the hydrophobic shell served to control its release from the system.

Gemcitabine is a deoxycytidine analog with a molecular weight of 299.66 g/mol and high water solubility (~38 mg/ml), and is approved for use in humans against non-small cell lung cancer, pancreatic, ovarian, bladder and breast cancer either alone or in combination with other anticancer agents (28). A major limitation associated with this molecule is its rapid metabolism in the plasma to inactive metabolite difluoro deoxyuridine (dFdU) offsetting its potential as an effective anti-neoplastic agent. Various approaches have been explored for delivering gemcitabine which include *in-situ* gel forming systems (29), liposomes (30), polymer drug conjugates (31) and nanoparticles (32,33). Owing to its low molecular weight and high water solubility, gemcitabine has a tendency to diffuse out from any nano-carrier system. Further, unlike other molecules such as doxorubicin, its solubility does not vary significantly on changing the pH of the solution. Gemcitabine hydrochloride salt has a solubility of 38 mg/ml while its base form has solubility of 22 mg/ml (unpublished data) which is still high for achieving encapsulation in nano-carrier system. In comparison to this, doxorubicin hydrochloride has a water solubility of 10 mg/ml which decreases appreciably on increasing pH enabling its encapsulation into a nano-carrier. Furthermore, gemcitabine does not carry any charged group, thus, ion pairing method could not be used for enhancing its encapsulation. It is having a log p of −1.4 indicating its high affinity to the water phase. Thus, none of the available formulation strategies are successful for delivery of gemcitabine at desired level and it is still delivered through infusion at commercial level. Thus, there is need to develop a delivery system for gemcitabine which can overcome the problems associated with these molecules.

Present study focuses on the development and *in vitro* evaluation of core-shell nanoparticles as hydrophilic drug carrier systems where bovine serum albumin (BSA) is used as the hydrophilic core containing gemcitabine surrounded by PLGA as the hydrophobic shell. These core-shell nanoparticles were prepared by double emulsion method and optimized for particle size and encapsulation efficiency. Formulations were characterized using TEM, confocal microscopy, XRD and DSC, and drug release was assessed in phosphate buffered saline (PBS) followed by *in vitro* evaluation in MG-63 osteosarcoma cell line. Qualitative uptake of the core-shell nanoparticles by the cells was studied using confocal microscopy and TEM and quantitative uptake was studied by analyzing the drug taken up by the cells with time. Drug loaded core-shell nanoparticles were then tested for efficacy in MG-63 cell line using MTT assay.

MATERIALS AND METHODS

Materials

Gemcitabine HCl was obtained as a kind gift from SMS Pharma (Hyderabad, India). PLGA was synthesized *in house* by ring opening polymerization of DL-lactide (PURAC, Germany) and glycolide (PURAC, Germany) (Mw- 90 KDa, Mn- 50 KDa, PDI- 1.78, lactide to glycolide ratio- 2.6:1). GEMCITE® was purchase from EliLilly and Co. India Pvt. Ltd., Gurgaon, India. Aerosol OT-100 was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India) and was dried prior to use. BSA was purchased from Sisco Research Laboratories Pvt. Ltd. (New Delhi, India). All other chemicals were of analytical grade and used as obtained without further purification.

Methods

Preparation and Optimization Core-Shell Nanoparticles

Core-shell nanoparticles were prepared by modified double-emulsion ($W_1/O/W_2$) solvent evaporation method (34). In this method, internal water phase (W_1) consisted of BSA and gemcitabine to form the core of the particle. Whereas, PLGA was dissolved in dichloromethane (DCM) as organic phase (O) to form the shell of the particle and aerosol OT-100 (2% w/v) was also added which served as a surfactant to stabilize W_1/O emulsion. The outer aqueous phase (W_2) consisted of polyvinyl alcohol (1% w/v) to stabilize the $W_1/O/W_2$ double emulsion. Sonication of the emulsion was performed at controlled amplitude of 80 for 1 min to reduce droplet size in the nano-size range.

For the preparation of nanoparticles, BSA (25 mg) and gemcitabine hydrochloride (5 mg) were dissolved in double distilled water (500 μl) and pH was adjusted to 8.5 using potassium hydroxide (0.5 M) to form the W_1 internal phase. It was then added to organic phase (O) consisted of DCM (5 ml) containing PLGA (100 mg) and aerosol OT-100 (2% w/v) with stirring at 1200 rpm followed by sonication at 80 amplitude for 1 min to form W_1/O emulsion. Sonication step was necessary to reduce the droplet size of the W_1/O emulsion. To this emulsion, glutaraldehyde (0.25 mmol) was added and allowed to stir for 30 min at 1200 rpm. During this step, glutaraldehyde being water soluble molecule, will diffuse into the internal aqueous phase leading to stabilization of BSA by cross-linking. The formed W_1/O emulsion was then added to outer aqueous phase, W_2 which consisted of polyvinyl alcohol solution, (1% w/v; 25 ml; aqueous phase) with stirring at 500 rpm to form $W_1/O/W_2$ double emulsion. The double emulsion was sonicated at amplitude of 80 for 1 min to reduce droplet size, followed by evaporation of organic phase (O) under vacuum to form core-shell nanoparticles. Nanoparticles were then centrifuged at 25,000 g for 5 min and washed with double distilled water to remove the free drug and adsorbed PVA from the surface of core-shell nanoparticles. Various process parameters (Table I) for nanoparticles preparation were optimized with respect to particle size and encapsulation efficiency.

Characterization of Core-Shell Nanoparticles

Particle Size and Size Distribution. Particle size and size distribution (PDI) of core-shell nanoparticles was determined using Zeta sizer (Malvern ZS, UK). Core-shell nanoparticles were dispersed in double distilled water and measurements were carried out in triplicate taking an average of 5 measurements. Zeta potential of core-shell nanoparticles was measured on the basis of electrophoretic mobility of nanoparticles under an electric field and was carried out in triplicate, each being an average of 30 measurements.

Drug Loading and Encapsulation Efficiency. For this purpose, core-shell nanoparticles suspension was centrifuged and dried under vacuum for 48 h. Weighed amount of the pellet was taken in a vial to which double distilled water was added and sonicated for 5 min at 80 amplitude to extract gemcitabine. The sample was filtered and drug content in the filtrate was determined by HPLC-UV analytical method at λ_{max} 268 nm using sodium acetate buffer (20 mM; pH-5.5) and methanol as mobile phase at a flow rate of 1 ml/min on Inertsil® ODS 3 column (4.6×250 mm; 5 μ). Drug loading and encapsulation efficiency were calculated using the following formulae:

$$Drug\ \ loading = \frac{Amount\ \ of\ \ drug\ \ encapsulated}{Total\ \ weight\ \ of\ \ nanoparticles} \times 100 \qquad (1)$$

$$Encapsulation\ \ Efficiency = \frac{Amount\ \ of\ \ drug\ \ encapsulated}{Total\ \ amount\ \ of\ \ drug\ \ taken} \times 100 \quad (2)$$

Morphology of Core-Shell Nanoparticles

Transmission Electron Microscopy (TEM)

TEM was used to determine the core–shell structure and shape of prepared nanoparticles. For this purpose, sample containing nanoparticles (10 μl) was appropriately diluted with double distilled water, loaded onto the copper grid (Formavar coated), allowed to dry in air followed by staining with phosphotungstic acid (2% w/v solution) and then examined using Technai® TEM F20 (FEI^{TM}, OR, USA) at an accelerating voltage of 160 kV.

Confocal Laser Scanning Microscopy

In this study, fluorescent labeled micron sized particles were prepared using similar procedure as that of core-shell nanoparticles except for the sonication step, to obtain particles in micron size range needed for visualization in confocal laser scanning microscope. In this process, FITC labeled BSA was used to get fluorescent core. For this, BSA solution (10 mg/ml in bicarbonate buffer (100 mM: pH-9.0)) was prepared to which FITC solution (1 mg/ml in DMSO) was continuously added in small increments and allowed to stir for 8 h in dark. The solution was then dialyzed against purified water through 2000 Da cut-off membrane to remove free FITC and the FITC conjugated BSA was freeze dried using Virtis freeze dryer (Wizard 2.0).

Table I Parameters for Optimization of Gemcitabine Loaded Core-Shell Nanoparticles

S.No.	Variable parameter	Levels	Remarks	Optimized value
1	BSA content (mg)	25, 50 and 75	Effects core size	25
2	PLGA content (mg)	50, 100 and 150	Varies the shell thickness; controls drug release	100
3	Glutaraldehyde amount (mmol)	0.125, 0.25 and 0.5	Stabilization of core	0.25
4	Drug content (mg)	2.5, 5 and 10	Affects pay load	5
5	Aerosol OT concentration (% w/v)	1, 2 and 3	Affects W_1/O emulsion stability	2
6	PVA concentration (% w/v)	0.5, 1 and 2	Affects $W_1/O/W_2$ emulsion stability	1
7	DCM Volume (ml)	2.5, 5 and 10	Affects droplet size	5

DSC and XRD Analysis

DSC thermograms of gemcitabine, PLGA, BSA, gemcitabine loaded core-shell nanoparticles, blank core-shell nanoparticles were obtained using a Perkin Elmer DSC instrument calibrated with Indium standards. For this purpose, core-shell nanoparticle suspension was centrifuged and pellet obtained was dried under vacuum for 48 h. Accurately weighed samples (4–6 mg) were sealed into standard aluminium pans and scanned from −10°C to 300°C at a heating rate of 10°C/min under inert atmosphere.

XRD analysis of gemcitabine loaded core-shell nanoparticles was performed to ascertain the nature of the entrapped drug. For this purpose, dried samples of gemcitabine, PLGA, BSA, gemcitabine loaded core-shell nanoparticles and blank core-shell nanoparticles were analyzed using powder XRD (Bruker axs D8, Germany).

Evaluation of Core-Shell Nanoparticles

In Vitro Drug Release Study

For this purpose, a dialysis bag (Molecular weight cutoff 10,000 Da) method was used wherein nanoparticle suspension (5 ml) was taken inside the dialysis bag which was then tied from both ends. This bag containing nanoparticles was then placed in vials containing release media (PBS; 20 ml; pH 7.4 and 5.5). . These vials were incubated in reciprocal shaking water bath maintained at 37°C/100 rpm. During incubation, gemcitabine was released from the nanoparticles and crosses the dialysis membrane to reach into the external media. At regular time intervals, 1 ml of external release medium was withdrawn and replaced with same amount of fresh medium. Amount of the drug released was then analyzed in the samples by using HPLC-UV method and percent cumulative release was plotted *v/s* time. In order to elucidate the mechanism of gemcitabine release form the core-shell nanoparticles, the release curves were fitted into different kinetic models such as zero order, first order, Higuchi model, Hixon-Crowell, and Baker-Lonsdale model. Best goodness-of-fit was determined from the linear regression co-efficient obtained from the model fitting of the data.

In Vitro Cell Culture Studies

Cell Culture and Maintenance

MG-63 (osteosarcoma cell line) was obtained from National Centre for Cell Sciences (NCCS), Pune, India. It was maintained at 37°C / 5% CO_2 in growth medium containing Dulbecco's Modified Eagel's Medium (DMEM, Biological Industries) supplemented with 10% Fetal Bovine Serum and 1% Antibiotics (Penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 mg/ml); Calbiochem, Germany).

Cell Uptake Study of Fluorescent Core-Shell Nanoparticles Using Confocal Laser Scanning Microscopy

To study the cell uptake, fluorescent core-shell nanoparticles were prepared using above mentioned method by replacing drug with a fluorescent dye (coumarin-6). Since coumerin-6 is a hydrophobic molecule and thus 1 mg of coumarin-6 was added to the DCM phase during preparation step. MG-63 cells (3×10^4) were seeded in 35 mm petri-dishes and allowed to attach for 24 h at 37°C/5% CO_2. After 24 h, the cells were incubated with coumarin-6 loaded core-shell nanoparticles for 2 h followed by washing of the cells for three times with PBS (pH 7.4). Washed cells were fixed using formaldehyde solution (4% v/v), permeabilized using triton X-100 (0.1% w/v) and labeled with propidium iodide dye to stain the nucleus. After staining, cells were washed thrice with PBS (pH 7.4) and analyzed using confocal laser scanning microscopy.

Cell Uptake Study of Core-Shell Nanoparticles Using TEM

Cells (2.5×10^5) were seeded in 25 cm^2 cell culture flask and incubated at 37°C/5%CO_2 for 24 h to allow cell

attachment. Attached cells were further incubated with predetermined amount of core-shell nanoparticles for 2 h and washed three times with PBS (pH 7.4). Cells were then trypsinized and fixed with glutaraldehyde (3% w/v) solution for 30 min followed by repeated washings with PBS (pH 7.4) and staining with osmium tetroxide solution (2% w/v) for 15 min. Cells were then washed with PBS and dehydrated using acetone:PBS mixture wherein, acetone ratio was gradually increased for 10% to 100%. Cells were then embedded into a low viscosity embedding media (Electron Microscopy Sciences, PA) consisting of noneyl succinic anhydride modified (NSA), DER 736 epoxy resin W.P.E. 184, ERL-4221 (3,4-epoxycyclohexane methyl 3′,4′-epoxycyclohexyl-carboxylate) and DMAE (2-(Dimethyl amino)ethanol) and kept at 70°C for 24 h for solidification. 60 nm thick sections were then cut using PowerTome Ultramicrotome (RMC products, USA) and loaded onto copper grids. Samples were then stained with uranyl acetate and observed under TEM at an accelerating voltage of 160 kV.

Quantitative Cell Uptake Study of Core-Shell Nanoparticles

Cells were seeded into 24 well cell culture plates (3×10^4 cells/well) and incubated for 24 h to allow cell attachment. After 24 h, media was removed and fresh media containing GEMCITE® (marketed formulation of gemcitabine) or gemcitabine loaded core-shell nanoparticles at equivalent drug concentration were added. At specific time points, media was removed and cells were washed with PBS (pH 7.4). The cellular uptake was analyzed indirectly by determining the amount of gemcitabine remaining in the media and washings by HPLC-UV analytical method and graphs of gemcitabine uptaken by the cells *versus* time were plotted.

In Vitro Cytotoxicity Assay

Cells (1×10^4 cells/well) were seeded in 96-well cell culture plates containing DMEM with 10% FBS as medium and incubated at 37°C/5%CO_2. After 24 h of incubation, the medium was replaced with 100 µl of the fresh medium containing different concentrations (0.1 to 100 µM) of GEMCITE® (marketed formulation of gemcitabine), gemcitabine loaded core-shell nanoparticles or blank core-shell nanoparticles and incubated for 24, 48, or 72 h. At each time point, cell viability was determined by MTT assay. For MTT assay, working solution of MTT (0.5 mg/ml) in PBS (pH-7.4) was prepared and added to each well and incubated for 4 h. After incubation, media was removed, formed formazan crystals were dissolved in DMSO and sample absorbance was measured with an ELISA microplate reader at 570 nm after correction for cell debris at 630 nm. Cell viability (%) was calculated with respect to control using the following equation:

$$Cell\ \ viability(\%) = \frac{Sample\ \ Absorbance}{Control\ \ Absorbance} \times 100 \qquad (3)$$

Statistical Analysis

All results are expressed as mean ± standard deviation. Statistical significances between data sets was determined by student *t*-test with a P value of <0.05 considered as statistically significant.

RESULTS

Proof of Concept for the Formation of Core-Shell Structure of Nanoparticles

Visualization Studies for the Core-Shell Structure

For this purpose, two studies were conducted using confocal laser scanning microscopy and transmission electron microscopy. In case of confocal studies, it was difficult to visualize the core-shell structure in nano range due to limitation of the instrument and thus florescent micron size range particles were prepared to enable direct observation of the prepared core-shell structure. However, visualization of nano size range particles was possible using TEM analysis and thus this analysis was performed using actual drug loaded core-shell nanoparticles.

Confocal Laser Scanning Microscopystudies

To visualize the formation of hydrophilic core and hydrophobic shell, FITC tagged BSA was loaded in the core and core-shell particles were visualized in confocal microscope by Z-sectioning where green color represented the core and dark circle surrounding the green color represents the shell of the particle. Figure 1a shows confocal photomicrographs of core-shell particles with all the particles having green fluorescence in the core (Fig. 1a (i)) whereas Z-sectioning (Fig. 1a (ii)) of a single particle indicated the green florescence of the core is surrounded by a darker hydrophobic shell made of colorless PLGA. As this study was to confirm the formation of core and shell within a particle in micron size range and thus, no further experiments were performed using this technique.

Transmission Electron Microscopy Studies

TEM studies can be used for visualization of particles in nano size range and also for quantitative size measurement. This technique can differentiate the regions of different materials and thus does not require fluorescent tagging.

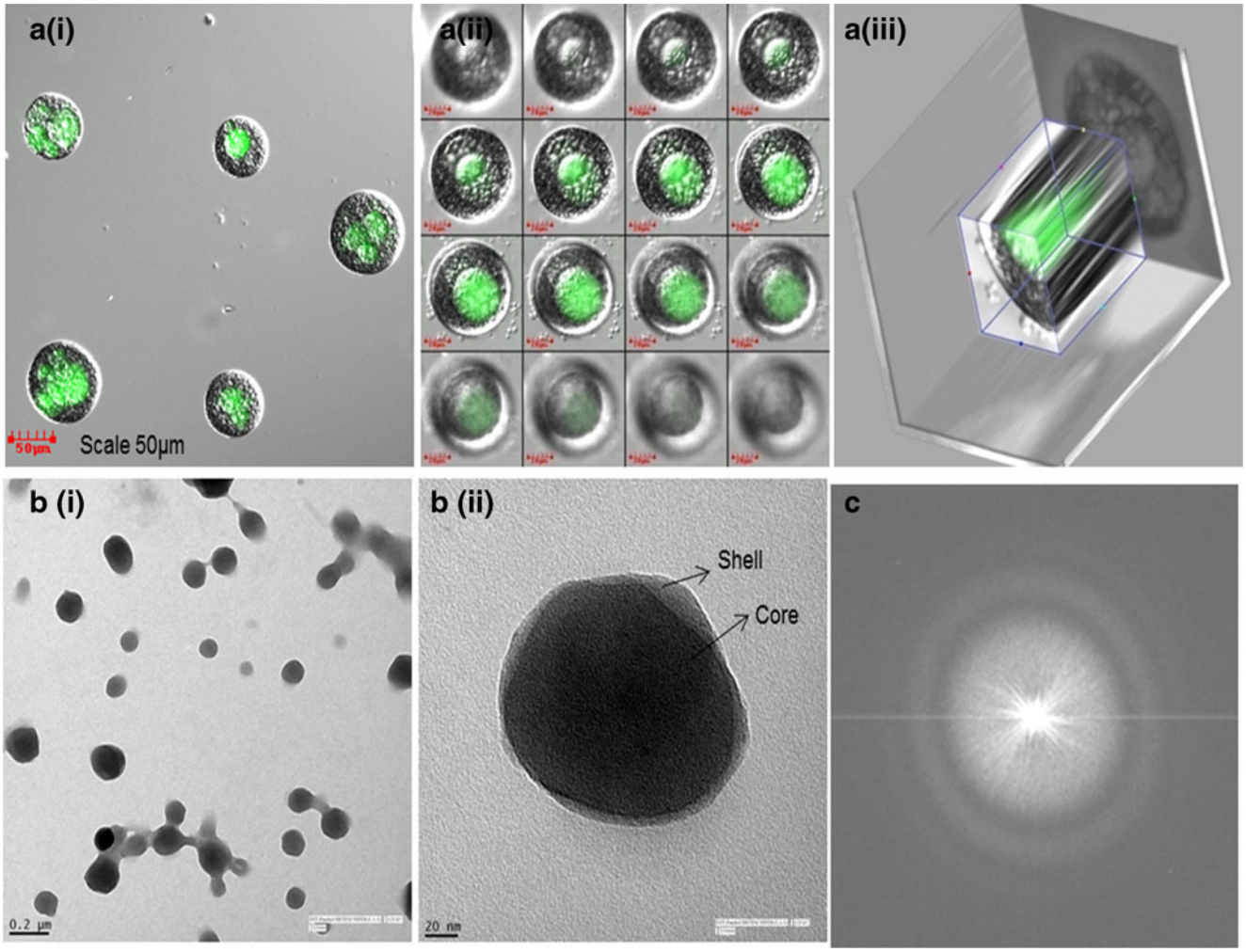

Fig. 1 Visual images of core-shell nanoparticles. (**a**) Confocal microscopy images of micron sized core-shell particles using BSA tagged with FITC i) overlay of DIC and fluorescent image, ii) Z-sectioning of single particle from top to bottom, iii) 3D view of single particle, (**b**) TEM images of gemcitabine loaded core-shell nanoparticles at magnification i) 5 K ii) 50 K, (**c**) FFT view of the single core-shell nanoparticle in TEM.

The difference in the material density also provides the inputs on its localization in the sample. Thus, TEM images captured at 5000X magnification (Fig. 1b (i)) showed that the core-shell nanoparticles were spherical and nano-meter sized. On further magnification at 50,000 X (Fig. 1b (ii)), the core-shell structure was clearly visible with core being more electron dense as compared to shell. Thickness of the outer shell was measured as 5–10 nm.

Characterization of Core-Shell Nanoparticles as a Drug Carrier

In order to assess the suitability of the developed core-shell nanoparticles as a hydrophilic drug carrier, a model drug molecule, gemcitabine, was selected and process was optimized for particle size and drug encapsulation. Total seven process variables were studied as shown in Table I and their effects on particle size and encapsulation efficiency are depicted in Fig. 2. The matrix of core-shell nanoparticles consisted of two polymers, BSA (as core-forming polymer) and PLGA (as shell forming polymer). In this method, initially aqueous core is formed using BSA as a matrix by W_1/O single emulsion and is stabilized using surfactant and cross-linking by glutaraldehyde followed by constructing a coat of PLGA as hydrophobic shell using $W_1/O/W_2$ double emulsion. The amount of both polymers can significantly affect the size and encapsulation of the drug in the formulation thus their contents were optimized using the parameters as depicted in Table I. On increasing BSA amount from 10 mg to 25 mg, particle size was increased from 185 nm to 231 nm whereas encapsulation efficiency increased from 6% to 21.5%, however, no further significant change was observed on both the parameters at albumin content of 50 mg. At PLGA content of 50, 100 and 150 mg, particle size was found to be 210, 243 and 311 nm, respectively while encapsulation efficiency first increased from 5.8% to 40.5% and then decreased to 36.4% at 150 mg PLGA content. Glutaraldehyde was used for crosslinking of encapsulated BSA

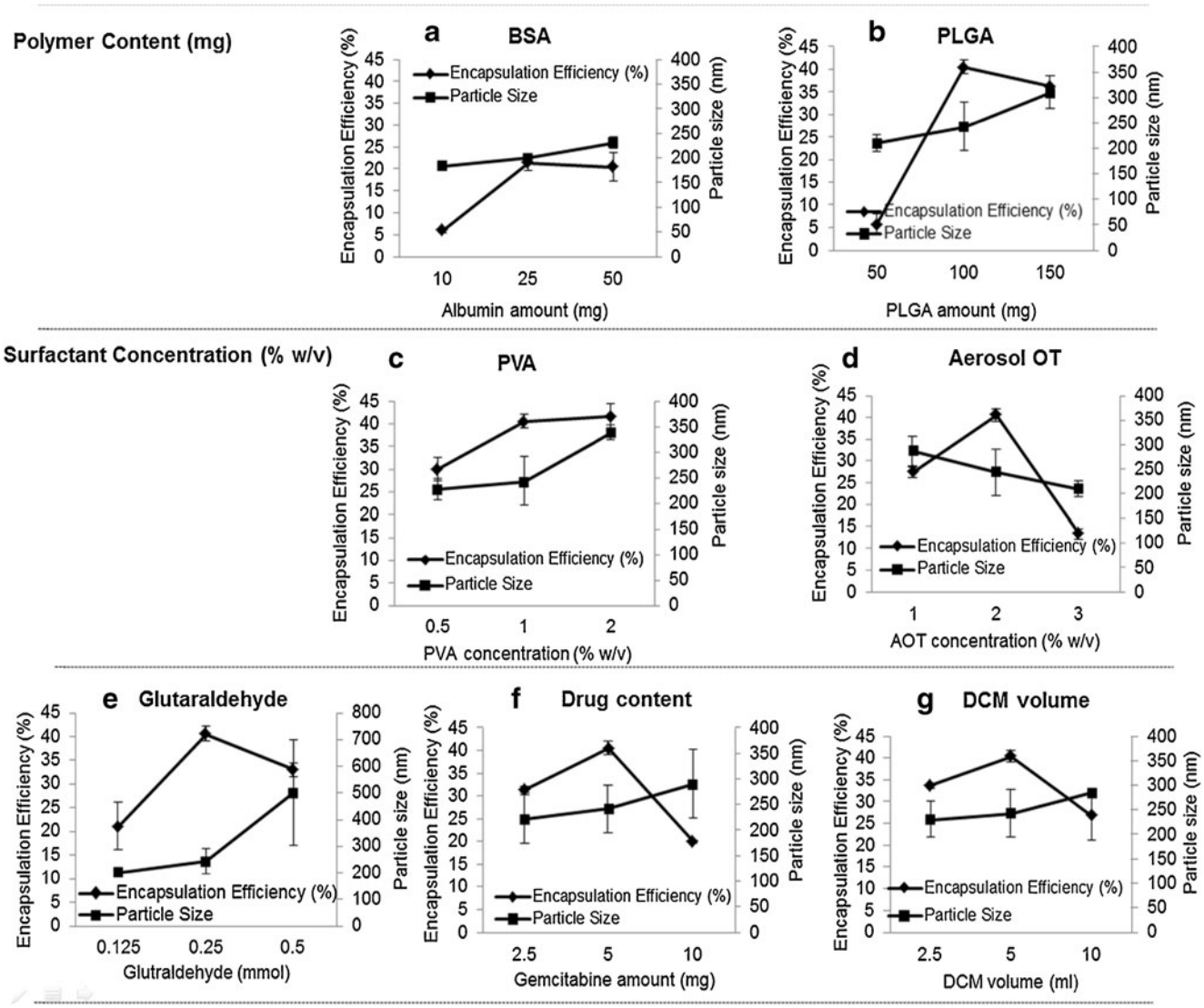

Fig. 2 Effect of process parameters as given in Table I on encapsulation efficiency and particles size of gemcitabine loaded core-shell nanoparticles.

to stabilize the core of the particle which also affects the particle size. At glutaraldehyde concentration of 0.125, 0.25 and 0.5 mmol, particle size was found to be 200, 243 and 500 nm with a PDI 0.17, 0.14 and 0.64 respectively. Similarly, encapsulation efficiency was increased from 21 to 40.5%, thereafter decreased to 30% at 200 µl glutaraldehyde concentration. Core-shell nanoparticles prepared without glutaraldehyde showed a particle size and encapsulation efficiency of 194.2 nm (PDI-0.14) and 5.7%, respectively.

A model drug gemcitabine was encapsulated in the core of the particle along with BSA and effect of varying drug concentration on size and encapsulation efficiency was observed. When the initial gemcitabine content was increased from 2.5 mg to 10 mg, particle size increased from 222.1 to 290.6 nm, however, encapsulation efficiency initially increased from 31.4 to 40.5% on increasing drug content from 2.5 mg to 5 mg and thereafter, decreased to 20.1% at 10 mg.

Effect of changing volume of organic phase in $W_1/O/W_2$ emulsion was assessed by varying the volume of DCM from 2.5 ml to 10 ml. In this study, particle size increased from 230.7 to 285.2 nm while encapsulation efficiency initially increased with increasing DCM from 2.5 to 5 ml and thereafter decreased at 10 ml of DCM. Formation of stable emulsions (W_1/O and $W_1/O/W_2$) was necessary to obtain core-shell structure and thus, two surfactants were used in the preparation method. If the formed emulsions are not stable then there are chances of formation of PLGA particles without any core of BSA. Thus, Aerosol-OT and PVA surfactants were used in organic and outer aqueous phase, respectively, to stabilize the emulsion and their concentration can also affect the droplet size. A decrease in particle size was observed on increasing the aerosol OT concentration whereas the encapsulation efficiency initially increased and then decreased on further increasing aerosol OT concentration. PVA is used

as surfactant in external aqueous phase to stabilize the $W_1/O/W_2$ double emulsion. Particle size was found to be increased from 227.1 to 339.1 nm with increasing PVA concentration from 0.5 to 2% w/v, however encapsulation efficiency initially increased from 30 to 40.5% as PVA concentration was increased from 0.5 to 1% and further remained constant at higher concentration (2% w/v) of PVA. Smallest particle size obtained during optimization of core-shell nanoparticles was 185 nm, however, gemcitabine encapsulation efficiency was only 6% at this particle size. Although, small sized particles should have better therapeutic use but these small sized particles have shown low encapsulation efficiency of the gemcitabine and thus, were not taken forward for efficacy testing. Highest encapsulation efficiency of 40.5% was obtained at a particle size of 243 nm with a drug loading of 8.5% w/w and this formulation was taken forward for further studies. All the optimized parameters for the final formulation are summarized in Table I.

Solid State Characterization of Gemcitabine in Core-Shell Nanoparticles

The state of the drug in core-shell nanoparticles was assessed using DSC and XRD techniques. Free form of gemcitabine is crystalline in nature and DSC studies revealed that free gemcitabine showed an endothermic melting peak at 270°C followed by an exothermic peak corresponding to its degradation. This melting peak is absent in DSC thermogram of drug loaded core-shell nanoparticles while a small endothermic peak appeared at 55°C corresponding to glass transition temperature of PLGA component of core-shell nanoparticles (Supplementary Material Fig. S1). In XRD analysis, free gemcitabine showed several sharp peaks corresponding to the crystalline regions of gemcitabine (Supplementary Material Fig. S2). However, in gemcitabine loaded core-shell nanoparticles, a hallow pattern was obtained without any crystalline regions indicating the amorphous nature of the encapsulated gemcitabine. Fast Fourier Transform (FFT) view of the single drug loaded nanoparticle using TEM was also captured to predict the nature of the entrapped drug. Circular rings were obtained in the FFT view (Fig. 1c) without any bright spots on the rings indicating amorphous nature of the loaded gemcitabine, and circular view indicates the hydrophobic regions at the periphery due to the presence of PLGA shell in the nanoparticles.

Evaluation of Core-Shell Nanoparticles as a Drug Carrier

For any drug carrier system to be effective, it is important to have a desired drug release from the carrier, drug/carrier uptake in the cells/tissues with maximum cytotoxicity/toxicity at disease site, but minimum to the normal cells. Thus, prepared carrier system was evaluated for drug release properties, drug/carrier uptake in a cancer cell line (MG-63) and efficacy with respect to cytotoxicity to the cells.

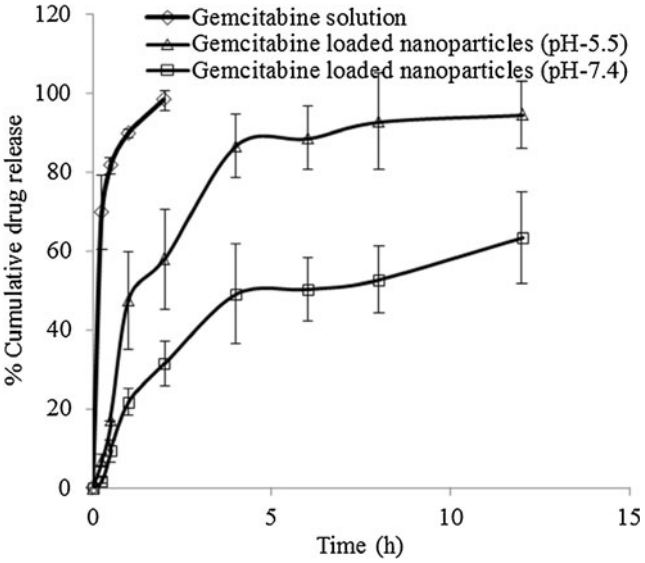

Fig. 3 *In vitro* release profile of gemcitabine from core-shell nanoparticles at pH 5.5 and 7.4.

In Vitro Drug Release Study

In vitro drug release study was performed to assess the ability of the core-shell nanoparticles to control the release of gemcitabine. These studies were conducted at pH 5.5 and 7.4 to mimic the tumor microenvironment and physiological conditions, respectively. In the present formulation, a controlled release of gemcitabine was observed for 12 h as shown in Fig. 3. At 5.5 pH, drug release was observed to be faster where 45% of gemcitabine was released in first 1 h thereafter drug release was continued till 12 h with a cumulative drug release of 85%. At pH 7.4, drug release was comparatively slower than at pH 5.5 where 20% of the drug was released in first 1 h followed by a slow release upto 12 h with a cumulative release of 60%. Drug release experiments were conducted in a dialysis membrane and thus it was also studied if there is any obstacle to the passage of the free drug from the membrane to bulk solution. Thus, the release of free drug to the bulk phase was studied and observed that there was a complete drug release to the bulk phase within 2 h where, 80% of the drug diffused to bulk phase in just 30 min. Model fitting of the drug release kinetics showed best fit in Higuchi model with R^2 value of 0.949 and 0.892 at pH 5.5 and 7.4, respectively.

Cell Uptake of Core-Shell Nanoparticles

The cellular uptake of core-shell nanoparticles was evaluated qualitatively using visual techniques confocal microscopy and TEM; and quantitatively by analyzing gemcitabine content taken-up by the cells after incubation with drug

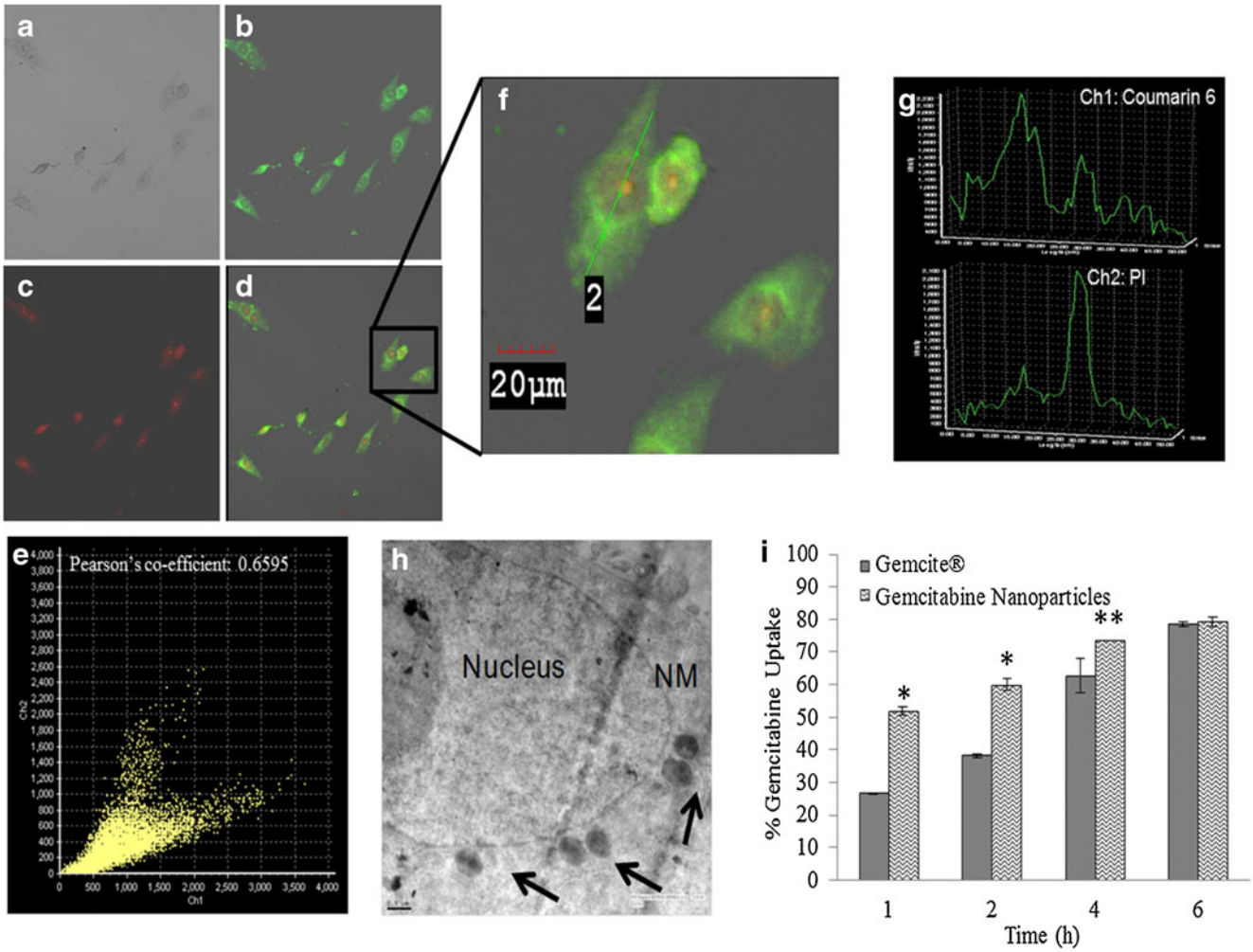

Fig. 4 Cell uptake of core-shell nanoparticles in MG-63 cell line by confocal microscopy (**a**, **b**, **c**, **e**, **f**, **g**) and TEM (**h**) and HPLC assay (**i**) (**a**) DIC image, (**b**) Coumarin-6 loaded core-shell nanoparticles in cells (*Green color*), (**c**) Cells (after fixing) stained with propidium iodide (*red color*), (**d**) Overlay of **a**, **b**, and **c**, (**e**) Pixel wise co-localization analysis, (**f** and **g**) line analysis of co-localization, (**h**) MG63 Cells after 2 h of incubation with gemcitabine loaded core-shell nanoparticles in TEM. Arrows represent gemcitabine core-shell nanoparticles within the cells. (**i**) Quantitative uptake (%) of gemcitabine in MG-63 cells using GEMCITE® and gemcitabine loaded core-shell nanoparticles. Gemcitabine loaded core-shell nanoparticles (*$p < 0.001$, ** $p < 0.05$ v/s GEMCITE®) showed significantly higher uptake than the GEMCITE®.

loaded nanoparticles using HPLC assay of gemcitabine. Qualitative analysis by confocal microscopy revealed that core-shell nanoparticles were efficiently taken-up by MG-63 cells within 2 h as depicted by green fluorescence (Fig. 4b). Further, to overcome the artifact of green fluorescence due to surface bound core shell nanoparticles, cells were counter stained with propidium iodide and optical sectioning was performed. It was observed that both red fluorescence (from propidium iodide) and green fluorescence (from C6 loaded nanoparticles) were present at the same optical plane (Fig. 4c) confirming the presence of nanoparticles inside the cells. Fig. 4d shows the overlap of green/red fluorescence. Co-localization (Fig. 4e) of green fluorescence of C6 and red fluorescence of PI was determined on six region of interests (ROIs) and pearson's co-efficient was found to be 0.6595. This indicated that both dyes are in partial co-relation for localization in cell. Line analysis was performed on the magnified overlay image (Fig. 4f) which showed that partial overlap of the signals of C6 and PI on the selected line passing through the length of single cell (Fig. 4g), however it is clearly visible that red fluorescence of PI is localized at the nucleus of the cell and green fluorescence of C6 is visible in the whole cell. That is the probable reason that pearson's co-efficient is less than 1.0 where value of 1.0 indicates for complete correlation of both dyes. TEM analysis (Fig. 4h) also revealed that the core-shell nanoparticles were taken up by the cells and were located in the cytoplasm of the cells within 2 h. Figure 4i shows the gemcitabine uptake (%) when cells were incubated with marketed formulation of gemcitabine (GEMCITE®) or equivalent gemcitabine loaded core-shell nanoparticles. The cell uptake was found to be time dependent and increased with

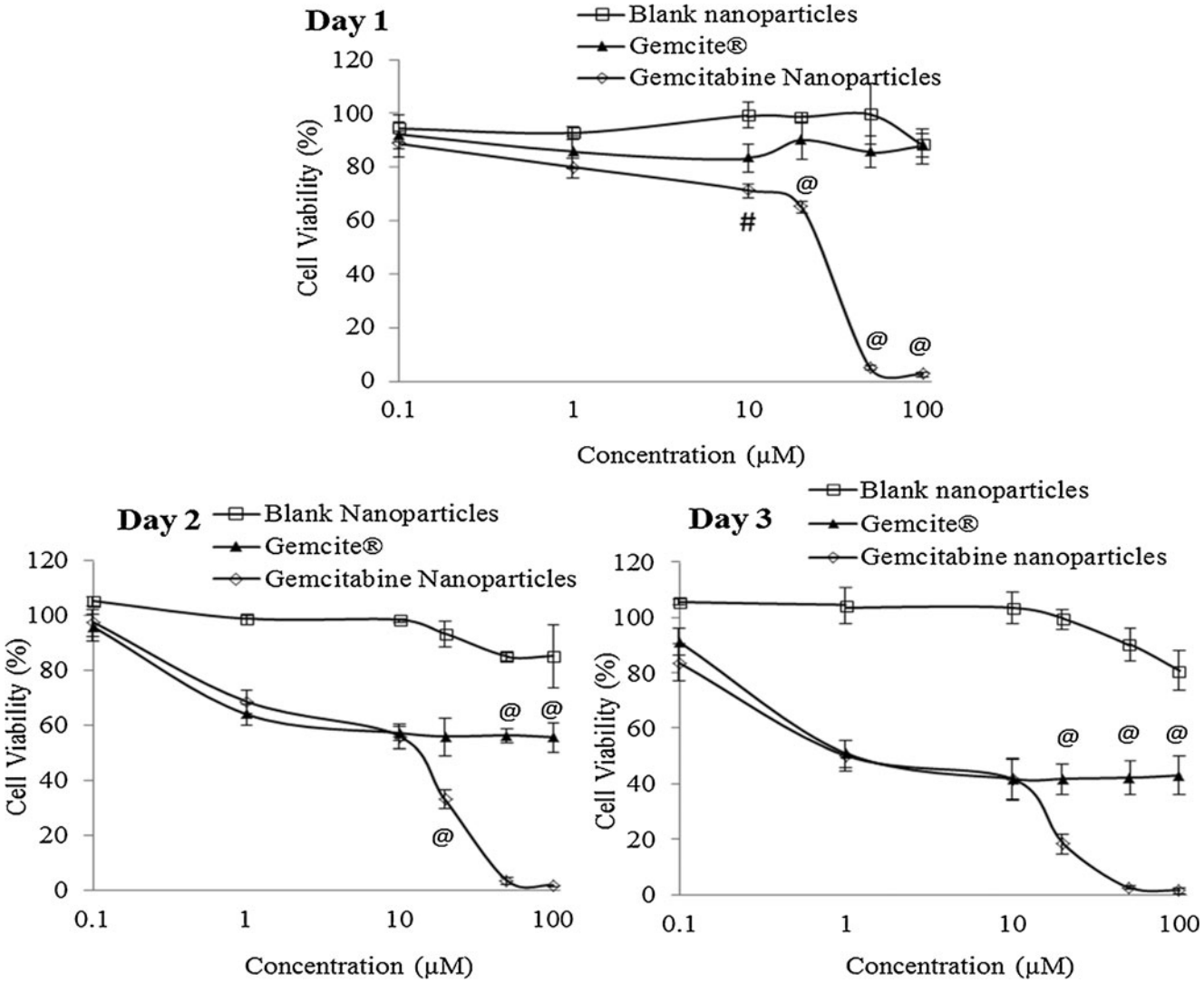

Fig. 5 Cytotoxicity of GEMCITE® and Gemcitabine loaded core-shell nanoparticles in MG-63 cells. Gemcitabine loaded core-shell nanoparticles showed statistically significant lower cell viability as compared to GEMCITE® indicating the enhanced efficacy of the core-shell nanoparticles ($^{\#}p < 0.01$, $^{@}p < 0.001$ v/s GEMCITE®).

incubation time. Further, it was observed that the gemcitabine uptake in 1 h from core-shell nanoparticles was significantly higher (52%) than that of GEMCITE® (27%).

Table II Cytotoxicity of Gemcitabine Loaded Core-Shell Nanoparticles and GEMCITE® in MG-63 Cell Line

Formulation	IC_{50} (μM) in MG-63 cell line		
	Day 1	Day 2	Day 3
Gemcitabine loaded core-shell nanoparticles	27.4	11.2	1.5
GEMCITE®	#	#	2.0

IC_{50} values could not be achieved

Cytotoxicity Evaluation in MG-63 Cell Line

To evaluate the efficacy of drug loaded core-shell nanoparticles, MG-63 osteo-sarcoma cells were incubated with different molar concentrations of drug loaded core-shell nanoparticles and GEMCITE® at 24, 48 and 72 h and cytotoxicity assays were performed as shown in Fig. 5. Blank core-shell nanoparticles were also studied at equivalent amount to that of gemcitabine loaded core-shell nanoparticles to evaluate the toxicity of the blank formulation. It was observed that the gemcitabine loaded core-shell nanoparticles were highly cytotoxic than GEMCITE® formulation at all the studied time points (Table II). Mean inhibitory concentration (IC_{50}) in case of GEMCITE® was reached only on third day while in case of gemcitabine loaded core-shell nanoparticles it was achieved on the first day. Further at

higher concentration levels, gemcitabine loaded core-shell nanoparticles resulted in much reduced cell viability as compared to GEMCITE® (1.5% *versus* 43.3% at 100 μM concentration). Furthermore, blank core-shell nanoparticles did not show any cytotoxic effect to MG-63 cells.

DISCUSSION

Several hydrophilic drugs have shown remarkable therapeutic benefit but their delivery is limited to conventional delivery strategies which result in poor patient compliance. On the other hand, designing carrier systems for these molecules is even more challenging due to their high water solubility. Efforts have been made to encapsulate these hydrophilic molecules into nano-carriers by varying pH conditions to decrease the drug solubility or using counter-ions to form comparatively less soluble drug complexes. Such strategies are largely dependent on the drug properties (such as pH solubility profiles, ionization, *etc.*) which change the physico-chemical profile of the native drug and thus affect the efficacy/toxicity of drug molecule. Hence, there is a need to develop a carrier system which can deliver hydrophilic molecules independent of their physico-chemical properties and more importantly retain their native therapeutic efficacy needed for effective treatment of the disease condition. While developing such systems, particularly for hydrophilic drugs, two important properties are needed *i.e.*; considerable payload of drug in the carrier of the desired size and its release from the system to ensure the desired therapeutic effect.

The present formulation of core-shell nanoparticles was designed keeping in view both of these parameters. The interior of the particles was made by hydrophilic polymer matrix (BSA) to provide a suitable environment for encapsulating water soluble drugs while shell was formed by hydrophobic polymer, PLGA, to control the release of the drug from the system.

For successful design of such a system, it is the first and foremost requirement to prove that core-shell structure is achieved in the developed carriers. For this purpose, a proof of concept was established by studying the formation of core-shell structure using confocal microscopy (visual and qualitative) and TEM (visual and quantitative) techniques as shown in Fig. 1. For confocal analysis, fluorescent BSA was obtained by tagging BSA with FITC and particles were prepared where fluorescent BSA shows green fluorescence in the core of the particles confirming its location in the core-shell nanoparticles. These results support the proposed hypothesis as BSA was taken in the internal water phase in $W_1/O/W_2$ double emulsion method for the preparation of core-shell nanoparticles. TEM images further confirmed formation of the core-shell structure wherein electron-dense core made of hydrophilic BSA was surrounded by a 5–10 nm thick shell of hydrophobic PLGA. In these particles, BSA core was very labile being hydrophilic in nature and thus it was stabilized by crosslinking with glutaraldehyde which were further covered by PLGA shell using $W_1/O/W_2$ double emulsion as seen in Fig. 1b (ii). Glutaraldehyde ia a homobifunctional crosslinker consisted of free aldehyde groups which can interact with the ε-amino group of lysyl residues of the BSA *via* schiff base and/or Michael-type addition reaction thereby resulting in inter- and intra-chain cross-linking and hence, stabilization of BSA. Further, drug loaded carriers were fabricated by loading a hydrophilic model drug gemcitabine in BSA core during the formulation of single emulsion and keeping other steps same as discussed earlier. It resulted in the formation of gemcitabine loaded core-shell nanoparticles where entrapped drug was observed to be amorphous in nature as indicated by the absence of bright spots on the circular rings in Fig. 1c, and by XRD (Fig. S1) and DSC (Fig. S2). These results are in coherence with the previous report showing conversion of crystalline drug to amorphous form after encapsulation in the polymeric matrix due to molecular dispersion of drug in the inner core of the particles (35).

Prepared core-shell particles were optimized for size and drug encapsulation based on seven required parameters as shown in Table I. Although these parameters could affect the core and shell properties such as their thickness which can influence the stability and drug release properties, however, particle size and encapsulation efficiency were selected as primary response parameters for optimization of drug carrier as both parameters dictate the therapeutic efficacy/toxicity of the formulation at desired site of action for effective treatment of the disease. A stepwise optimization of size and drug encapsulation of prepared particles was done based on 7 listed parameters by varying one parameter at a time and keeping others constant as shown in Table I. On increasing BSA content, particle size was increased due to increasing core volume and drug encapsulation was also increased. It is reported that gemcitabine interacts with albumin *via* non-covalent linkages during co-encapsulation (36) which might also be the reason for obtaining higher encapsulation of gemcitabine at higher BSA content. To minimize the drug release from BSA matrix/droplet at double emulsion stage, glutaraldehyde was added to W_1/O single emulsion so that BSA matrix/droplet can be stabilized by crosslinking with glutaraldehyde and the release of hydrophilic drug into aqueous media can be minimized at double emulsion stage. Glutaraldehyde is slouble in both water and DCM, thus it could be added either in the internal aqueous phase (W_1) or after formation of W_1/O emulsion. However, it was observed that addition of glutaraldehyde in W_1 leads to aggregation of BSA and hence, for successful formulation of nanoparticles, it was added in the W_1/O emulsion. It is observed that

increasing glutaraldehyde concentration enhances the encapsulation efficiency of gemcitabine and particle size of the core-shell nanoparticles was also enhanced. Increasing particles size and drug encapsulation also indicate that higher amount of glutaraldehyde lead to higher interaction between albumin chains (both intra and inter-molecular) leading to formation of larger albumin aggregates in the core, thereby increasing the particle size as well as PDI. Further, it is expected that GTA can affect the core properties and can lead to collapse of the core. In the present study, it was observed that higher GTA concentration (*i.e.* above 0.5 mmol) results in aggregation and precipitation of BSA during W_1/O emulsion stage. On increasing PLGA content in the DCM phase at double emulsion stage, particle size was further increased which could be due to the increased viscosity of the DCM phase (37) thereby increasing the droplet size in the double emulsion. This increased viscosity may reduce the diffusion of drug to the external phase resulting in an increase in encapsulation efficiency. On increasing the amount of gemcitabine from 2.5 mg to 5 mg in the feed, particle size and encapsulation efficiency also increased due to the increased core volume, however at higher gemcitabine content (10 mg), encapsulation efficiency again decreased due to limiting holding capacity of the polymeric matrix. It is observed that all the above tested parameters had increased the particle size of the formulation and thus it was necessary to reduce the particle size so that desired size can be achieved. For this purpose, surfactants were used as they can reduce the droplet size of emulsion during the preparation process and thereby can reduce the size of final formulation. It is also important to note that in the current formulation two heterogeneous matrices are used to form core-shell nanoparticles and thus two different surfactants are necessary to stabilized both matrices and thus aerosol OT as a hydrophobic surfactant was employed to stabilize the W_1/O single emulsion and PVA as a hydrophilic surfactant was taken in the outer aqueous phase for stabilizing $W_1/O/W_2$ emulsion. During the preparation of the formulation at higher PVA concentration, particle size was increased due to increased viscosity of the external phase and hence larger droplet size was obtained resulting in bigger size of the particles (38,39). It is also observed that organic phase volume does not affect the particle size significantly on the tested values, however it resulted in reduction of drug encapsulation efficiency.

After the proof of concept and characterization/optimization of drug loaded core-shell nanoparticles, it is important to demonstrate that these drug carriers can release the drug and can also be taken-up by the cells/tissue for desired therapeutic effect. Gemcitabine is a hydrophilic molecule with a water solubility of 38 mg/ml. Such high water solubility poses challenge to control the release of the drug from carriers which diffuses at a faster rate into the external aqueous environment. In the present formulation, PLGA shell was used to control the drug release from the hydrophilic core of BSA within the core-shell nanoparticles. Due to the rapid metabolism of gemcitabine in biological environment, it is also desirable to control the release of gemcitabine from the formulation so that its metabolism by cytidine deaminase enzyme present in plasma can be reduced and thereby it may result in higher efficacy even at the lower dose of the formulation. Further, it is reported in literature that a better anticancer activity of gemcitabine can be achieved by prolonged IV infusion as compared to bolus administration (40) and the same can be achieved from a controlled release formulation like the present formulation. In case of core-shell nanoparticles, sufficient time would be available for the extravasation into the tumor compartment which is not in the case of free gemcitabine due to faster metabolism in biological environment. A controlled release for 12 h was obtained from the core-shell nanoparticles which can be delivered through IV injection as a replacement of continuous IV infusion. In this way, infusion can be avoided and an enhanced efficacy can be achieved owing to its controlled release/nanoparticulate nature. In the present study, drug release pattern was studied in PBS at pH 7.4 and 5.5 to mimic the physiological and tumor microenvironment, respectively. A faster release of gemcitabine was observed at pH 5.5 in comparison to the pH 7.4 which further ensures the higher release of drug in the tumor micro-environment than in the physiological conditions and thus beneficial for faster treatment. Drug release from nano-carriers generally occurs through several mechanisms such as diffusion through the polymeric matrix, release by polymer degradation and solubilization, diffusion through micro-channels that exist in polymeric matrix or are generated during polymer hydration. Further, gemcitabine release pattern showed best-fit in Higuchi model indicating that the release is mainly governed by the diffusion of the drug molecules. This is also expected since gemcitabine being a hydrophilic molecule has a high tendency for diffusion. Secondly, the outer shell is made up of high molecular weight PLGA which is not expected to undergo significant degradation during the initial 12 h. However, there may be formation of diffusion channels in the outer shell of PLGA leading to outward movement of drug molecules. A rapid release of gemcitabine under acidic conditions might have resulted from the faster creation of diffusion channels in the outer PLGA shell due to hydration and cleavage of ester bonds under acidic environment. An initial burst release of gemcitabine was observed from the nanoparticles which could be due to the drug molecules adsorbed in the PLGA shell and/or dispersing close to the nanoparticle surface, which can rapidly diffuse in the initial incubation time.

In vitro efficacy of the formulation was tested in MG-63 cancer cells by studying their uptake to the cells and cytotoxicity. In cancer cells, nanoparticles are generally uptaken *via* energy driven endocytic pathway in a time and dose dependent manner. These pathways may be categorized into clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin

and caveolae-independent endocytosis and macropinocytosis (41). It has been reported that nanoparticles may initially get associated with the membrane proteins and lipids, subsequently be delivered to the endocytic machinery, and finally, escape from the endosomal compartments into cytoplasm. The efficacy could be greatly enhanced by using core-shell nanoparticles due to higher surface energy of hydrophobic shell and release of the therapeutic molecules directly in the vicinity to the cell nucleus. In the present study, both confocal microscopy and TEM techniques indicated that core-shell nanoparticles were efficiently taken up by the cancer cells within 2 h and are present in the vicinity of the nucleus. Further, higher uptake of core-shell nanoparticles ensure higher uptake of gemcitabine as compared to its marketed formulation which is given in solution form. Gemcitabine uptake from solution relies mainly on the active transport by nucleoside transporters present on the cell membrane to enter the cell and reach the target site (42) while gemcitabine loaded core-shell nanoparticles uptake is majorly contributed by the endocytic pathway as mentioned earlier. The enhanced cytotoxicity of gemcitabine loaded core-shell nanoparticles could also be related to its more rapid and enhanced uptake by the MG-63 cells.

CONCLUSIONS

In summary, core-shell nanoparticles having a hydrophilic core of BSA and hydrophobic shell of PLGA were developed as new carrier system for water-soluble drugs. The proof of concept of core-shell nanoparticles and their applicability for carrying the therapeutic payload was established by taking gemcitabine as a water soluble model drug. These results clearly indicate the effectiveness of the system in encapsulating gemcitabine with a desired particle size, drug release profile and an efficacy in cell culture model. The present core-shell nanoparticles have the potential to load other hydrophilic molecules and could be used as a promising platform for developing nanomedicines of hydrophilic molecules for wider applications in drug delivery.

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