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Enhanced cellular delivery of idarubicin by surface modification of propyl starch nanoparticles employing pteroic acid conjugated polyvinyl alcohol

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ABSTRACT

Enhanced intracellular internalization of the anti-cancer active idarubicin (IDA) was achieved through appropriate surface modification of IDA loaded propyl starch nanoparticles. This was conducted by synthesizing pteroic acid modified polyvinyl alcohol (ptPVA) and employing this stabilizer for formulating the said nanoparticles. Pteroic acid attached at the nanoparticles improved the surface protein adsorption of the nanoparticle, a condition which the nanoparticles would largely experience *in vitro* and *in vivo* and hence improve their cellular internalization.

Spherical, homogenous IDA nanoparticles (214 ± 5 nm) with surface modified by ptPVA were formulated using the solvent emulsification–diffusion technique. The encapsulation efficiency and drug loading amounted around 85%. *In vitro* release studies indicated a controlled release of IDA. Safety and efficacy of the nanoparticles was confirmed by suitable cellular cytotoxicity assays. Protein binding studies indicated a higher adsorption of the model protein on nanoparticles formulated with ptPVA as compared to PVA. Cellular uptake studies by confocal laser scanning microscopy revealed a higher cellular uptake of ptPVA stabilized nanoparticles thus confirming the proposed hypothesis of higher protein adsorption being responsible for higher cellular internalization.

1. Introduction

Polymeric nanoparticles (NPs), amongst the numerous other nanoparticulate systems currently being investigated, have been proven for their efficiency to deliver anti-cancer agents. This has been primarily attributed to their ability to transport large quantities of therapeutic molecules, prolonged circulation times, passive accumulation in tumour tissues and subsequent slow release of the drug (Kulratne and Low, 2010; Ma et al., 2009). Over the years, numerous polymeric materials including polyethylene glycol (PEG), proteins, polysaccharides, polylactic acids, poloxamines, and poloxamers have been investigated by researchers to impart desirable attributes like enhanced biocompatibility, biodegradability, cellular uptake and pharmacokinetics to the resulting NPs (Ma et al., 2009; Ringsdorf, 1975; Moghimi and Hunter, 2000; Park et al., 2005). Amongst the polysaccharides, NPs based on starch and its derivatives are receiving increasing importance due to the biocompatibility, biodegradability and non-toxicity of this polymer backbone. However its high hydrophilicity restricts its scope in this area thus inviting approaches like synthesis of hydrophobic starch derivatives to overcome this drawback (Santander-Ortega et al., 2010; Thiele et al., 2011). Furthermore such an association of hydrophobic counterparts with the hydrophilic copolymers allows efficient encapsulation of drugs and hence an enhancement in their payloads (Kulratne and Low, 2010). Our research group has previously demonstrated the effectiveness of this approach in delivering both hydrophilic and hydrophobic drugs employing NPs formulated with the propyl derivative of starch (Santander-Ortega et al., 2010). The current investigation also utilizes this starch derivative to encapsulate the anthracycline anti-cancer agent, idarubicin (IDA).

IDA, a synthetic analogue of daunorubicin, acts by intercalating between DNA base pairs and inhibiting topoisomerase II (Takimoto and Calvo, 2008; Minotti et al., 2004). Additionally, it induces free oxygen radicals leading to destruction of DNA and cell membrane (Minotti et al., 2004). IDA differs from daunorubicin due to its

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lack of methoxy group at position 4 of the D ring of the aglycone (Gubernator et al., 2010; Hande, 2008). This synthetic modification leads to high lipophilicity, better DNA binding, and greater cytotoxicity. IDA is currently combined with cytosine arabinoside as a first line treatment of acute myeloid leukemia (Çelik and Arinç, 2008). Also, IDA has been recognized as one of the best antiglioma agent amongst the anthacyclines (Boogerd et al., 1999). However despite its efficiency IDA possesses various adverse effects like myelosuppression, neutropenia, induction of secondary tumours and disease relapse due to multiple drug resistance (MDR). This necessitates its formulation into novel delivery vehicles like polymeric NPs to exploit its benefits in minimizing its systemic exposure and thus reducing non-specific toxicity, but retaining its efficacy at the tumour site (Ma et al., 2009).

In the present investigation IDA (base) loaded propyl starch NPs were formulated using the solvent emulsification–diffusion technique. Amongst the various emulsion stabilizers used polyvinyl alcohol (PVA) presents the most interesting option due to its ability to form small and homogenous emulsion droplets and hence similar NPs on subsequent solvent evaporation. However this stabilizer which forms an interconnected network at the polymer surface has been reported to reduce the cellular internalization of NPs (Sahoo et al., 2002). The aim of this investigation was to improve the cellular uptake of IDA loaded propyl starch NPs by employing pteroic acid conjugated polyvinyl alcohol (ptPVA) as the formulation stabilizer. This surfactant has been synthesized and utilized for improvising NP uptake for the very first time, to the best of our knowledge. This modification was hypothesized to promote an increased binding of the formulated NPs to serum proteins (Rothenberg et al., 1975). The principal proteins that have been reported to bind most strongly to polymeric NPs include albumin, immunoglobulins, complement, fibrinogen and apolipoproteins. The latter in turn have been reported to promote cellular uptake through formation of spontaneous protein corona resulting in biophysicochemical interactions at the cellular interface (Nel et al., 2009). This along with the passive accumulation of NPs in the tumour tissue due to their 'enhanced permeability retention effect' (Maeda, 2001) was hypothesized to improve their cellular uptake.

2. Materials and methods

2.1. Materials

Maize Starch polymer with an amylose content of 25 wt.% was kindly gifted by BASF SE, Ludwigshafen, Germany. Ethyl acetate (Fluka Chemie GmbH, Buchs, Switzerland), polyvinyl alcohol (PVA; molecular weight M_w = 31,000 g mol^{-1}, residual content of acetyl 12 mol%, Mowiol 4-88; Kuraray Specialties Europe GmbH, Frankfurt, Germany), cellulose membrane M_w 12–14 kDa (Medicell International Ltd., London, UK) were used as obtained from the suppliers. Idarubicin hydrochloride (IDA·HCl) was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). All other solvents and chemicals used were of the highest grade commercially available. Deionized water (Milli Q Plus system, Millipore, Bedford, MA, USA) was used throughout the investigation. The propyl starch used was synthesized as previously reported by our group (Santander-Ortega et al., 2010), but with a higher degree of substitution of DS = 2.0. *N*-(6-Aminohexyl)-pteroylamide was synthesized as recently described by Thiele et al. (2011).

2.2. Synthesis of pteroic acid modified polyvinyl alcohol

Modification of PVA was performed following the coupling procedure from Ossipov and Hilborn (2006). 2.0 g (45 mmol) of PVA was dissolved in 40 mL dimethylsulfoxide (DMSO) under nitrogen, further 3.65 g (22.5 mmol) 1,1′-carbonyldiimidazole (CDI) was added into this solution and stirred for 2 h at 25 °C. Afterwards 0.81 g (1.972 mmol) *N*-(6-aminohexyl)-pteroylamide was added into the reaction mixture and stirred for another 24 h. Then the reaction mixture was quenched unreacted by addition of 5.0 mL conc. NH_3. After removal of the solvent by vacuum distillation the residue was dissolved again in water, purified by ultrafiltration (Celgard membrane P20F, cut off 20 kDa), and then freeze dried to give a brown foam, 1.8 g (90%,). It has to be stored at 8 °C under exclusion of light. The degree of substitution of pteroate groups DS_{pt} was determined by UV spectroscopy (d = 1 cm. Lambda 2, Perkin Elmer, Germany) from solutions in 0.5 M HCl, taking an extinction coefficient ε = 6804 M^{-1} cm^{-1} at λ = 305 nm, determined for *N*-(6-aminohexyl)-pteroylamide in the same solvent. From the measured extinction E = 1.585 at λ = 305 nm for a polymer concentration of 1.0 mg mL^{-1} equivalent to c_{poly} = 22.7 mM, a DS_{pt} = 1.0% was determined according to equation 1. Other ptPVA samples with DS_{pt} ranging from 0.2% to 4% were synthesized accordingly.

$$DS_{pt} = \frac{c_{pt}}{c_{poly}} = \frac{E}{\varepsilon d c_{poly}} \quad (1)$$

2.3. Preparation of idarubicin NPs using the idarubicin base

Idarubicin NPs were formulated using the solvent emulsion diffusion method (Ravi Kumar et al., 2004) with slight modifications. 1 mL of a solution of IDA base in ethyl acetate (1.0 mg mL^{-1}) was mixed with 1 mL of a propyl starch solution in ethyl acetate (1.0 mg mL^{-1}) and thoroughly stirred at 5000 RPM using high speed homogenizer (Ultra Turrax® Ika®, Staufen, Germany). 5 mL of an aqueous solution of polyvinyl alcohol (PVA; 1 mg mL^{-1}) was added into this mixture at 14,500 RPM and emulsified for 10 min. Further, water was added into the mixture to promote diffusion of organic phase into the aqueous phase. The resulting NPs were stirred for 10 h at 25 °C to allow complete evaporation of the organic solvent. The final volume of the NP dispersion was adjusted by water to 10 mL, equivalent to a content of IDA of 0.1 mg mL^{-1}.

2.4. Formulation optimization

The drug loaded NPs were optimized with respect to various formulation parameters including IDA to propyl starch ratio, effect of various surfactants. Each parameter was varied one at a time maintaining the others constant. For each variation, the particle size and distribution of the NPs were considered as the end points for the selection of the optimum formulation. IDA to propyl starch ratios were altered ranging from 1:1 to 1:3 (w/w). Finally, the ratio of IDA to PVA was varied ranging from 1:1 to 1:10 (w/w) since this stabilizer resulted in the most optimum formulation.

2.5. Analytical method

IDA was analyzed by a validated reversed phase HPLC method. The method was modified from a previously reported procedure (Kuhlmann et al., 1999). Validation was carried out using HPLC apparatus consisting of Dionex Pump P680 (Idstein, Germany) equipped with a Hitachi Fluorescence detector L-2480 (Darmstadt, Germany), Dionex ASI-100 Automate Sample Injector (Idstein, Germany) and a Chromeleon Chromatography Data System (Version 6.8). The analyses were performed using LichroCART® 125-4 RP-18 column (4.6 mm × 250 mm, 5 μm from Merck, Germany). The mobile phase employed was water, acetonitrile, triethylamine, ortho-phosphoric acid (624:370:4:2, v/v), with the flow rate set at 1.0 mL min^{-1}. The column was maintained at 25 °C and the excitation/emission wavelengths used were 485 and 542 nm, respectively. The injection volume used was 10 μL. The mobile

phase was freshly prepared on the day of use, filtered through 0.45 μm filter (Pall Life Sciences, Germany) and degassed by degasser.

2.6. Size and zeta potential

Size and ζ-potential of the NPs were analyzed by photon correlation spectroscopy (PCS) using a Nano-ZS (Malvern Instruments, Malvern, UK).

2.7. ESEM and wet-STEM analysis

The morphological characteristics of the NPs were examined using a high resolution environmental scanning electron microscope (ESEM, FEI Quanta 400 FEG). For ESEM observations one drop of dilute NPs solution was placed on a small piece of Silicon wafer and dried in the SEM chamber. Images were recorded at room temperature with an accelerating voltage of 20 kV and a pressure $p = 100$ Pa water. Wet-STEM investigations were done with a 2 μL drop of dilute NPs solution placed on a carbon coated copper TEM grid at an accelerating voltage of 30 kV and a pressure $p = 800$ Pa ($T = 276$ K).

2.8. Encapsulation efficiency and drug content

The optimised NPs were freed from the not encapsulated drug by ultracentrifugation using Centrisart I® (MWCO: 300 kDa; Sartorius AG, Göttingen, Germany) at 3500 × *g* for 30 min (Universal 30RF, Hettich Centrifuges, Tuttlingen, Germany). Subsequently the content of not encapsulated IDA in the supernatant was analyzed by HPLC, as described earlier, after sufficient dilution with the mobile phase.

The encapsulation efficiency was then determined by the following equation:

$$\%EE = \frac{M_{\text{initial-drug}} - M_{\text{free-drug}}}{M_{\text{initial-drug}}} \times 100 \quad (2)$$

where "$M_{\text{initial-drug}}$" is the amount of initial drug used for the formulation and the "$M_{\text{free-drug}}$" is the amount of free drug detected in the supernatant after centrifugation.

For determining the drug content, the purified NPs obtained following the ultracentrifugation, as explained before, were completely dissolved in a mixture of DMSO, ethyl acetate and mobile phase (1:1:1; v/v) before injecting into HPLC. Both the encapsulation efficiency and the drug content studies were conducted in triplicates.

2.9. Drug release

The release of IDA from PSNPs was performed in phosphate buffer saline (PBS, 154 mM, pH 7.4) containing 1%(w/v) sodium lauryl sulphate (SLS) at 37 °C at 100 rpm in an orbital shaker. SLS was employed in the buffer saline not only to maintain a sink condition but also to provide solubility for IDA base in aqueous phase. In detail, NPs equivalent to 0.1 mg mL^{-1} of free drug was transferred into a QuixSep Micro Dialyzer (Roth, Karlsuhe, Germany) with a cellulose membrane M_w 12–14 kDa (Medicell International Ltd., London, UK) and immersed into a 100 mL polypropylene container containing 100 mL of PBS. At predetermined intervals, samples were collected. The IDA concentrations in the released samples were determined using HPLC.

2.10. Cell culture

A549 cells (Human lung adenocarcinoma epithelial cell line, CCL-185; ATCC, Manassas, VA, USA) were maintained as monolayer cultures in RPMI-1640 with L-glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% FBS (Gibco, Karlsuhe, Germany). Caco-2 cell lines were (ATCC No CRL-2102) cultured in DMEM supplemented with 1% non-essential amino acids (both from Gibco, Karlsruhe, Germany) and 10% FBS (Sigma–Aldrich, Taufkirchen, Germany). HT-29 cells (Human colon adenocarcinoma cell line) were cultivated in folate depleted medium to enable suitable expression of folate receptors. These cells were maintained as monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM) medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal bovine serum (Gibco, Karlsuhe, Germany) All cells were kept at 37 °C in a humidified atmosphere (5% CO_2).

2.10.1. MTT cytotoxicity assay

To evaluate cytotoxicity of NPs, MTT assay (Dandekar et al., 2009) was carried out using standard protocol and the details of method are described in Supporting information.

2.10.2. ATP (ViaLight® Plus) assay

The efficacy of the IDA NPs in comparison to IDA alone was investigated by determining the cell viability in Caco-2 cells using ViaLight® Plus Cell Proliferation and Cytotoxicity BioAssay Kit (Lonza, Wuppertal, Germany) based upon the bioluminescent measurement of ATP (Crouch et al., 1993). IDA NPs prepared using non-modified PVA and unloaded NPs were maintained as controls. The experiments were performed in white opaque walled 96-well plates (Corning, Costar, NY) as per the manufacturer's instructions. For the ATP assay, 20,000 Caco-2 cells per well were plated and treated with different concentrations of IDA and IDA NPs at equivalent concentrations (0.1–100 μM) for 24 h. Cell viability was calculated as per the following equation:

$$\%\text{cell-viability} = \frac{Abs_{\text{exp}} - Abs_{\text{low-control}}}{Abs_{\text{high-control}} - Abs_{\text{low-control}}} \times 100 \quad (3)$$

2.11. Protein binding studies

Protein binding studies were performed to understand the affinity of NPs for the protein. 1 mL of propyl starch NPs formulated using PVA and ptPVA were incubated with three different concentrations of bovine serum albumin (BSA; 200, 400, and 600 μg) for 6 h. Three different batches of each NP formulation were employed herein. Later, these NPs were centrifuged at 15,000 RPM for 1 h. The amount of protein present in the supernatant was quantified using BCA protein estimation kit (Sigma–Aldrich, Taufkirchen, Germany) as per the manufacturer's protocol.

2.12. Cellular uptake by confocal laser scanning microscope (CLSM)

A549 and HT-29 cells were seeded in 24 well plate 24 h prior to the experiment. A549 cells were seeded at a density of 10,000 cells per well. HT-29 cells were seeded at a density of 20,000 cells per well. All cells were kept under the conditions described in Section 2.10. For uptake experiments cells were treated with FITC-loaded propyl starch NPs. The method of preparation of FITC loaded NPs was performed as described before, with the modification of IDA being partly replaced by FITC (50 wt.%). The NP suspension was double diluted with Krebs Ringer Buffer (KRB) (pH 7.4). Cells were incubated with this medium for 6 h in the incubator. Afterwards, the incubation medium was replaced by normal cell culture medium without NPs. FITC-loaded propyl starch NPs prepared using non-modified PVA were used as a control. After 24 h of incubation, the cells were fixed and stained (Supporting information). The cells were stored in 500 μL/well PBS till further analysis at 4 °C.

The imaging was performed using confocal laser scanning microscopy CLSM (LSM 510; Zeiss, Jena, Germany) equipped with an argon/neon laser and a 63× water immersion objective. Zeiss LSM510 software was employed for all the measurements and analyses.

3. Results and discussion

Increased cellular uptake, retention and subsequent intervention of the cellular replication are the major factors governing the efficacy of anti-cancer agents. This holds special importance specifically for drugs like IDA where inherent pharmacokinetic properties accompanied by MDR effect often prevent sufficient drug concentrations at the action site (Duffy et al., 1996; Kessel et al., 1999). The present investigation was an attempt to address both of these facts through formulation of polymeric NPs of IDA with suitable surface properties to facilitate increased cellular uptake and drug efficiency and possible reduction in systemic exposure and non-specific toxicity.

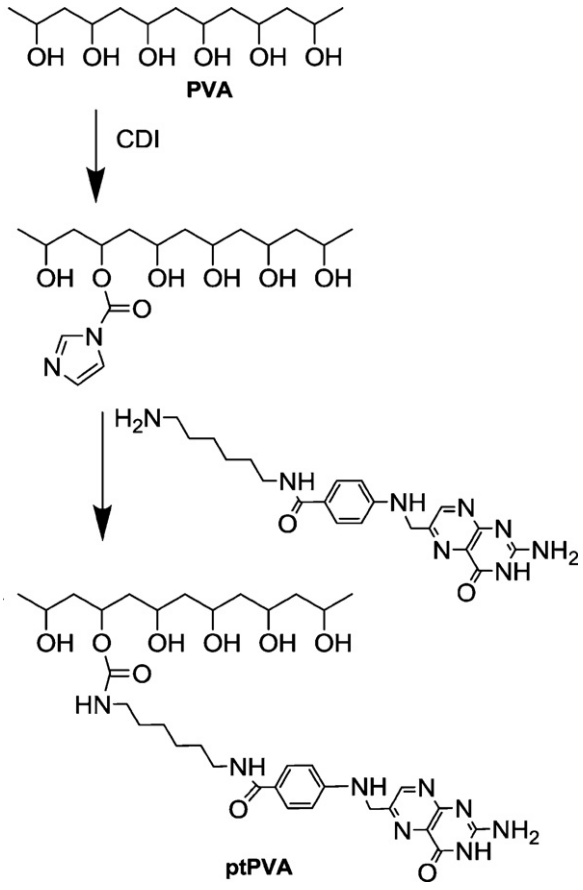
3.1. Synthesis of pteroic acid modified polyvinyl alcohol (ptPVA)

Modification of PVA with pteroic acid was carried out by a two step procedure. At first PVA was activated by reaction of its hydroxyl groups with 1,1′-carbonyldiimidazole (CDI) in DMSO solution leading to the *O*-(1-imidazolyl-carbonyl)derivative of PVA as described previously (Ossipov and Hilborn, 2006). Secondly, *N*-(6-aminohexyl)-pteroylamide was coupled to the activated PVA via a stable urethane linker. Pteroate moieties were chosen instead of folate groups, because mono-functionalization of pteroate with amino groups was easier to perform than the same functionalization of folate. (Thiele et al., 2011). The reaction scheme has been depicted in Scheme 1. Finally, residual *O*-(1-imidazolyl-carbonyl) groups were quenched to hydroxyl groups by reaction with aqueous ammonia. The degree of substitution DS_{pt} of pteroate groups linked to the PVA backbone within the final product was determined by UV spectroscopy ($\varepsilon = 6804\ M^{-1}\ cm^{-1}$ at $\lambda = 305$ nm) and was found to be $DS_{pt} = 1.0\%$. The DS of pteroate could be varied by the stoichiometry of *N*-(6-aminohexyl)-pteroylamide relative to PVA hydroxyl groups between 0.2% and 4 (Supporting information). The $DS_{pt} = 1.0\%$ was found to be the optimum, because solubility in water decreased significantly for higher DS_{pt} values. For lower values the effect on cellular uptake became less. This well controllable low degree of functionalization of the hydroxyl groups of PVA with its resultant chemical stability and structural homogeneity holds particular importance for biological applications (Ossipov and Hilborn, 2006; Jahann, 2009).

3.2. Preparation of IDA NPs

IDA hydrochloride was converted to the corresponding base (Supporting information), the extraction efficiency was calculated to 96.77 ± 3.33%. This conversion resulted in a more hydrophobic form of IDA as against the water soluble hydrochloride salt which was anticipated to increase its encapsulation within the hydrophobic starch derivative used in the current investigation. Our previous experience with other form of this polymer with a lower degree of propyl substituents has confirmed its ability to encapsulate higher amounts of hydrophobic drugs over the hydrophilic ones.

The choice of the method (solvent emulsification–diffusion method) of formulation of IDA loaded NPs was governed by its numerous benefits including high yield, encapsulation, reproducibility, scalability and control over the oil/water phase ratio and hence the particle size and distribution (Santander-Ortega et al., 2010).



Scheme 1. Scheme depicting synthesis of pteroylated PVA (ptPVA) by activation of PVA with 1,1′-carbonydimidazole (CDI) and subsequent reaction with *N*-(6-aminohexyl)-pteroylamide.

The stabilizer employed in the all the formulation and optimization studies was ptPVA, a derivative of PVA synthesized for the first time, to the best of our knowledge. The native PVA is one of the most widely used stabilizers for the formation of NPs by emulsification–diffusion–evaporation method due its ability to result in small and homogenous emulsion droplets. These features govern the size and distribution of the NPs formed on subsequent evaporation of the organic solvent from the emulsion. However the hydrophilic nature of PVA coupled with its ability to form an interfacial network, even after washing the NPs, may hinder their interaction with the hydrophobic cell membrane and hence uptake into the cells (Sahoo et al., 2002). One of the measures to overcome this possibility is to provide the stabilized NPs with conditions conducive for adsorbing higher amounts of proteins, a condition which the NPs would naturally encounter once inside the body. Numerous researchers have established the role of protein adsorption in improving the cellular association and consequent uptake and trafficking of the NPs. Furthermore studies have also proved that non-specific interactions between a wide variety of proteins usually encountered in the body dominate their attachment to the cell surface or that there exists no dominant receptor specific interaction in a complex environment like cell culture or blood. Researchers report diverse possibilities upon the attachment of protein on particle surface to strengthen this bond and prevent the protein displacement. The simplest one may be lateral diffusion as observed in case of albumin, a protein incapable of enabling secondary binding of another protein. The lateral mobility of proteins overcomes the factors that may cause its dissociation form NP surface and leads to stronger protein-NP interaction. Once stably adsorbed, the protein may in turn

guide either the endocytic uptake of the NP by means of specific receptor, if present on the cell surface, or by non-specific events like pinocytosis and phagocytosis, which are commonly observed in case of non-designed NPs present in nature. In either case this protein "corona" is thus responsible for a higher cellular association and uptake of the NPs (Nel et al., 2009; Lynch and Dawson, 2008; Aggarwal et al., 2009; Ehrenberg et al., 2009). In the present investigation modification of PVA with amino functionalized pteroic acid was used as the strategy to promote higher protein binding to the surface of the resulting NPs. The rationale behind this hypothesis was the experimental evidence of pteroic acid, the parent compound of folic acid, binding to serum and milk proteins (Rothenberg et al., 1975).

3.3. Optimization of IDA NPs

The morphological features of the final formulation were tuned through optimization of various process parameters to enable a good reproducibility and possibly bioavailability (Dandekar et al., 2009). Each parameter was evaluated one at a time, keeping the others constant. The selection of the optimum formulation was conducted on the basis of the particle size and distribution of the NPs.

Various propyl starch to ptPVA ratios ranging from 1:1 to 1:10 (w/w) were evaluated for their influence on the NPs as represented in Fig. 1A. As indicative from the figure, the lowest particle size and PI (218.5 ± 6.12 nm; 0.067 ± 0.04) were obtained at the propyl starch to ptPVA of 1:5. Further increase in ptPVA concentration led to a slight increase in particle size and distribution. This might be due to the increase in viscosity of the aqueous phase due to the higher amount of ptPVA used leading to a subsequent increase in the size of emulsion droplets and hence the particle size (Dandekar et al., 2009). ptPVA in this case forms a polymer network on particle interface shielding the charge on the particle surface and

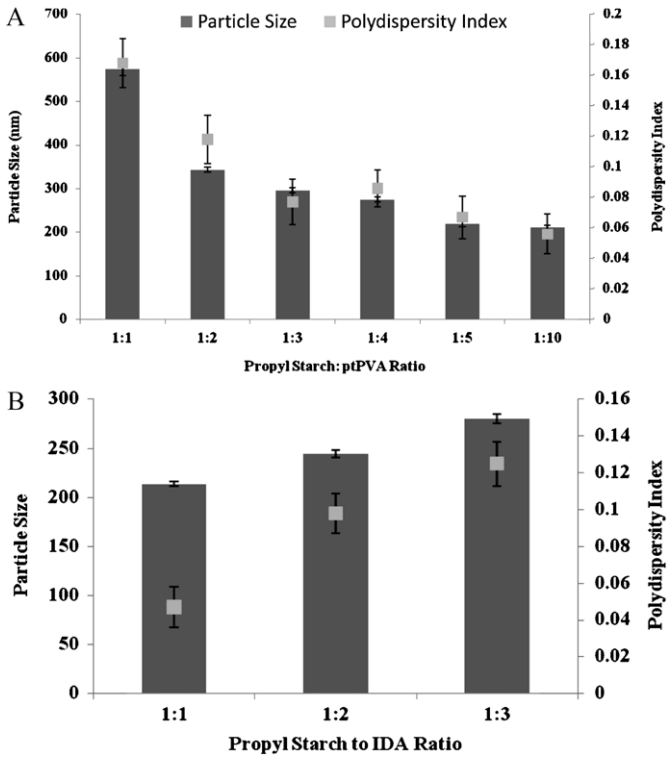


Fig. 1. (A) Optimization of nanoparticle formulation: propyl starch to ptPVA ratio and (B) optimization of nanoparticle formulation: propyl starch to IDA ratio ($n = 3$, mean ± S.D.; ratio, w/w).

stabilizing the emulsion by steric repulsion between the individual particles. This role of amphiphilic polymers like PVA and polyvinyl pyrrolidone in stabilizing the NP dispersions has been proved by researchers earlier (Dandekar et al., 2009).

The influence of propyl starch to IDA ratios ranging from 1:1 to 1:3 (w/w) on the characteristics of final formulation has been depicted in Fig. 1B. As seen from the figure, the lowest particle size and PI (213.6 ± 5.34 nm; 0.047 ± 0.038) were obtained at the ratio of 1:1. Further increase in the polymer concentration resulted in larger particles with a wider distribution which may be attributed to the excess polymer present in the NP dispersion. Hence these ratios were used for further studies.

3.4. Particle size, morphology, surface charge and stability of the IDA NPs

The optimized formulation exhibited a low particle size of 218.5 ± 6.12 nm and narrow distribution as indicated by a low PI value of 0.067 ± 0.04. This low size is of specific relevance for the delivery of agents like IDA as it provides the possibility of easy penetration across cellular barriers and retention at the site of action. This is likely to result in a more pronounced action of the drug within the tumour cells, sparing the unaffected cells of its hazardous effects (Dandekar et al., 2009). The surface charge of the optimized NPs was found to be -5.54 ± 0.88 mV. Though this absolute value is lower than that required resulting in stable NPs ($\sim \pm 30$ mV), the zeta potential does not play a major role in stabilization of the present system. In this case the NPs are stabilized by steric stabilization due to the inter-individual repulsion attributed to the presence of the formulation stabilizer (Dandekar et al., 2009). ESEM and WET-STEM images of the NPs have been depicted in Fig. 2. Particle size and morphology of the polymeric NPs are similar after drying (Fig. 2A) and in the liquid film (Supporting information). The contrast seen in ESEM images is much higher than in the WET-STEM images (Fig. 2B). This is due to the secondary electron contrast used in the ESEM which shows the edges of the polymeric NPs much sharper as the transmitted electrons detected with a solid state detector in STEM mode. Though ESEM (WET-STEM) does not offer advantage of a very good contrast the technique is still relevant especially for polymeric NPs since it is the only technique which offers visualization without physical distortment of the latter. This is important for polymeric NPs since they may show an alteration in their morphology upon complete evaporation of the liquid film. All the subdivisions of Fig. 2 depict spherical, uniform NPs with the homogenous distribution. This is likely to facilitate their easy passage through the tumour vasculature (Prabu et al., 2009). The NPs were also investigated for their stability at room temperature over a period of sixty days (Fig. 3A). Particle size and PI of the NP measured at the time points of 15, 30 and 60 days revealed no significant difference in these parameters indicating their stable nature over the time period tested. This is especially important as the NPs tend to be retained in tumour vasculature over extended time periods slowly releasing the encapsulated drug, a property of the polymer used in this study (Santander-Ortega et al., 2010).

3.5. Encapsulation efficiency and drug content

The encapsulation efficiency (EE) indicative of the ratio of the amount of drug actually incorporated in the NPs to the amount initially added to the formulation was determined by estimating the free drug in the dispersion medium. An encapsulation efficiency of $84.2 \pm 3.23\%$ was obtained which may be due to the low size of the particles resulting in drug loss by diffusion to the aqueous external phase (Feng et al., 2002). The drug content of the formulation as estimated after their complete solubilisation after separation from the not encapsulated drug was found to be $82 \pm 4\%$. These results

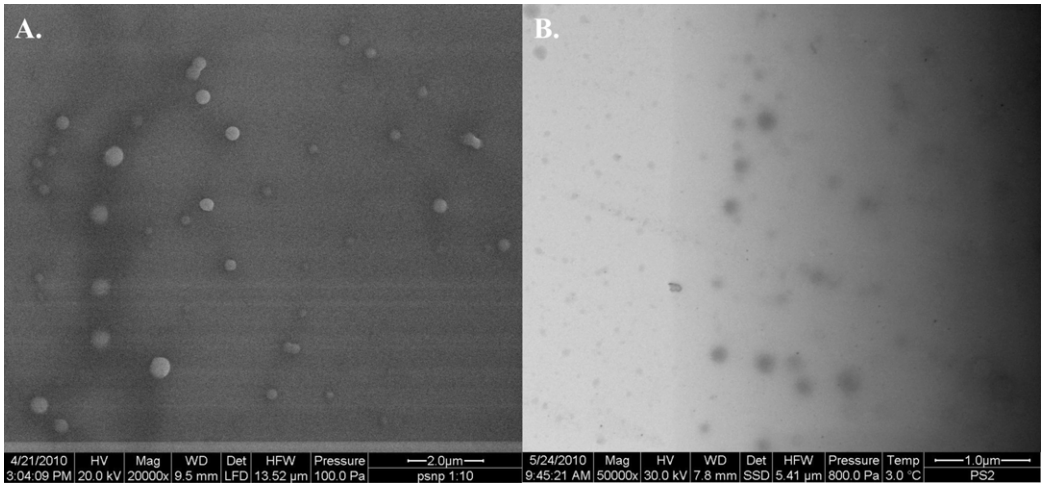


Fig. 2. (A) ESEM photomicrograph of the propyl starch NPs in natural dried and (B) wet-STEM photomicrograph of propyl starch NPs shows NPs in aqueous medium in the edge of the drop.

indicate an agreement between the values obtained by estimation of the unassociated drug in the external dispersion medium and the one associated with the NPs.

Also the encapsulation efficiency was evaluated at various IDA to propyl starch to ptPVA ratios as shown in Fig. 3B, where the highest encapsulation of IDA was observed at the ratio of 1:1:5 (w/w) indicating the suitability of the optimized formulation for encapsulating IDA.

3.6. Drug release and protein binding studies

The *in vitro* release profile of IDA loaded NPs was investigated at 37 °C in PBS with 1% SLS to provide sufficient sink conditions. Moreover the use of the surfactant in the dissolution medium prevents the adherence of the released drug to the apparatus facilitating a more accurate estimation on subsequent analysis. Thus appropriate measures were adopted to tackle the reported tendency of IDA to adhere to various experimental surfaces (Chawla and Amiji, 2002).

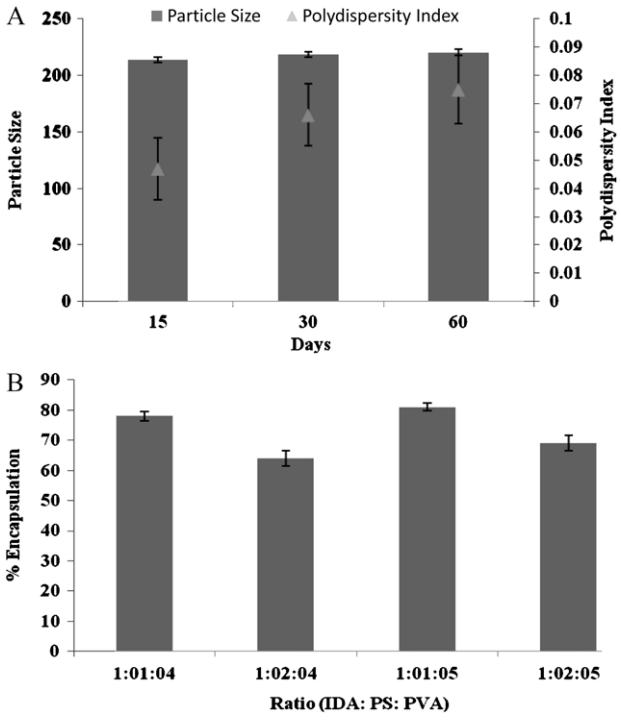


Fig. 3. (A) Stability studies of the developed propyl starch NPs in PBS (pH 7.4) (particle size = nm) and (B) encapsulation efficiency of the developed propyl starch NPs ($n = 3$, mean ± S.D.; ratio, w/w).

The cumulative percentage release has been depicted in Fig. 4. After the initial burst release for about 4 h, the NPs exhibited a further sustained drug release. IDA released in the first 3 h was equivalent to 31.6 ± 3.6% of the initial drug load of NPs. After 7 days, the amount of cumulative IDA release was 46.7 ± 3.2%. The burst release of IDA may be due to the dissolution and diffusion of the drug that was poorly entrapped in the polymer matrix, while the slower and continuous release may be attributed to the diffusion of the drug localized in the core of the NPs. Similar type of release profiles have been previously observed for other hydrophobic anti-cancer agents (Mu and Feng, 2003; Danhier et al., 2009).

The results of the protein binding studies have been depicted in Fig. 5. As can be seen, the propyl starch NPs formulated with native PVA adsorbed significantly lower amounts of the model protein (BSA) as compared with the NPs formulated with ptPVA. The ptPVA NPs adsorbed as high as 80% BSA as against around 40% observed with PVA NPs. This higher protein adsorption provides a possibility of increased cellular attachment to cellular surfaces and hence a possibility of improved internalization as described earlier in the manuscript. Other cellular investigations like cytotoxicity studies and uptake studies by CLSM were conducted to throw more light on these observations.

3.7. MTT assay and ATP assay

The MTT assay is based on the ability of the mitochondrial dehydrogenases of the living cells to reduce MTT, a yellow tetrazolium salt to purple formazan crystals. It is one of the most widely employed direct indicators of cellular death which depends on the compound toxicity. Such assays are especially important for nanoparticulate carriers due to the possibility of their long term cellular accumulation, retention and hence probable toxicity (Dandekar et al., 2009, 2010).

The results of the MTT assay of the unloaded NPs at various test concentrations have been represented in Supporting information. The results indicated that the unloaded NPs exhibited no significant toxicity at the highest concentration tested. Furthermore at lower concentrations the cells were almost completely viable, this being particularly relevant because of similar doses being tested in further cellular investigations. Thus the developed NPs were

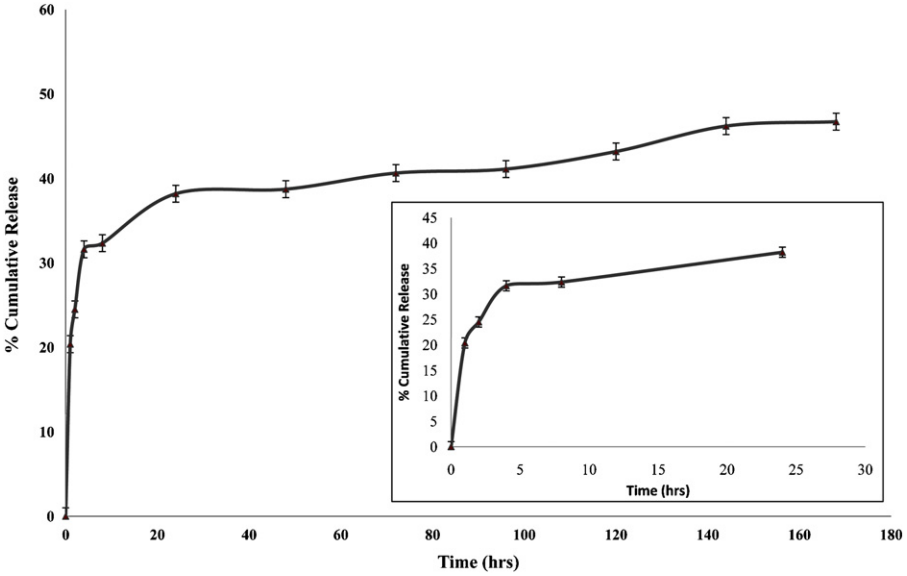


Fig. 4. *In vitro* release profile of IDA loaded ptPVA modified propyl starch NPs. The inset graph indicated release profile in first 24 h.

non-toxic to the cells at the concentrations evaluated and the studies indicated their potential for further cellular evaluations.

A comparison in the cellular cytotoxicity of free IDA and the one encapsulated within NPs, at equivalent concentrations, was considered as an indicator of the efficiency of the developed NPs. The cytotoxicity studies were conducted by ATP assay, a more precise method for determining the number of viable, metabolically active cells in a culture based on quantification of the ATP concentration since ATP is required for all cell functions. Any form of cell injury results in a rapid decrease in cytoplasmic ATP levels which can be detected utilizing the luciferase enzyme to catalyze the formation of light from ATP and luciferin. The procedure involves addition of an equal volume of reagent to the medium in test wells, which in a single step generates a luminescent signal proportional to the concentration of ATP present in cells. The reagent contains detergents to break the cell membrane, causing ATP release in the medium and ATPase inhibitors to stabilize the released ATP. The observed luminescence is considered proportional to the quantity of ATP in cells (Crouch et al., 1993). A comparison of the IC_{50} values (concentrations causing 50% reduction in cell viability) of the individual test compounds was used to estimate the efficiency of the NPs.

The percent cell inhibition at various test concentrations has been shown in Fig. 6. Calculation of IC_{50} values indicated that more amount of free IDA was required to inhibit 50% cells (46.44 ± 2.95 μM) as compared to the NPs (38.82 ± 3.80 μM) at the end of 24 h. This time point was chosen for its ability to give a clear indication of cell death and hence compound toxicity, the cells being in exponential growth phase at this time point. When the results were compared with the IC_{50} value of the NPs prepared with non-modified PVA, it was found that the value was higher than the free drug (48.92 ± 6.09 μM). At the same test concentrations the cells retained almost complete viability when incubated with the unloaded NPs.

The significant efficacy ($p < 0.05$) of the ptPVA NPs over free IDA may be attributed to the increased cellular uptake of the former due to higher protein binding resulting from ptPVA network on the NPs in turn resulting in higher intracellular drug concentrations. The influence of size on cellular uptake is yet another factor which may contribute to the nanoparticle superiority (Dandekar et al., 2009). The results partly confirmed our hypothesis of improved cellular uptake due to the employment of ptPVA in formulating the IDA NPs. However at the same test concentrations, the NPs prepared

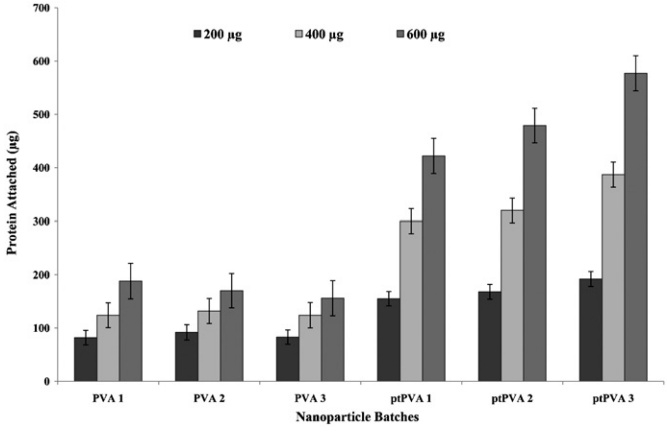


Fig. 5. Protein binding studies using BSA as a model protein with three different batches of propyl starch NPs formulated with PVA (PVA 1–3) and ptPVA (ptPVA 1–3).

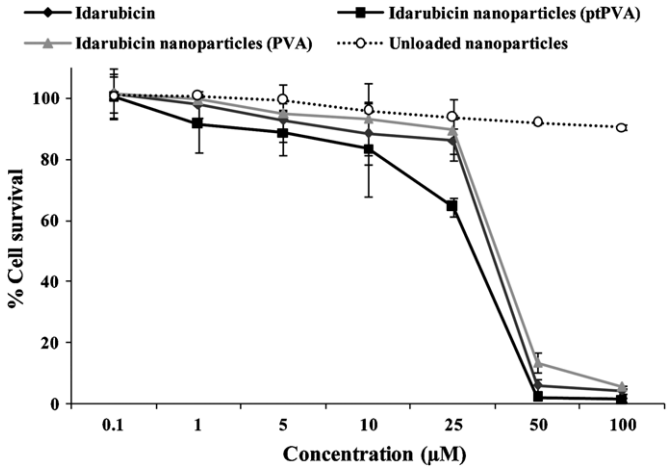


Fig. 6. Results of ATPase assay in Caco-2 cells. The results are expressed as mean values ± standard deviation of 3 measurements of two independent experiments.

using non-modified PVA were less effective ($p < 0.05$) than free IDA. This may be attributed to the fact that their cellular association was lower as compared to the ptPVA modified NPs, thus negatively affecting their uptake and efficacy. CLSM studies were further used to confirm this conjecture.

3.8. Cellular uptake studies by confocal laser scanning microscope (CLSM)

To further confirm our hypothesis and confirm our interpretation of cellular cytotoxicity, CLSM was performed to visualize the uptake of NPs formulated with ptPVA using the NPs made with non-modified PVA as the control. The latter had similar size and distribution to the former (data not shown).

Furthermore to study the possibility of receptor mediated endocytosis the studies were conducted in HT 29 cells cultivated with medium deficient in folic acid to facilitate expression of folate receptors (Pellis et al., 2008). A549 cells were used as a folate receptor negative control (Canal et al., 2010). Folate receptor was particularly selected for studying the possibility of receptor mediated uptake due the fact of pteroic acid being its direct precursor. Both the nanoparticulate systems were loaded with FITC to provide suitable visualization of fluorescent green particles in comparison to the red cell membranes and blue nuclei. The CLSM images have been shown in Fig. 7. NPs formulated with non-modified PVA did not exhibit any noticeable cellular uptake (Fig. 7C). This may be due to the hydrophilicity of PVA network providing insufficient interaction or displacement from the hydrophobic cell membrane (Sahoo et al., 2002). These results were in compliance with the results of the ATPase assay, wherein the NPs formulated using non-modified PVA exhibited a lower toxicity than the drug and ptPVA modified NPs due to their significantly lower uptake into the cells.

Another interesting fact observed was that there was no significant difference between the extent of ptPVA NPs taken up the HT 29 cells and A 549 cells. This indicates reduced possibility of cellular uptake by folate receptor mediated pathway or the possibility of numerous diverse mechanisms of uptake like micropinocytosis (Lynch and Dawson, 2008; Aggarwal et al., 2009; Ehrenberg et al., 2009). However, the studies once again supported our hypothesis of ptPVA being responsible for increased protein adsorption on the NP surface and hence an improved cellular internalization.

Though, the clearance of particles by the reticuloendothelial system (RES) is known as a major barrier for efficient particulate drug delivery system. Recent strategies to engineer long circulating particles are focused on surface modifications like grafting hydrophilic polymers for steric hindrance of opsonization with proteins like IgG, fibronectin, complement protein C3, which activated the phagocytosis. For interaction with the RES system the nature of bound protein is important. Proteins are classified in opsoninsand dysopsonins with regard to their effect on clearance by phagocytic cells. Serum albumin is described as non-adhesive protein (Meng et al., 2004), as mild opsonin (Vyas and Sihorkar, 2010) or even suggested as dysopsonin (Furumoto et al., 2007). A preferential binding of albumin is therefore an advantage as it can prevent binding of strong opsonins. We hypothesized a similar mechanism to prevent or cause negligible clearance of particles by RES system, thus allowing their efficient internalization by the cancer cells.

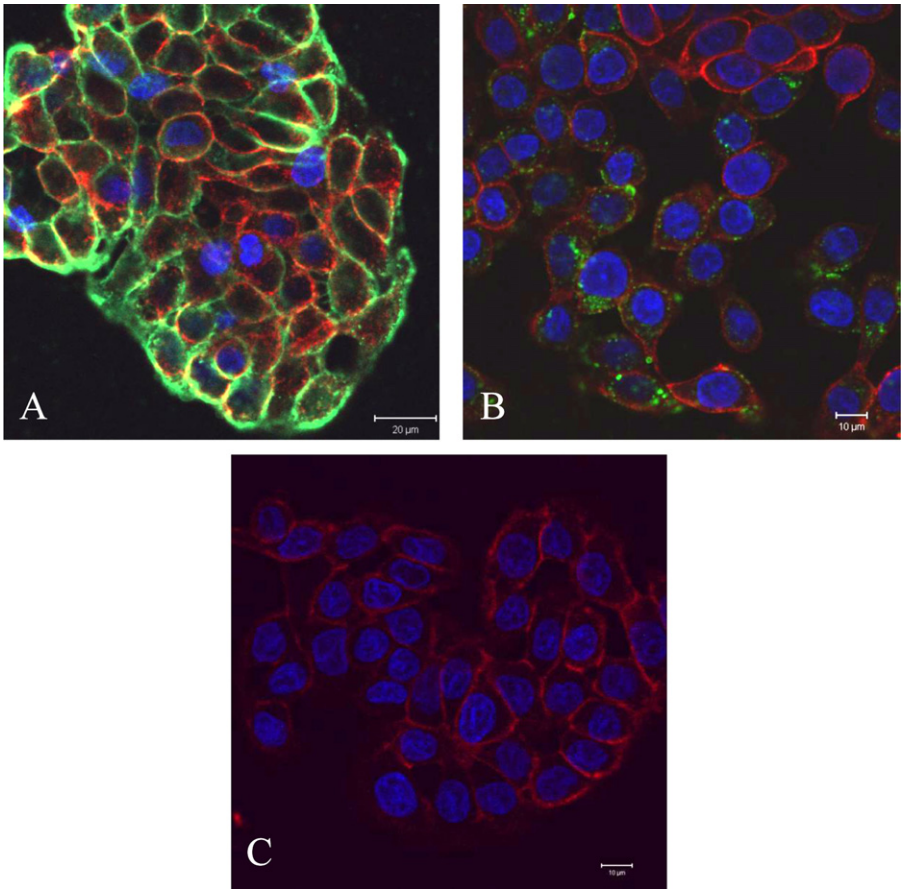


Fig. 7. HT-29 cell uptake studies of propyl starch NPs prepared with ptPVA. Cell membranes stained with RRCA indicated by red fluorescence, cell nuclei stained with DAPI indicated by blue fluorescence. NPs are attached to the cell membrane after 2 h (A) with progressively higher uptake at 4 h (B). No uptake was observed with propyl starch NPs formulated with non-modified PVA (C).

4. Conclusion

The present investigation confirms the possible role of protein adsorption being responsible for improved cellular internalization of NPs. This is particularly important since NPs encounter a plethora of protein materials inside the body. Such an improved uptake of NPs and the resulting improved efficiency strengthen the medical potential of such systems.

In the present study IDA NPs developed using ptPVA showed an improved efficiency over the parent drug. Also CLSM studies indicated an improved uptake of ptPVA NPs as compared to those formulated with native PVA. These investigations along with the observed experimental cellular safety indicate the potential of the NPs for further investigations in animal models to confirm the cellular evaluations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.08.030.

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