International Journal of Pharmaceutics 409 (2011) 111–120

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Enhanced cellular association of paclitaxel delivered in chitosan-PLGA particles

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ARTICLE INFO

Article history:
Received 20 October 2010
Received in revised form
30 December 2010
Accepted 21 February 2011
Available online 26 February 2011

Keywords:
PLGA
Chitosan
Paclitaxel
Nanoparticles
Microparticles

ABSTRACT

We have previously demonstrated that the cellular association, cytotoxicity, and *in vivo* anti-tumor efficacy of paclitaxel are significantly greater when delivered in PLGA microparticles compared to nanoparticles. The purpose of this research is to test the hypothesis that mucoadhesive chitosan promotes adhesion of PLGA particles to mucus on the tumor epithelium, resulting in enhanced cellular association and cytotoxicity of paclitaxel. PLGA particles containing paclitaxel or Bodipy® were prepared and chitosan was either adsorbed or chemically conjugated to the particle surface. The cellular association and cytotoxicity of paclitaxel in 4T1 cells was determined. A 4–10 fold increase in cellular association of paclitaxel was observed when chitosan was adsorbed or conjugated to the PLGA particles. Chitosan-conjugated PLGA microparticles were most cytotoxic with an IC_{50} value of 0.77 μM. Confocal microscopy demonstrated that chitosan-PLGA microparticles adhered to the surface of 4T1 cells. Pretreatment of either 4T1 cells or chitosan-PLGA particles with mucin resulted in significant increase in cellular association of paclitaxel. A linear correlation was established between theoretical amount of chitosan used and experimentally determined amount of chitosan adsorbed or conjugated to PLGA nanoparticles. In conclusion, cellular association and cytotoxicity of paclitaxel was significantly enhanced when delivered in chitosan-PLGA particles.

1. Introduction

Breast cancer accounts for about 26% of the newly diagnosed cases of cancer, and is expected to be the second most common cause of cancer-related death in women (Jemal et al., 2009). The discovery of anti-tumor property in paclitaxel represented an important milestone in the chemotherapy of breast cancer. This drug stabilizes microtubules by inhibiting their depolymerization, resulting in mitotic arrest. However, the two major challenges to successful chemotherapy with paclitaxel are drug resistance of tumors to paclitaxel and low aqueous solubility. The commercial products of paclitaxel include the Cremophor® EL-based formulation Taxol®, and the albumin-bound nanoparticulate suspension, Abraxane®. Numerous other approaches to improve the aqueous solubility of paclitaxel can be broadly classified as (a) use of co-solvents, (b) synthesis of pro-drugs, or (c) solubilization in hydrophobic components of drug delivery systems including liposomes, micelles, emulsions, topical pastes, niosomes, polymeric nano- and microparticles, and cyclodextrins.

Biodegradable and biocompatible polymers such as PLGA and PLA have been extensively investigated in drug delivery as nanoparticles, microparticles and in tissue engineering as scaffolds, sutures, and skeletal implants (Bala et al., 2004; Chakravarthi and Robinson, 2009). Previously we reported that when paclitaxel was delivered in PLGA microparticles, cellular accumulation and cytotoxicity of the drug significantly increased compared to nanoparticles in three mucus-secreting epithelial tumor cell lines (De et al., 2005). Confocal microscopy experiments revealed that, while nanoparticles were internalized, the PLGA microparticles predominantly adhered to the surface (De et al., 2005). These results were confirmed in a subsequent *in vivo* efficacy study using a mouse xenograft model of mammary carcinoma where it was shown that, when delivered in 1 μm and 10 μm microparticles, paclitaxel arrested tumor growth over 13 days (Chakravarthi et al., 2010). Conversely, a 1.5-fold increase in tumor volume was observed in mice treated with paclitaxel administered as nanoparticles of mean diameter 315 nm. The cellular association of paclitaxel delivered in PLGA particles increased 1.5 fold when either the 4T1 cells or the particles were coated with mucin (Chakravarthi et al., 2010). Based on these results, it was hypothesized that PLGA microparticles adhere to mucus on the cell membrane, and the extent of adhesion of PLGA particles to mucus can be further enhanced by modifying the

Abbreviations: PLGA, poly-[D,L-(lactide-co-glycolide)]; PVA, polyvinyl alcohol; MES, 2-(N-morpholino) ethanesulfonic acid; EDC, 1-(3-dimethyl aminopropyl)-3-3-ethylcarbodiimide hydrochloride; NHS, N-hydroxy succinimide; SEM, scanning electron microscopy; DMSO, dimethyl sulfoxide; PLA, poly-(lactic acid).

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doi:10.1016/j.ijpharm.2011.02.034

particle surface with a mucoadhesive polymer such as chitosan. Chitosan, (β-(1, 4) 2-amino-2-D-glucose), is a biodegradable, biocompatible, cationic polymer and has been extensively used as a mucoadhesive polymer in the formulation of several delivery systems including nano- and microparticles, hydrogels, fibers, and polyelectrolyte complexes (Chakravarthi and Robinson, 2008).

As documented in the examples below, the advantages of modifying the surface of PLGA particles with a mucoadhesive polymer, such as chitosan, may potentially include: (a) decreased burst effect of the encapsulated drug, (b) increased stability of macromolecules such as, proteins, (c) reversal of zeta potential promoting cellular adhesion and retention of the delivery system at the site, and (d) conjugation of targeting ligands to the free amine groups on chitosan. Coating PLGA particles with chitosan reduced the burst release of haloperidol from 70% to 36% (Budhian et al., 2008) and bovine serum albumin by 50% (Zheng et al., 2004, 2005). Yamamoto et al., modified the surface of PLGA nanoparticles with chitosan and PVA and after pulmonary administration, these positively charged particles were retained at the bronchial mucus tissue and significantly enhanced the delivery of calcitonin (Yamamoto et al., 2005). The nasal deposition of larger microparticles was significantly enhanced after coating their surface with chitosan resulting in a more efficient delivery of recombinant protein to the lungs (Zhao et al., 2006). Enhanced mucoadhesion of chitosan-coated PLGA nanoparticles to the intestinal mucosa was observed compared to unmodified particles (Kawashima et al., 2000). To demonstrate the potential for targeted delivery of chitosan-coated microparticles, biotin-labeled particles were prepared by conjugating biotin to the amine groups on chitosan and were targeted to fluorescent streptavidin (Fischer et al., 2006). Covalent grafting of chitosan to an immobilized PLGA surface also significantly enhanced the cellular attachment and hepatocyte proliferation demonstrating its use in tissue engineering (Wang et al., 2003).

Chitosan can be incorporated into or onto PLGA by one of three methods: (1) the use of an acidic aqueous solution of chitosan during the o/w emulsification step used to prepare PLGA particles/films; (2) coating the surface of the PLGA particles or films using an aqueous solution of chitosan; and, (3) chemical conjugation of chitosan to the PLGA particles or films using crosslinkers such as, carbodiimide. While each of these methods have been independently adopted for the surface modification of PLGA, our hypothesis that mucoadhesion of PLGA microparticles increased cellular association of paclitaxel delivered intratumorally has not been reported. Further, the effect of coating versus chemical conjugation of PLGA surface with chitosan on the cellular association of paclitaxel has not been investigated. This research aims to compare the effect of modifying PLGA particles by either physically adsorbing or chemically conjugating chitosan to their surface. Further, the cellular mechanism underlying the increased accumulation of paclitaxel delivered by chitosan-PLGA nano- and microparticles was investigated. Because of the difference in surface area of nanoparticles and the microparticles, the extent of adsorption/conjugation of chitosan to nanoparticles and microparticles was also determined to evaluate the strength of mucoadhesion.

The specific aims of this research were to: (1) prepare and characterize paclitaxel-PLGA nanoparticles and microparticles that have chitosan physically adsorbed and chemically conjugated to the surface, (2) compare the cellular association and cytotoxicity of paclitaxel in 4T1 cells when delivered in chitosan-adsorbed or chitosan-conjugated PLGA nanoparticles and microparticles, (3) visualize the surface deposition of the chitosan-PLGA nano- and microparticles using confocal microscopy, (4) quantify the cellular association of paclitaxel into 4T1 cells delivered in chitosan-PLGA particles, after coating either the 4T1 cells or the chitosan-PLGA particles with porcine mucin, (5) to establish a correlation between the initial amount of chitosan used in the preparation of the chitosan-PLGA particles and the experimentally determined amount of chitosan adsorbed or conjugated to the particles, and (6) determine the relationship between the concentration of chitosan used in the preparation of particles and their zeta potential.

2. Materials and methods

2.1. Materials

Poly-(D,L-lactide-co-glycolide) (PLGA 50:50, inherent viscosity: 0.63 dL/g) was purchased from Birmingham Polymers, Inc. Paclitaxel, PVA, chitosan, sodium hydroxide, sodium carbonate, 2-(N-morpholino) ethanesulfonic acid (MES), HEPES, ninhydrin reagent (2%, w/v solution) and porcine mucin were purchased from Sigma–Aldrich, St. Louis, MO. 1-(3-Dimethyl aminopropyl)-3-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxy succinimide (NHS) were purchased from Acros Organics, Morris Plains, NJ. The 4T1, murine mouse mammary carcinoma, cell line was purchased from ATCC, and the BCA® Protein Assay Kit was purchased from Pierce, Rockford, IL.

2.2. Methods

2.2.1. Preparation of chitosan solution

A 0.2% (w/v) solution of chitosan (p*Ka* = 6.3–7.0) was prepared by dissolving 0.1 mg of the biopolymer in 50 mL of 1% (v/v) aqueous acetic acid. This solution was used to both physically adsorb and chemically conjugate the polymer onto PLGA particles as subsequently described. To covalently conjugate chitosan to PLGA, the polymer was first precipitated from solution by increasing the pH to ~7.2 with 1.0 M sodium hydroxide and then the precipitate was sonicated using a Misonix® Probe Sonicator at a power setting of 5.0 for 1 min before being used for the chemical conjugation to PLGA.

2.2.2. Preparation of paclitaxel-containing PLGA particles

PLGA nanoparticles and microparticles were prepared by the conventional o/w emulsion-solvent evaporation method as described previously (Chakravarthi et al., 2010). Briefly, paclitaxel (2 mg) was dissolved in a polymeric solution of PLGA (90 mg) in dichloromethane (3 mL) and emulsified into 25 mL of an aqueous 1.5% (w/v) PVA solution. The size of the nanoparticles and microparticles was controlled by the energy of emulsification. The emulsions were stirred overnight to evaporate the dichloromethane to produce a suspension of nanoparticles or microparticles in the aqueous PVA solution. Prior to surface modification by chitosan, the particles were washed and recovered by the following processes. The nanoparticles were recovered by centrifugation at 22,000 rpm using Beckman Ultracentrifuge with a 50-2Ti rotor for 20 min at 4 °C, washed twice with carbonate buffer (100 mM, pH 9.0) and MES buffer (100 mM, pH 5.5), respectively. After each wash, the particles were recovered by centrifugation at 20,000 rpm for 20 min at 4 °C. After the second wash, the nanoparticles were suspended in MES buffer and mildly sonicated using a Misonix® Probe Sonicator at a power setting of 1.5 for 0.5 min to disperse the agglomerates. The microparticles were recovered using an identical procedure, except that they were centrifuged thrice at a constant speed of 25,000 rpm for 20 min at 4 °C followed by resuspension in the appropriate buffer as described above.

2.2.3. Adsorption of chitosan onto PLGA particles

Three milliliters of 0.2% (w/v) chitosan solution in 1% (w/v) aqueous acetic acid (pH ~3.7) was added to the suspension of PLGA nanoparticles in MES buffer. After stirring for 12 h, the chitosan-coated nanoparticles were recovered by centrifugation at 12,000 rpm for 20 min at 4 °C, washed twice with water before

recovering by centrifugation and then lyophilized. The supernatants from both the centrifugation steps were combined to determine the residual chitosan concentration. Chitosan-adsorbed microparticles were prepared using a similar protocol. Control nanoparticles and microparticles were subjected to identical processes except that 3 mL of MES buffer replaced the chitosan solution.

2.2.4. Chemical conjugation of chitosan onto PLGA particles

The carboxyl groups on the PLGA particles were activated by first preparing a 0.1 M solution of N-hydroxy succinimide (NHS) and 1-(3-dimethyl aminopropyl)-3-3-ethylcarbodiimide hydrochloride (EDC) by dissolving 0.0575 g of NHS and 0.095 g of EDC in 5 mL of HEPES/NaOH buffer (20 mM, pH 7.4). To the nanoparticle suspension in MES buffer, 2 mL of 0.1 M solution of NHS and EDC was added, stirred for 3 h at room temperature, and then centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was discarded and the nanoparticles washed in 5 mL of distilled water and finally resuspended in 5 mL of HEPES/NaOH buffer. The suspension was sonicated using a Misonix® Probe Sonicator at a power setting of 1.5 for 0.5 min. Three milliliters of chitosan suspension was added to the nanoparticle suspension, stirred for 12 h at room temperature, and the chitosan-conjugated nanoparticles recovered by centrifugation at 12,000 rpm for 20 min at 4 °C. After washing twice with distilled water, the particles were lyophilized and stored at 4 °C until used. The supernatants were collected and residual chitosan determined. The chitosan-conjugated microparticles were prepared by an identical protocol. Control nanoparticles and microparticles were prepared using an similar procedure except that the EDC/NHS and chitosan solutions were replaced with 2 mL of MES buffer and 3 mL of HEPES/NaOH buffer, respectively.

Bodipy®-loaded PLGA nanoparticles and microparticles were prepared by an identical procedure except that a 100 μL of a methanolic solution of Bodipy® was added into the organic phase prior to emulsification. Acetic-acid washed PLGA particles were prepared by stirring the chitosan-PLGA nanoparticles in 10 mL of 1% (v/v) aqueous acetic acid solution for about 20 min prior to lyophilization. These particles were recovered as described previously.

2.2.5. Confocal microscopy of chitosan-PLGA particles containing Bodipy®

One milliliter of 4T1 cells (75,000 cells/mL) were seeded onto Bioptech® plates and incubated at 37 °C with 5% CO_2. After achieving confluency in 16 h, the media was replaced with fresh media containing 250 μg of chitosan-PLGA nanoparticles and microparticles containing Bodipy® then incubated at 5% (v/v) CO_2 at 37 °C for 90 min. The media was then discarded and the cells washed thrice with ice-cold PBS. One milliliter of fresh 4T1 cell media was added and the surface deposition of Bodipy® (λ_{ex} = 495 nm and λ_{em} = 511 nm) observed by monitoring the fluorescence with a confocal microscope using a filter of 488 nm and cut-off filter at 515 nm.

2.2.6. Effect of pre-treatment of 4T1 cells and PLGA-chitosan particles with porcine mucin on their in vitro cellular association

Two methods were used to study the effect of mucus on the cellular association of paclitaxel. The first method involved precoating the 4T1 cells with mucus whereas in the second method, PLGA nanoparticles and microparticles were coated with mucus. The cellular association of the particles onto 4T1 cells was quantified using both methods. Uncoated 4T1 cells and PLGA particles were used as controls.

2.2.7. Effect of the initial concentration of chitosan on the amount of polymer adsorbed or conjugated onto PLGA nanoparticles

Solutions of chitosan in 1% (v/v) aqueous acetic acid were prepared in concentrations of 0.2, 0.5, 0.75, and 1% (w/v). Three milliliters of each chitosan solution was used to adsorb to or conjugate the surface of PLGA nanoparticles as described previously. After physical adsorption or chemical conjugation of chitosan to the PLGA nanoparticles, the supernatants were separately collected and the chitosan content quantified using ninhydrin. The chitosan-PLGA particles were lyophilized and their zeta potential determined.

2.2.8. Characterization of delivery systems

The control as well as chitosan-PLGA nanoparticles and microparticles were coated with chromium at 95 mA and visualized under the scanning electron microscope (Hitachi, S4700 Field-Emission SEM). The number average particle size was determined from a selected 5 cm × 5 cm field SEM image. The zeta potentials were determined using dynamic light scattering after suspending 5–10 mg of the control and chitosan-PLGA nanoparticles and microparticles in water with mild sonication using Misonix® Probe Sonicator at a power setting of 0.1 for 0.5 min. The zeta potential was reported as the average of six readings over two cycles using the Zeta Plus Particle Size Analyzer (Brookhaven Instrument Corporation, Huntsville, NY).

The drug content and loading efficiency were determined in triplicate using the HPLC assay described below. To extract the drug, accurately known weights (~2 mg) of the particles were dissolved in 1 mL of dichloromethane and 0.5 mL of acetonitrile, vortexed, then agitated at 37 °C for 30 min to ensure dissolution of polymer. The solvent was evaporated by flushing with nitrogen (20 psi). Acetonitrile (5 mL) was added to the extract and agitated overnight in a reciprocal shaking bath at 37 °C and 50 rpm to dissolve paclitaxel. The drug in the acetonitrile was quantified by reverse-phase HPLC fitted with a C-18 column (Curosil® PFP) using a mobile phase of 60:40 (v/v) of acetonitrile and water at a flow rate of 1 mL/min. A calibration curve spanning a paclitaxel concentration range of 2.5–12.5 μg/mL was used to determine the drug content.

2.2.9. Drug release from chitosan-PLGA particles

Nanoparticles or microparticles, containing the equivalent of 25 μg of paclitaxel, were suspended in 25 mL of 0.1% (v/v) aqueous Tween® 80 solution and sonicated with a Misonix® Probe Sonicator at a power setting of 0.5 for 20 s. One milliliter aliquots of each of the particulate suspensions was transferred to microcentrifuge tubes, sealed, and incubated at 37 °C in a reciprocating shaking bath. At predetermined time intervals, triplicate samples of the control particles, chitosan-PLGA nanoparticles or chitosan-PLGA microparticles were centrifuged at 10,000 rpm for 30 min. The supernatant was separated, lyophilized and the amount of paclitaxel in the supernatant determined by HPLC as described.

2.2.10. Determination of the amount of chitosan adsorbed or conjugated to the PLGA particles

Ninhydrin reagent (triketohydrindane hydrate) has been commonly used to detect primary amines and to determine the degree of deacetylation of chitosan (Khan et al., 2002). The amount of chitosan adsorbed or conjugated to PLGA particles was calculated by spectroscopically quantifying the amount of chitosan remaining in the supernatant collected after preparation of the particles. One milliliter of each of the supernatants, along with 1 mL of standard solutions of chitosan at concentrations of 0.5, 1, 1.5, and 2 mg/mL were individually transferred into test tubes, mixed with 0.5 mL of ninhydrin reagent, and then boiled for 10 min on a water bath. After

appropriate dilution, 100 μL samples of the standards and supernatants were transferred into a 96-well plate and the absorbance recorded on a microplate reader at λ_{max} of 550 nm. The concentration of chitosan in the supernatants extrapolated from a standard plot.

2.2.11. FTIR spectra of chitosan-PLGA particles to confirm chemical conjugation of chitosan to PLGA

The control PLGA and chitosan-PLGA particles were spread onto a glass slide and placed on the stage of IlluminatIR® Infrared microspectroscope (Smiths Detection, Boston, MA). The IR spectra of the samples were obtained using the reflection absorption spectroscopy technique involving the use of an ARO (all reflective objective) lens while simultaneously viewing under a 10× eyepiece. A total of 32 scans were coadded for each sample at a resolution of 4 cm^{-1} and the spectra recorded from 500 to 4000 cm^{-1}. The spectral data was collected using the SynchronizIR® software and the numerical values transferred to Microsoft Excel® software for graphical representation. The IR fingerprint region of the amide bond was obtained from the literature and compared with the IR spectrum of chitosan-conjugated particles.

2.2.12. Cellular association of control PLGA and chitosan-PLGA nano- and microparticles onto 4T1 cells

Murine, metastatic breast carcinoma (4T1) cells were used to compare the cellular concentration of paclitaxel following delivery in chitosan-PLGA nanoparticles or microparticles as described elsewhere (Chakravarthi et al., 2010).

2.2.13. Cytotoxicity of control and chitosan-PLGA particles in 4T1 cells

A suspension of control PLGA or chitosan-PLGA particles containing 0.064 mM paclitaxel was prepared in Iscove's Modified Dulbecco's media. This suspension was diluted with media to prepare 1 mL suspensions containing the following concentrations of paclitaxel: 32, 10, 6.4, 3.2, 1.0, 0.64, 0.32, 1, 0.064, 0.032, and 0.1 μM. Each stock suspension was mildly sonicated before dilution to ensure that the particles were homogenously dispersed. Identical concentrations of paclitaxel solutions in media containing 0.1% (v/v) dimethyl sulfoxide (DMSO) were prepared as positive controls. The 4T1 cells were seeded into 96-well plates at 5000 cells/well and incubated for 16 h at 37 °C in an atmosphere of 5% CO_2 to obtain approximately 20% confluence. The media was aspirated and replaced with 100 μL of fresh particle suspension in media/well of control PLGA and chitosan-PLGA particles containing varying concentrations of paclitaxel. Untreated 4T1 cells and those treated with paclitaxel solution served as negative and positive controls, respectively. After 8-h incubation at 37 °C and 5% CO_2, the media containing the PLGA or chitosan-PLGA particles were aspirated and the cells washed twice with 100 μL/well of fresh media. Finally, 200 μL of fresh culture medium was added to the each well and incubated for 72 h at 37 °C and 5% CO_2.

The cell viability was determined by an MTT assay. Briefly, 4T1 cells were incubated with 100 μL/well of culture media and 25 μL/well of MTT reagent and after 2 h, 100 μL/well of a co-solvent (20%, w/v sodium dodecyl sulfate in 1:1 dimethyl formamide:water, pH 4.7) was added and then incubated for 8 h at 37 °C and 5% CO_2. The optical density was quantified using a microplate reader at 550 nm. The absorbance of untreated 4T1 cells was considered 100% viability and the viability of the treatment groups calculated as a percentage of the optical density of untreated cells. The IC_{50} values were obtained by non-linear regression curve-fitting of the cell viability data using Microcal™ Origin (Microcal Software, Northampton, MA).

Table 1
Particle size and zeta potential of control and chitosan-PLGA particles.

Formulation	Particle size (mean ± std.dev.)	Zeta potential (mV)
Control nanoparticles	390 ± 0.1 nm	−28.5
Chitosan-adsorbed nanoparticles	386 ± 20 nm	−9.2
Chitosan-conjugated nanoparticles	357 ± 12 nm	−6.6
Control microparticles	1.80 ± 0.5 μm	−23.6
Chitosan-adsorbed microparticles	1.9 ± 0.2 μm	−12.0
Chitosan-conjugated microparticles	2.2 ± 0.4 μm	−14.9

3. Results

3.1. Particle size and zeta potential of PLGA and chitosan-PLGA particles

The average diameters of the PLGA nanoparticles and microparticles used for these studies were 390 nm and 1.8 μm, respectively (Table 1). The particle size of the nanoparticles and microparticles after adsorption or conjugation of chitosan did not significantly increase although the polydispersity significantly increased. The zeta potential of the control PLGA nanoparticles and microparticles was negative and following adsorption or conjugation of chitosan, the zeta potential was less negative although not reversed at the concentration of chitosan used.

3.2. Amide bond detection by Fourier-transform infrared spectroscopy (FTIR)

The covalent conjugation of chitosan to PLGA via the formation of the amide bond was confirmed by observing the weak band at 1642 cm^{-1} and a strong band at 1575 cm^{-1} corresponding to the Amide I and Amide II bands, respectively (Fig. 1). These Amide I and II bands were not observed when chitosan was adsorbed to the particles. The bands at 1153, 1071, and 1029 cm^{-1} in both chitosan-adsorbed and chitosan-conjugated particles confirm the saccharide structure of chitosan (Wan et al., 2006).

3.3. Efficiency of the adsorption or conjugation of chitosan to PLGA particles

The ninhydrin method is a rapid, sensitive, precise, reproducible, and inexpensive technique for quantification of chitosan (Curotto and Aros, 1993). Standard plots generated by colorimetric determination of chitosan in the concentration range 0–60 μg/mL were linear with a regression coefficient of 0.999. The percentage of chitosan physically adsorbed or chemically conjugated to the par-

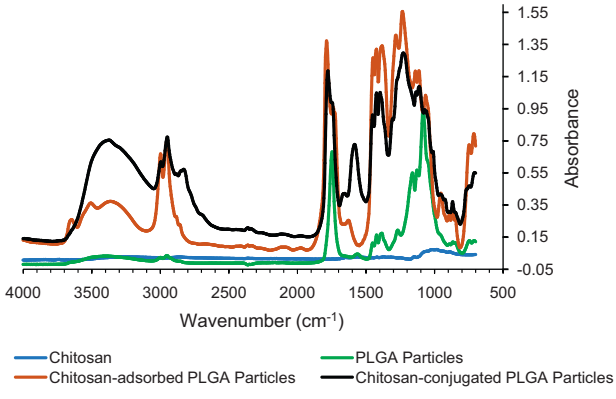

Fig. 1. FTIR spectrum of control and chitosan-PLGA particles (peak at 1600 cm^{-1} represents Amide II band. NP: nanoparticles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

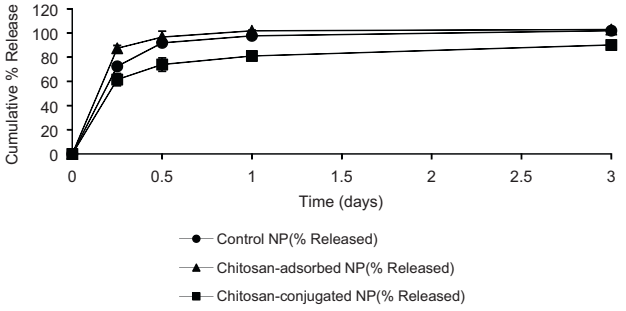

Fig. 2. Dissolution of paclitaxel from chitosan-PLGA nanoparticles in 0.1% (v/v) aqueous Tween® 80. NP: nanoparticles ($n = 3$).

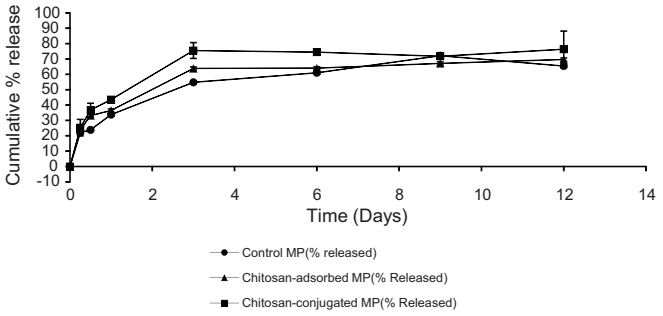

Fig. 3. Dissolution of paclitaxel from chitosan-PLGA microparticles in 0.1% (v/v) aqueous Tween® 80. MP: microparticles ($n = 3$).

ticles was between 3.3 and 6.6% (w/w) (wt.% of chitosan relative to the weight of PLGA particle). While the adsorption efficiency was 80–81%, the conjugation efficiency was significantly greater at 98% confirming that carbodiimide mediates a strong chemical reaction between the carboxyl groups of PLGA and amine groups of chitosan, forming an amide bond.

3.4. Release of paclitaxel from chitosan-PLGA particles

A significant burst effect was observed in nanoparticles with about 77% of the drug released from the control nanoparticles within 6 h (Fig. 2). After the surface had been modified with chitosan, 87% and 61% of the drug released in 6 h from chitosan-adsorbed and chitosan-conjugated PLGA nanoparticles, respectively. During a three day incubation period, complete release of paclitaxel was observed from both the control and chitosan-adsorbed nanoparticles, whereas only 80% of the drug was released from chitosan-conjugated nanoparticles. A linear correlation ($R^2 = 0.91$) was obtained when the percentage of paclitaxel released was plotted against the square root of time indicating that the mechanism of release was by diffusion. As expected, the burst release of paclitaxel from microparticles was significantly lower than for the nanoparticles with only 22–25% of the drug released from the control and chitosan-PLGA microparticles after 6 h. Modification of the surface of PLGA microparticles with chitosan did not reduce the burst release of paclitaxel relative to uncoated control microparticles (Fig. 3). After 12 days, 65%, 69%, and 75% of the drug was released from control, chitosan-adsorbed and chitosan-conjugated microparticles, respectively (Fig. 3). The release of paclitaxel from the PLGA or chitosan-PLGA microparticles did not fit either first or zero-order kinetics.

3.5. Cellular association of paclitaxel delivered in control and chitosan-PLGA nanoparticles and microparticles

The cellular association of paclitaxel with 4T1 cells when delivered in PLGA and chitosan-PLGA nanoparticles and microparticles is illustrated in Fig. 4 and quantified in Table 2 from which the following conclusions were made. (1) Compared to control PLGA nanoparticles, the cellular association of paclitaxel increased between 4.5 and 10.3 times when chitosan was adsorbed or conjugated respectively to the surface of the PLGA nanoparticles; (2) a 4.1 and 5.9 fold increase in cellular association of paclitaxel was observed when delivered from chitosan-adsorbed or chitosan-conjugated PLGA microparticles compared to control PLGA microparticles; (3) after chitosan was adsorbed or conjugated to PLGA microparticles, there was a 2.5 and 1.4 fold increase in cellular association of paclitaxel compared to nanoparticles modified with chitosan, confirming that there is a size-dependent increase in cellular association of paclitaxel. To test whether the observed increase in cellular association and cytotoxicity of chitosan-PLGA particles was due to free or unconjugated chitosan, chitosan-PLGA nanoparticles were rinsed with 1% (v/v) acetic acid solution to dissolve the unadsorbed or unconjugated chitosan. The cellular association of chitosan-PLGA nanoparticles was unaffected by washing the particles with 1% (v/v) acetic acid solution indicating that: (a) the observed increase in cellular association and cytotoxicity is not as a result of unadsorbed or unconjugated chitosan, and (b) adsorbed or conjugated chitosan was not dissolved and dislodged from the particle surface using this acid treatment.

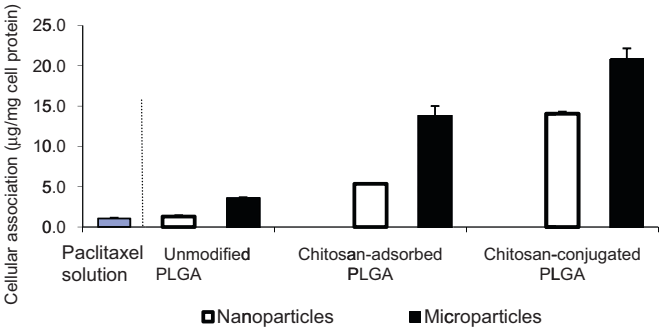

Fig. 4. Cellular association of paclitaxel into 4T1 cells delivered as (a) solution in 0.1% (v/v) DMSO, (b) control, (c) chitosan-adsorbed PLGA, and (d) chitosan-conjugated PLGA nano- and microparticles ($n = 3$).

Table 2
Relative increase in cellular association of paclitaxel into 4T1 cells compared to paclitaxel solution, control PLGA nanoparticles and microparticles.

Particle type	Increase in cellular association of paclitaxel		
	Relative to paclitaxel solution	Relative to control PLGA particles	Relative to PLGA nanoparticles
Control nanoparticles	1.3	1.0	–
Chitosan-adsorbed nanoparticles	5.1	4.5	–
Chitosan-conjugated nanoparticles	13.4	10.3	–
Control microparticles	3.4	1.0	2.6
Chitosan-adsorbed microparticles	16.9	4.1	2.5
Chitosan-conjugated microparticles	19.8	5.9	1.4

Table 3
Inhibitory concentrations (IC_{50}) of paclitaxel solution, control PLGA and chitosan-modified PLGA nanoparticles and microparticles.

Type of delivery system	IC_{50} (μM)	Factor change in cytotoxicity of paclitaxel	
		Compared to paclitaxel solution	Compared to control PLGA particles
Paclitaxel solution	7.34	1	–
PLGA nanoparticles	3.49	2.1	1
Chitosan-adsorbed PLGA nanoparticles	3.85	1.9	1.1
Chitosan-conjugated PLGA nanoparticles	1.40	5.2	2.5
Control PLGA microparticles	2.86	2.5	1
Chitosan-adsorbed PLGA microparticles	1.50	4.8	2
Chitosan-conjugated PLGA microparticles	0.77	9.5	3.7

3.6. Cytotoxicity of chitosan-PLGA particles

The cytotoxicity of control PLGA, chitosan-adsorbed PLGA, and chitosan-conjugated PLGA nanoparticles and microparticles is shown in Figs. 5 and 6 and summarized in Table 3. The lowest cytotoxicity or highest IC_{50} was obtained for paclitaxel in solution. The IC_{50} value of paclitaxel delivered in the control PLGA nanoparticles was 3.4 μM, which is a 2.1 fold increase in the cytotoxicity of paclitaxel compared to the solution. These studies demonstrated that the adsorption of chitosan on the surface of PLGA nanoparticles did not significantly increase the cytotoxicity of paclitaxel compared to the control, uncoated PLGA nanoparticles. Importantly, the conjugation of chitosan to PLGA nanoparticles decreased the IC_{50} 2.5 fold compared to control nanoparticles again showing that the mucoadhesive polymer promotes accumulation of drug into or onto the cells (Table 3).

These data also show that the control PLGA microparticles lowered the IC_{50} values of paclitaxel 1.3 and 2.5 fold compared to the control PLGA nanoparticles and paclitaxel solution, respectively. These findings reaffirm that paclitaxel was significantly more cytotoxic when delivered in larger microparticles compared to the smaller nanoparticles or as a solution. Adsorption of chitosan to the surface of PLGA microparticles further lowered the IC_{50} of paclitaxel 1.9 fold compared to the control PLGA microparticles. Paclitaxel was most cytotoxic (IC_{50} = 0.77 μM) when delivered in PLGA microparticles that had chitosan chemically conjugated to their surface.

3.7. Visualization of PLGA and chitosan-PLGA particles containing Bodipy® using confocal microscopy

Fig. 7 illustrates that, while chitosan-modified nanoparticles adhered to the cell surface, control PLGA nanoparticles did not. Aggregates of chitosan-PLGA microparticles were also observed to adhere to the cell surface and could not be removed with repetitive washings. Although the larger microparticles were not internalized, it is proposed that adhesion of these particles to the cell surface increased the local concentration of paclitaxel and enhanced cellular association of the drug by passive diffusion resulting in greater cytotoxicity of the larger particles.

3.8. Effect of pre-treatment of 4T1 cells and PLGA particles with mucus

Fig. 8 illustrates that pre-treatment of 4T1 cells with mucus significantly increased the cellular association of chitosan-modified nanoparticles. Specifically, when either the 4T1 cells, or the chitosan-adsorbed PLGA nanoparticles were pre-treated with mucus, the cellular association of paclitaxel increased 1.8 or 2 fold, respectively. Similarly, pre-treatment of the 4T1 cells or chitosan-conjugated PLGA nanoparticles with mucus increased the cellular association of drug 1.6-fold (Table 4). Pretreatment of 4T1 cells or chitosan-adsorbed PLGA microparticles increased the cellular association 1.2 and 1.4 times, respectively (Fig. 9 and Table 5). However, chitosan-conjugated microparticles did not significantly increase

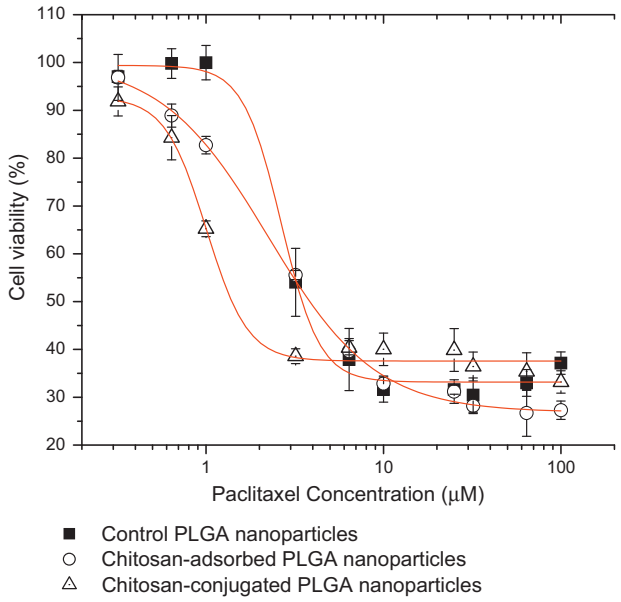

Fig. 5. Cytotoxicity of paclitaxel delivered in PLGA and chitosan-PLGA nanoparticles (n = 3).

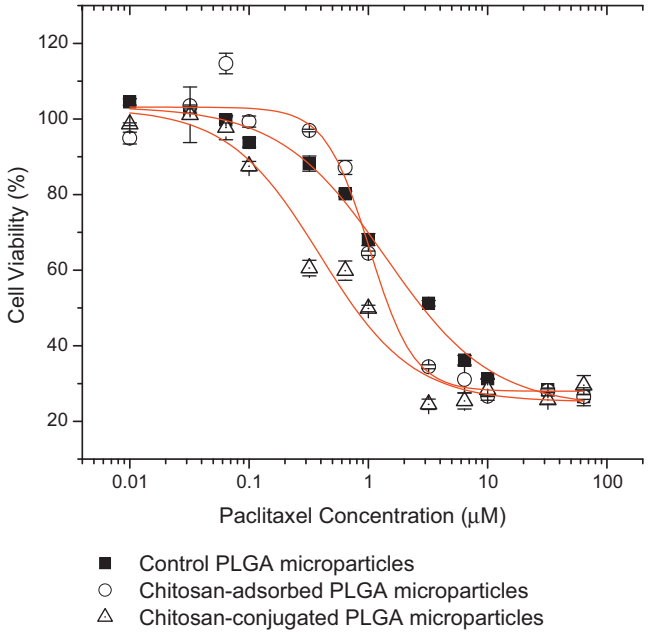

Fig. 6. Cytotoxicity of paclitaxel delivered in PLGA and chitosan-PLGA microparticles (n = 3).

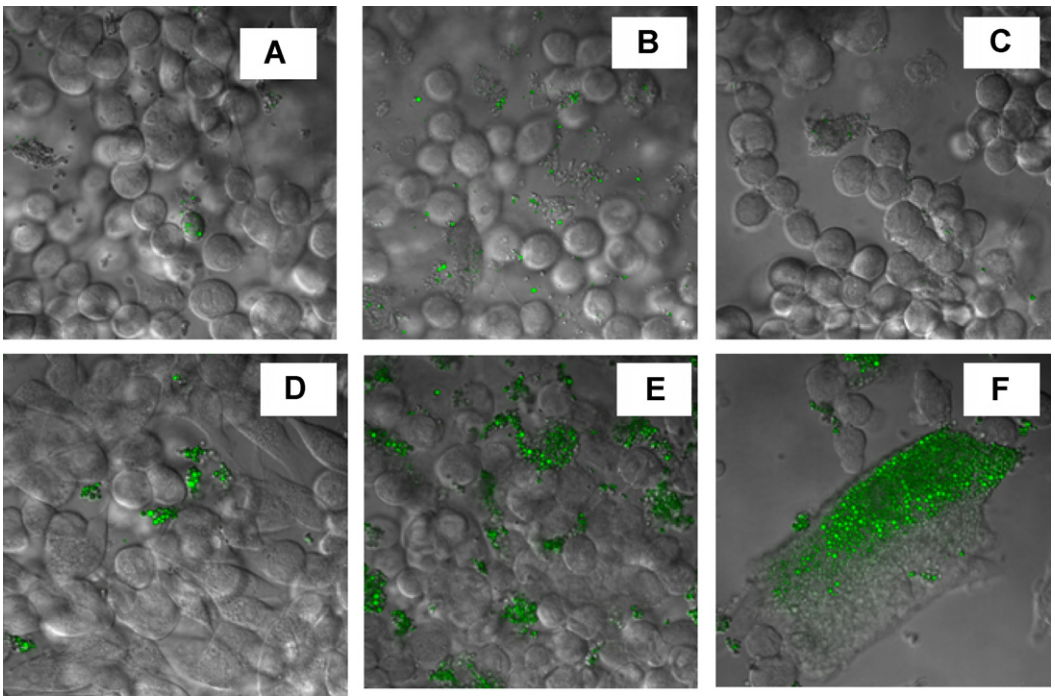

Fig. 7. Confocal photomicrographs illustrating the surface deposition of control PLGA, chitosan-adsorbed PLGA, and chitosan-conjugated PLGA nanoparticles and microparticles onto 4T1 cells. (A) Control PLGA nanoparticles; (B) chitosan-adsorbed PLGA nanoparticles; (C) chitosan-conjugated PLGA nanoparticles; (D) control PLGA microparticles; (E) chitosan-adsorbed PLGA microparticles; (F) chitosan-conjugated PLGA microparticles.

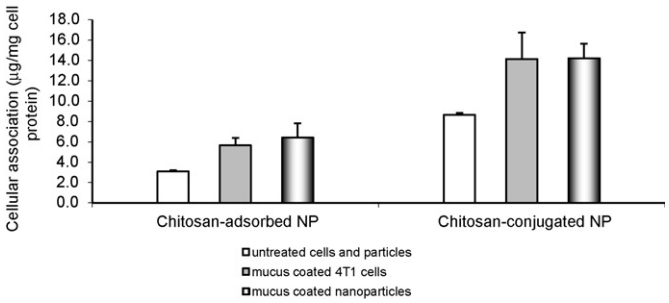

Fig. 8. Effect of mucus pre-treatment of the particles or 4T1 cells on the cellular association of paclitaxel delivered in control PLGA and chitosan-modified PLGA nanoparticles. NP: nanoparticles.

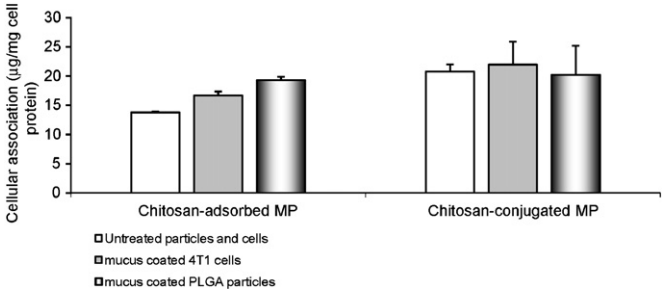

Fig. 9. Effect of mucus pre-treatment of the particles or 4T1 cells on cellular association of paclitaxel delivered in control PLGA and chitosan-modified PLGA microparticles. MP: Microparticles.

cellular association of paclitaxel after either the particles or the 4T1 cells were treated with mucus.

3.9. Correlation between the initial amount of chitosan used and the extent of adsorption and conjugation

The efficiency of adsorption or conjugation of chitosan was calculated as ratio of the experimentally determined amount of chitosan adsorbed or conjugated to the PLGA particles relative to the known amount of chitosan used. An adsorption efficiency of 80–81% was obtained for the nanoparticles and microparticles, whereas the efficiency of conjugation was higher at 98%. Table 6 summarizes the relationship between the known, and experimentally determined, amounts of chitosan that is adsorbed or chemically conjugated to the PLGA particles and the adsorption or conjugation efficiencies. The adsorption efficiency of chitosan decreased from 81% to 60% as the initial amount of chitosan increased from 0.2% (w/v) to 1% (w/v). In contrast, the conjugation efficiency remained constant at 98% when the concentration of chitosan used increased from 0.2% (w/v) to 1% (w/v), suggesting a stronger interaction between chitosan and PLGA. A linear relationship was observed between the theoretical amount of chitosan

Table 4
Relative increase in the cellular association of paclitaxel delivered in mucus coated chitosan-PLGA nanoparticles compared to untreated PLGA nanoparticles.

Treatment/type	Chitosan-adsorbed nanoparticles	Chitosan-conjugated nanoparticles
Untreated cells/particles	1	1
Mucus coated 4T1 cells	1.83	1.64
Mucus coated PLGA particles	2.08	1.65

Table 5
Relative increase in the cellular association of paclitaxel delivered in mucus coated chitosan-PLGA microparticles compared to untreated PLGA microparticles.

Treatment/type	Chitosan-adsorbed microparticles	Chitosan-conjugated microparticles
Untreated cells/particles	1	1
Mucus coated 4T1 cells	1.21	1.06
Mucus coated particles	1.40	0.97

Table 6
Efficiency of adsorption or conjugation of different concentrations of chitosan to PLGA nanoparticles.

Type	Known conc. of chitosan added[a] (%, w/v)	Experimentally determined amount adsorbed or conjugated (mg)	Adsorption or conjugation efficiency (%)
Chitosan-adsorbed nanoparticles	0.2	4.87	81.22
	0.5	3.09	61.88
	0.75	4.37	58.29
	1	5.98	59.85
Chitosan-conjugated nanoparticles	0.2	5.92	98.62
	0.5	4.96	99.13
	0.75	7.32	97.67
	1	9.93	98.62

[a] 3 mL of chitosan solution of each concentration was added to 90 mg of PLGA.

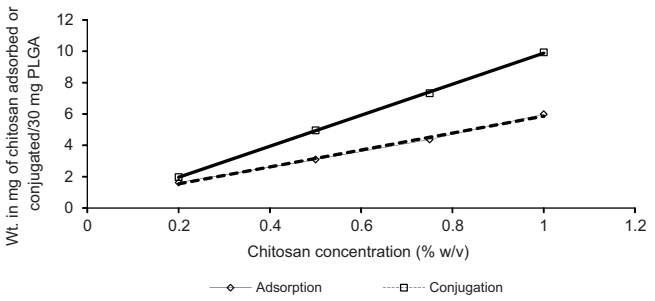

Fig. 10. Correlation between amount of chitosan added initially and the weight of chitosan adsorbed or conjugated to PLGA nanoparticles.

added to PLGA and the amount of chitosan adsorbed or conjugated onto the nanoparticles (Fig. 10). These results demonstrate that, within the concentration range tested, there is a direct correlation of the extent of adsorption or conjugation of chitosan to PLGA particles as the initial amount of chitosan increased. Further, as illustrated in Table 7, when the known amount of chitosan exceeded 0.5% (w/v), the zeta potential of the particles is reversed to a positive surface charge.

4. Discussion

It has been previously demonstrated that, when paclitaxel and etoposode were delivered in PLGA microparticles of size 2077 nm, the cellular association of paclitaxel in 4T1 cells was enhanced 4.8 and 29-fold, respectively, compared to PLGA nanoparticles of size 310 nm (De et al., 2005). Similar findings were obtained using the mucus-secreting cell lines, including Caco-2 and Cor-L23/R. These observations were confirmed in an *in vivo* tumor efficacy study that demonstrated that 1 μm and 10 μm PLGA microparticles arrested tumor growth in mouse xenografts more effectively than nanoparticles.

Table 7
Effect of initial concentration of chitosan on the zeta potential of surface modified PLGA nanoparticles.

Type	Initial chitosan concentration (%, w/v)	Zeta potential (mV)
Chitosan-adsorbed nanoparticles	0.2	−9.2
	0.5	3.5
	0.75	6.0
	1	3.4
Chitosan-conjugated nanoparticles	0.2	−8.3
	0.5	7.4
	0.75	1.2
	1	14.0

The effect of particle size on cellular association of PLGA particles has been investigated in the literature. Contrary to our observations, cellular uptake of PLGA nanoparticles into Caco-2 cells was reported to decrease as particle size increased (Desai et al., 1997). However, using confocal microscopy, these authors demonstrated that, while PLGA nanoparticles of size 0.1 μm and 1 μm were predominantly internalized into the cytoplasm and the endosomes, the larger 10 μm microparticles adhered to the cell surface. This observation was consistent with our finding that PLGA microparticles adhere to the surface of the 4T1 cells. Similarly, it has been reported that cellular uptake of PLGA and PLGA-chitosan nanoparticles decreases with an increase in particle size (Tahara et al., 2009). Using confocal microscopy, these authors concluded that the PLGA nanoparticles of average diameter 200 nm were predominantly internalized, with limited surface adhesion although they did not study the surface adhesion of larger PLGA particles. In addition, these authors demonstrated that coating the PLGA particles with chitosan significantly enhanced cellular uptake independent of particle size. This observation is consistent with our findings that demonstrated that surface modification of PLGA nanoparticles and microparticles with chitosan significantly enhanced the cellular association of the drug.

Although the amount of chitosan adsorbed or conjugated to PLGA was similar, chitosan-conjugated PLGA microparticles were most cytotoxic. One plausible explanation is that the biopolymer is weakly adsorbed to the PLGA polymer. However, quantitative studies are required to demonstrate the relative binding strength. In addition, quantitation of the mucoadhesive force between the PLGA-chitosan particles and the cell membrane using techniques such as atomic force microscopy is expected to give an insight into the extent of mucoadhesion and the specific mechanism of interaction of the PLGA-chitosan particles. Further, it is expected that microparticles remain adherent to the cell surface and release paclitaxel for a longer time. Conversely, nanoparticles demonstrated a burst release of drug. These particles may either be internalized or have a weak affinity to mucus, resulting in lower effective permeation of the encapsulated drug.

The p*K*a of lactic acid and oligomeric poly-lactic acid is 3.1 and that of glycolic acid is 3.83 (Gresser et al., 2003; Siparsky et al., 1998). Therefore, when suspended in MES buffer, pH 5.5, the carboxyl groups on the polymer are ionized and the particles have a net negative surface charge. Further, the p*K*a of the primary amine group of chitosan is reported to be between 6.3 and 7.0, and the polymer is only water-soluble at a pH below its p*K*a (Beppu and Santana, 2002). Therefore, chitosan is soluble in MES buffer and its protonated amine groups give the polymer a net positive charge. Adsorption of chitosan onto the surface of PLGA is due to the electrostatic interaction between the negatively charged PLGA particles and the positively charged chitosan. Chitosan also reacts with the activated surface carboxyl groups on the PLGA particle suspension to form an amide bond. Preliminary experiments using turbidimetry confirmed that at pH of 6.4 and above, the polymer precipitated out of solution with a 160-fold increase in turbidity at a pH of 7.1. Covalent conjugation of PLGA to chitosan was mediated using EDC to react with the carboxyl group on the PLGA to form o-acylisourea, an amine-reactive intermediate. The unstable intermediate is stabilized in the aqueous solution by NHS through the formation of an active ester intermediate that reacts with the primary amine groups on chitosan to form an amide bond. As the rate of reaction is fastest in the pH range of 7.0–8.0, the reaction was carried out in HEPES/NaOH buffer (pH 7.4). At this pH, chitosan is insoluble in water and precipitates as a flocculate. It has been reported that 92% of the primary amine groups are uncharged in HEPES/NaOH buffer, pH 7.4 (Beppu and Santana, 2002).

FTIR is commonly used to confirm the secondary structure of proteins and polypeptides. The characteristic IR peak of chitosan was observed as a strong band at 1580–1650 cm^{-1} that results from NH_2 deformation vibrations. Further, a broad band at 3450 cm^{-1} is due to the NH_2 symmetric stretching vibrations (Wang et al., 2004). The amide I band in the FTIR spectrum is a result of the stretching vibrations of the C=O bond whereas, the amide II band is formed as a result of the bending vibrations of the N–H bond. The amides I and II bands were absent in the FTIR spectra of PLGA and deacetylated chitosan alone. However, in the FTIR spectrum of acetylated chitosan (chitin), amide -I, II, and III bands are observed at 1649–1655, 1554, and 1322–1325 cm^{-1}, respectively, suggesting that Amide bands are a result of covalent modification of the primary amine in chitosan (Wang et al., 2004).

While surface modification of PLGA with chitosan has been widely reported, the effect of physical adsorption and chemical conjugation of chitosan on the size of PLGA particles has not been reported. Recently, chitosan has been coated or chemically conjugated to PLGA nanoparticles of average diameter 248 nm (Chen et al., 2009). However, the effect of surface modification of the PLGA nanoparticles on the *in vitro* cellular association of the drug has not been investigated.

Confocal microscopy is commonly used to demonstrate cellular localization of fluorescently tagged compounds (de la Fuente et al., 2008), and this technique has been used to confirm the adsorption of chitosan to the PLGA particles (Fischer et al., 2006). Further, mucoadhesion of chitosan can also be quantified by determining of the residual weight of chitosan binding to mucus following deposition; the use of a resonant mirror biosensor, or a modified tensiometer (Bravo-Osuna et al., 2007; Sigurdsson et al., 2006; Snyman et al., 2003). Increased mucus content on the 4T1 cells or the chitosan-PLGA particles may enhance the adhesion of these particles to further increase cellular association and cytotoxicity. In addition, it is widely reported that surface modification of PLGA particles with chitosan results in a positive zeta potential which favors mucoadhesion (Guan et al., 2008; Pamujula et al., 2008).

The increased *in vitro* cellular association of paclitaxel into 4T1 cells when chitosan was either adsorbed or conjugated to the surface supports our hypothesis that the mucoadhesive properties of chitosan enhances paclitaxel concentration into or onto 4T1 cells. When expressed as a percentage of the initial amount of drug incubated with the cells, 5.2% of the initial amount of paclitaxel added was associated with the 4T1 cells when delivered in control nanoparticles, compared to a 14.1% increase observed with control PLGA microparticles. Similarly, there was a 21–56% increase in cellular association of paclitaxel when delivered in chitosan-PLGA nanoparticles relative to the initial amount of paclitaxel. Importantly, the cellular association of paclitaxel was highest (55–83%) when delivered in chitosan-PLGA microparticles.

As widely described in the literature, when in solution, paclitaxel is rapidly effluxed from the tumor cells decreasing the cellular accumulation and cytotoxicity of the drug. Further, based on these cytotoxicity results, it can be concluded that: (1) modifying the surface of PLGA particles with mucoadhesive chitosan significantly enhanced the cytotoxicity of both nanoparticles and microparticles and, (2) the IC_{50} values of paclitaxel delivered in PLGA microparticles was significantly lower after chitosan had been adsorbed or conjugated to their surface. These data support our hypothesis that modification of the surface of the PLGA particles with chitosan significantly enhances the *in vitro* cellular association and cytotoxicity of paclitaxel because of enhanced mucoadhesion of the particles to the tumor epithelium.

5. Conclusions

The size of PLGA particles was not significantly altered after modifying the surface with chitosan. The ninhydrin assay confirmed that about 6% (w/w) of chitosan is adsorbed or conjugated to the PLGA particles which corresponded to an adsorption or conjugation efficiency of 81% and 98%, respectively. FTIR spectroscopy confirmed that chitosan is conjugated to the PLGA particle surface. The cationic chitosan lowered the zeta potential of the PLGA particles although at the concentration used; did not reverse the surface charge. The rate and extent of release of paclitaxel from the nanoparticles and microparticles was not significantly altered after modification with chitosan. The cellular association of paclitaxel into 4T1 cells increased 4.5–10 times when chitosan was adsorbed or conjugated to PLGA nanoparticles. Similarly, a 4–6 fold increase in cellular association was observed when chitosan was adsorbed or conjugated to PLGA microparticles. Confocal microscopy confirmed that adsorption or conjugation of chitosan to the PLGA particles significantly enhances the cellular association, particularly with microparticles. Pre-treating 4T1 cells or chitosan-PLGA particles with mucus demonstrated that, after modification with chitosan, nanoparticles and microparticles predominantly adhere to the mucus resulting in increased cellular association. The amount of chitosan adsorbed or conjugated to the PLGA particles was correlated linearly with the initial amount of chitosan added. These results prove that the cellular association and cytotoxicity of paclitaxel is significantly enhanced by delivering in chitosan-PLGA particles.

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