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# Novel in Situ Gel Drug Delivery System for Breast Cancer Treatment

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INTRODUCTION:

The rationale of this study was to utilize the overproduction of mucin in cancerous cells as a drug targeting strategy to develop a safe and effective delivery system for taxol. Since the chemotherapeutic agents do not discriminate cancer cells and normal tissue, highly effective cancer treatment agents such as taxol cause major toxicity to normal tissue. This toxicity can be fatal if not prevented. The hypothesis for this project was a mucoadhesive in situ gel delivery system containing paclitaxel can be targeted to the cancerous cells where MUC1 gene is overexpressed as compared to normal cells and substantially reduce its toxicity to normal cells. The primary objective of this investigation is to develop a sustained release novel in situ gel delivery system for the targeted local delivery of taxol. The delivery system was designed so that when injected close to the site of tumor, at the biological pH (7.4), the ionic polymer used in the delivery system would deprotonate and turn into an instant gel at the site of injection. This will provide a sustained release of paclitaxel (PTX) from the gel at and around the site of cancer while the systemic drug concentration will be negligible. The specific aims of this study are: 1) formulation, and physicochemical characterization of the in situ gel delivery system, and 2) evaluation of the effectiveness of the targeted local in situ gel delivery system verses the systemic delivery and determination of the local tissue and organ toxicity of the delivery system.

BODY:

Physiochemical Characterization of In Situ Gel Drug Delivery System:

The initial phase of this research project was to determine the physiochemical properties of a novel in situ gel delivery system for the sustained delivery of paclitaxel in breast cancer therapy. The formulation and physiochemical properties of the in situ gel are well characterized and published in the appended manuscript (Jauhari and Dash, 2006). Briefly, the formulated in situ gel delivery system was developed using chitosan (3% w/v) and glyceryl monooleate (3% w/v) (GMO) that exists as a viscous solution at low pH. When this viscous solution is introduced to a matrix at biological pH (7.4), the viscous solution becomes deprotonated turning into a gelatinous sustained drug delivery vehicle. The major challenge in this application was the homogenous incorporation of paclitaxel in the gel matrix. The optimal formulation conditions were to disperse a known amount of paclitaxel in the citric acid solution followed by the addition of chitosan (3% w/v), and then this solution was mixed with melted GMO. In addition, a rapid HPLC method was developed and validated to provide analytical support for the rest of the studies. The release of paclitaxel from the gel matrix followed a matrix diffusion controlled mechanism that was dependent on concentration. In the initial phase of this project, a considerable obstacle became evident. The low pH of the in situ gel design without PTX demonstrated a significant mitogenic cytotoxicity profile when introduced into an in vitro model of human breast cancer MDA-MB-231 cells (data not shown). At this point of the projects attempts were made to balance the low pH of the gel design with sodium hydroxide or high pH (10) phosphate buffered saline, this proved to be problematic at best. The addition of either sodium hydroxide or PBS alone or in combination with citric acid caused significant cytotoxicity without the chitosan and GMO constituents of the in situ gel design (data not shown). Therefore, a new novel approach was needed to circumvent this major obstacle.
Physiochemical Characterization of the Nanoparticulate Drug Delivery System:

The novel approach was to incorporate hydrophobic drugs like PTX into a chitosan and GMO nanoparticulate system. This new direction allowed the development of a neutral bioadhesive drug delivery system by evaporating the excessive hydrogen ions under freeze drying conditions that could be stored as a free flowing powder and easily be resuspended into an aqueous matrix. The nanoparticulate drug delivery system was prepared by a multiple emulsion method. GMO was melted in to a fluid phase at 40°C, a known amount of PTX (4.5% w/w) was incorporated into the fluid phase of GMO and an emulsion comprised of (14% v/v) GMO and 0.5% aqueous polyvinyl alcohol (m.w 30000-70000) was ultrasonicated for 2 minutes at 18Watts. Chitosan (2.4% w/v) was dissolved in citric acid (2.4% w/v) was added to the oil-water emulsion and ultrasonicated for 2 minutes at 18 Watts. The final oil-water emulsion is frozen prior to freeze drying (-52 °C and < 10 μm mercury pressure). The compounds of interest incorporated into the novel nanoparticulate delivery system for the remainder of these studies are: paclitaxel, dexamethasone (control compound), and osmium tetroxide (electron dense compound for transmission or scanning electron microscopy).

The initial phase of this new approach was to characterize the physiochemical properties and determine the bio-compatibility of this novel nanoparticulate DDS. The in vitro release of a similar model compound (dexamethasone) or PTX from three different batches has shown an initial burst release to obtain a local therapeutic level at the site of action along with a sustaining release profile (Figures 1a and 1b). The release of paclitaxel or dexamethasone from the formulations was determined by a USP method. Briefly, a known quantity of the formulation was added to 40 ml of PBS (pH 7.4), shaken in a bath incubator at 37°C and 80 rpm. At predetermined time intervals 200μl of the sample was withdrawn and replaced with equal amount of phosphate buffer. The amount of PTX or DEX was determined by HPLC. The HPLC method for PTX was previously described (appended manuscript) (Jauhari and Dash, 2006). The HPLC method for DEX consisted of a mobile phase, methanol/0.1M ammonium acetate 60:40 (v/v), maintained at a flow rate of 1.2 ml/min A C18 Luna column (4.6 mm, 250 mm, 5um), Phenomenix, CA) was used with UV detection at 254 nm. The release of two model drugs was studied in a USP release apparatus. The release profile has a sustained release followed by an initial burst release. The maximal release from dexamethasone was 58 percent in the 4-hour study period. The release profile of paclitaxel was similar to dexamethasone; however the amount of paclitaxel released was significantly reduced compared dexamethasone (12% compared to 58%)(Figures 1a and 1b).

**Figures 1a and 1b:** The in vitro release profiles from three different batches of dexamethasone (4.5% w/w) (Figure 1a) and Paclitaxel (4.5% w/w) encapsulated in a chitosan/GMO nanoparticulate formulation. The data is expressed as mean ± SEM and n=9.
**Physiochemical Characterization of the Nanoparticulate Drug Delivery System:**

The physiochemical properties were further evaluated and found the formulation and preparation to yield particles in the nano-sized range with a polycationic surface charge (Table 1). The particles were suspended in deionized water and the measurements were carried out by a Zeta meter (Brookhaven instruments). In addition, the properties were compared to the charges associated with a suspension of human breast cancer cells (MDA-231). The cell suspension showed a significant negative charge (Table 1). These data suggest that the particles may have a significant ionic attraction to the human breast cancer cells (MDA 231). Furthermore, the proposed ionic attraction may aid in the biohesion of the particles increasing the resonance time of the drug at the site of action.

**Table 1**  
Comparison of Human Breast Cancer Cell line Surface Charge and Formulation Particle Size and Surface Charge  

<table>
<thead>
<tr>
<th>Groups</th>
<th>Particle Size (nm) Mean ± SEM, n=3</th>
<th>Particle Charge (mV) Mean ± SEM, n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-231 cell suspension</td>
<td>N/A</td>
<td>-21.32 ± 6.63</td>
</tr>
<tr>
<td>Blank Chitosan/GMO</td>
<td>676.0 ± 16.3</td>
<td>+31.78 ± 0.54</td>
</tr>
<tr>
<td>Osmium Tetroxide Chitosan/GMO</td>
<td>532.2 ± 39.3</td>
<td>+25.33 ± 1.46</td>
</tr>
<tr>
<td>4.5% Dexamethasone Chitosan/GMO</td>
<td>454.5 ± 43.7</td>
<td>+26.66 ± 0.87</td>
</tr>
<tr>
<td>4.5% Paclitaxel Chitosan/GMO</td>
<td>432.5 ± 37.1</td>
<td>+33.17 ± 1.52</td>
</tr>
</tbody>
</table>

**Nanoparticle Evaluation by Transmission Electron Microscopy:**

Chitosan/GMO nanoparticles were loaded with osmium tetroxide (electron dense compound for transmission electron microscopy and evaluated for size, homogeneity and surface morphology) (Figures 2a and 2b). A known amount (4 mg/ml) of the DDS was resuspended in de-ionized water and 20 microliters was placed on a copper formar grid and allowed to air dry over night. The mounted particles were viewed by Transmission Electron Microscopy at various magnifications (10,000-50,000X) at Creighton University EM Center. The electron density around the particles suggests that the nanoparticles appear to have a hydrophobic core surrounded by a thin layer of hydrophilic material (Figure 2a). The particle size appear to be rather poly disperse and heterogeneous in nature (Figure 2b). The particle size and distribution is qualitatively similar as observed from the zetapotential readings reported in Table 1. The particle composition appears to be soft and gelatin in nature suggesting that they may have in situ gel forming nature (Figure 2b). The surface morphology appears smooth and non-porous in nature (Figure 2b).
Figures 2a and 2b: Chitosan/GMO nanoparticles were loaded with osmium tetroxide (Figures 2a and 2b). The mounted particles were viewed by Transmission Electron Microscopy at 10,000X magnification (Figure 2a) and 51,300X magnification (Figure 2b) at Creighton University EM Center.

The Physical State of Drug Incorporated in Chitosan/GMO Nanoparticles:

The physical state of the drug incorporated in the drug delivery system was evaluated by both x-ray diffraction (Figure 3) and scanning differential calorimetry (data not shown). The drug was observed to be in the soluble form in the polymer matrix as observed by the absence of remarkable peak findings in the x-ray diffraction profile for blank particles, PTX loaded particles, DEX loaded particles, coumarin-6 loaded particles and rhodamine 123 loaded particles (Figure 3). This observation was also confirmed in separate scanning differential calorimetry studies by the absence of any remarkable endothermic or exothermic events (data not shown).

Figure 3: X-Ray Diffraction of Several Different Chitosan/GMO Nanoparticles.
In Vitro Drug Delivery Model:

The Bioadhesive Properties of Chitosan/GMO Nanoparticles:

The in vitro bioadhesion and targeting efficiency of the delivery system were evaluated in MDA-MB-231 human breast cancer cells (Figures 3a and 3b). In these studies, cell monolayers were cultured on Thermofax® cover-slips. The cell monolayers were treated with the surface modified polycationic nano-sized delivery system loaded with osmium tetroxide as a function of time (15-to-30 minutes). The cell monolayers were washed three times in ice cold PBS, fixed with glutaraldehyde (3%) and dehydrated with successive alcohol solutions (50-to-100 percent) prior to mounting on a stub for critical point drying and gold sputter coating for scanning electron microscopy (SEM) imaging. The SEM images confirmed the particle size and demonstrated the bio-adhesive properties of the polycationic nanoparticle formulations to the inherent negative cell surface-charge of the MDA-MB-231 cells (Figures 3a and 3b). The formulation particles appear to be in a swollen hydrated state attached to the cellular surface. In addition, the expression of integral protein appears high in the cell surface morphology of the human MDA-MB-231 cells. Furthermore, the particle adherence also appears to have destabilized the cell surface morphology due to the charge-charge interactions of the particles with the cellular surface proteins. These data suggest that this formulation can adhere to the carbohydrates/glycoconjugate sites expressed on cancerous cells. This further suggests that the formulation may have a preference for the over expressed mucopolysaccharides on the cell surface of cancerous cells. However, further investigation is needed to determine the exact nature of the bioadhesive forces involved here.

Figures 3a and 3b: The Bioadhesive Properties of the Chitosan/GMO Sustained Release Formulation. Scanning electron microscopy images are shown of a single human breast cancer cell (MDA-231). The control cells were treated with the particle suspension medium alone for 30 minutes (Figure 3a), while the test cells were treated with the chitosan/GMO sustained release formulation for 30 minutes (Figure 3b).
The Cellular Association of Paclitaxel in MDA-MB-231 Cells:

The intracellular uptake and extracellular association of the nanoparticle delivery system in MDA-MB-231 human breast cancer cells were evaluated by the HPLC method previously mentioned (Figure 4). In these studies, the cell monolayers were cultured in standard 6-well tissue culture plates at a seeding density of 500,000 cells per square centimeter and cultured until confluence in a humidified chamber at 37°C in RPMI-1640 growth media supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. Confluent cell monolayers were treated with a single bolus solution of paclitaxel or the nanoparticulate delivery system loaded with paclitaxel for various times (15-to-60 minutes). The cell monolayers were washed three times with ice cold PBS and lysed with 1% triton-X. The cell monolayer lysates were assayed for total protein content by the BCA method prior to freeze-drying. The freeze dried cell monolayer lysates were re-suspended in acetonitrile and centrifuged at 14,000 RPM in a microcentrifuge. The supernatant was analyzed by the HPLC and the amount of paclitaxel was determined. The cellular uptake data was presented as amount paclitaxel per mg protein. The cellular association and uptake of paclitaxel was significantly increased with the nanoparticle formulation when compared to a solution of free paclitaxel. Furthermore, the increase was 4 fold higher in the paclitaxel formulation when compared to the free form of paclitaxel.

![Graph showing cellular association of paclitaxel](image)

**Figure 4: The effects of Chitosan/GMO nanoparticles on Cellular Uptake of Paclitaxel.** Confluent MDA-231 monolayers were exposed to paclitaxel solution (1 uM) or chitosan/GMO nanoparticles containing paclitaxel (1uM free fraction) at various time intervals. The data is expressed as mean ± SEM of three MDA-231 monolayers. The data was considered statistically significant when *p*-value < 0.05 compared to paclitaxel solution.
MTT Cell Viability Assay:

The viability of MDA-MB-231 human breast cancer cells were determined using MTT cytotoxicity analysis (Figures 5a, 5b and 5c). Briefly, the cell monolayers were seeded in a 96-well cell culture plate at a density of 10,000 cells per well and incubated overnight in a humidified chamber at 37°C in RPMI-1640 growth media supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. The cells were treated with various concentrations in a single bolus with a solution of paclitaxel or the nanoparticulate delivery system loaded with and without paclitaxel or with dexamethasone for 4 hours, then washed three times with PBS and supplied with fresh growth media (48-to-96 hours). After the incubation period, the cells were treated with fresh MTT reagent and further incubated for 2-hours, then treated with a fresh DMF and SDS. The absorbance was read on a microplate reader at 550 nm. The absorbance data was analyzed and presented as percent survival of control monolayers receiving media alone. The MTT cytotoxicity dose-response studies revealed that the placebo (no drug added nanoparticles) or drug loaded nanoparticles ≤1 mg/ml showed a 100 percent cell-survival of these cells. These dose-response studies further revealed that MDA-MB-231 cells exposed to the same dose (PTX solution versus amount released PTX from the formulation) of paclitaxel for 4 hours demonstrated a significant decrease in the cell survival associated with the formulation (Figures 5a, 5b and 5c). The fold decrease in ED$_{50}$ for the formulation was 650, 500, 1000 at 48, 72 and 96 hours post treatment when compared to the PAC solution alone (Figures 5a, 5b and 5c). The significance of these data are that the bioadhesive and sustained delivery properties of the nanoparticulate formulation increases the residence time of the drug and thus, increases the duration of chemotherapeutic effect of PTX.

**Figure 5a:**
The Cytotoxicity effects of Chitosan/GMO nanoparticles at 48-hours post treatment. Confluent MDA-231 monolayers were exposed to various concentrations of paclitaxel solution or nanoparticles containing paclitaxel. The data is expressed as mean ± SEM of three MDA-231 monolayers. The data was considered statistically significant when *p*-value <0.05 when compared to placebo or **p*-value < 0.05 compared to paclitaxel solution and placebo.
Figure 5b: The Cytotoxicity effects of Chitosan/GMO nanoparticles at 72-hours post treatment.
Confluent MDA-231 monolayers were exposed to various concentrations of paclitaxel solution or nanoparticles containing paclitaxel. The data is expressed as mean ± SEM of three MDA-231 monolayers. The data was considered statistically significant when *p-value < 0.05 when compared to placebo or **p-value < 0.05 compared to paclitaxel solution and placebo.

Figure 5c: The Cytotoxicity effects of Chitosan/GMO nanoparticles at 96-hours post treatment.
Confluent MDA-231 monolayers were exposed to various concentrations of paclitaxel solution or nanoparticles containing paclitaxel. The data is expressed as mean ± SEM of three MDA-231 monolayers. The data was considered statistically significant when *p-value < 0.05 when compared to placebo or **p-value < 0.05 compared to paclitaxel solution and placebo.
Sustained Release of Paclitaxel from Chitosan/GMO nanoparticles.

The extent of paclitaxel release from chitosan/GMO nanoparticles was studied by an in vitro USP method (Figure 6). The release of paclitaxel from chitosan/GMO nanoparticle formulation in the presence of Tween 80 was significantly increased (3.6 fold) when compared to PBS release media over the 96-hour study. The significant increase in the amount of PTX released in the presence of a surfactant (Tween 80) demonstrates that this formulation has significant amounts (72%) of drug in reserve for the sustained release of PTX.

![Paclitaxel Release from Chitosan/GMO](image)

**Figure 6:** The In Vitro Release profile of Paclitaxel from Chitosan/GMO nanoparticles. The data is expressed as mean ± SEM of three independent samples.

**In Vivo Comparison of the Effectiveness of Local Delivery versus Systemic Administration:**

The safety and efficacy of the localized PTX nanoparticulate DDS was compared to the conventional route for PTX administration in an in vivo model of human breast cancer (Figures 7a and 7b). The rationale for using an in vivo model is to mimic the clinical situation of localized disease i.e. carcinoma in situ and flank malignant tumor growth. Briefly, in these studies, FOX Chase SCID Female Mice with CB17 background (7 weeks old) mice were purchased from Charles Rivers Laboratories. MDA-MB-231 human breast cancer cells were cultured as previously described. On the day of injections, a MDA-MB-231 were collected and resuspended (30 million cells/ml) in RPMI 1640 serum free media. The mice received an injection (0.1 ml) in the fourth inguinal mammary pad and another subcutaneous injection (0.1 ml) in the ipsilateral flank. Both the mammary pad and the flank tumor development along with the animal weight were monitored at various intervals throughout the entire study. On day 14, the mice were randomly separated into four groups for treatment as follows: control (no treatment), PTX standard clinical IV solution (15 mg/kg) tail vein, one dose each day for 3 days), placebo (blank nanoparticle formulation) (15 mg/kg, total formulation weight), PTX (45% w/w) nanoparticle formulation (15 mg/kg, total formulation weight). Therefore, the total PTX dose for the nanoparticle formulation was determined to be 0.625 mg/kg. The nanoparticle formulations were suspended in sterile water just prior to injection. On day 14, each animal received the respective treatment either intravenous or localized intratumoral injection in both tumors. On day 21, a second dose was administered. The data is expressed as mean ± SEM, n=6.
After the initial MDA-MB-231 cell injection, tumor development was visible after 6 days and measurable on day 9 (Figures 7a and 7b). The tumor diameter increased at a constant rate for all the groups between day 7 and day 14 (Figures 7a and 7b). After a single intratumoral bolus dose of the PTX formulated nanoparticles, a significant decrease (50%) in tumor diameter in both the mammary pad and the flank was observed on day 15 when compared to control, placebo and PTX administered intravenous (Figures 7a and 7b). At four days post treatment, the tumor diameter reached the maximal decrease in diameter to approximately 72% in both the mammary pad and the flank when compared to control, placebo and PTX administered intravenous (Figures 7a and 7b). Even though, the tumor shrinkage reached a significant reduction in diameter by day 18 in both the mammary pad and the flank, the difference was reduced to approximately 50% by day 21 in both the mammary pad and the flank when compared to control, placebo and PTX administered intravenous (Figures 7a and 7b). At this point in the study, all the groups received a second treatment on day 21 (Figures 7a and 7b).

**Figures 7a and 7b:** *In Vivo Comparison of the Effectiveness of Local Delivery versus Systemic Administration.* The effectiveness of localized PTX formulated in chitosan/GMO nanoparticles was compared to systemic administration in SCID mice. Control mice (solid squares) received no treatment, PTX IV mice (solid diamonds) received PTX solution tail vein (15 mg/kg, for 3 days), Placebo group (solid triangles) received a single bolus local injection of chitosan/GMO nanoparticles without PTX (15 mg/kg formulation weight), and PTX formulation group (crosses) received a single bolus local injection of chitosan/GMO nanoparticles with PTX (15 mg/kg formulation weight). The tumor diameter data is expressed as mean ± SEM, n=6 animals.
KEY RESEARCH ACCOMPLISHMENTS:

- Proof of a novel concept that DDS consisting of Chitosan and GMO can form nano-particles with significant bio-adhesive properties and sustained release profiles that are easily re-suspended in an aqueous matrix.
- These bio-adhesive properties have significantly increased the effectiveness of paclitaxel \textit{in vitro} and \textit{in vivo} by increasing the amount of the drug at the therapeutic site using less chemotherapeutics as well as prolonging the therapeutic duration and thus possibly reducing significant side effects associated chemotherapy.
- DDS consisting of Chitosan and GMO are non-toxic to SICD mice \textit{in vivo} and MDA-231 cells \textit{in vitro}.

REPORTABLE OUTCOMES:

**Contributed Presentations with Abstracts**


**Full Manuscripts**


11

**Degrees Awarded**
S. Jauhari, Masters Degree, Creighton University 2006

**Patent Applications:**
Dash, A.K. and Trickler, W.J.; U.S. Patent Pending, Mucoadhesive Nanoparticles for Cancer Treatment; October 26, 2006

**Grants Applied For still Pending:**
N/A

**Grants Applied For Not Funded:**
DOD Concept Grant: BCO52458 SURFACE MODIFIED MUCOADHESIVE NANO-DELIVERY SYSTEM FOR BREAST CANCER TREATMENT.

Nebraska Smoking Grant LB506 SURFACE MODIFIED MUCOADHESIVE NANO-DELIVERY SYSTEM FOR BREAST CANCER TREATMENT

DOD idea Grant W81XWH-06-BCRP-IDEA TARGETED MUCOADHESIVE NANO-DELIVERY SYSTEM FOR MULTIDRUG RESISTANCE IN BREAST CANCER THERAPY.


**CONCLUSION:**
In conclusion, this work provides a significant foundation and proof of concept that chitosan and glyceryl monooctate can form a nanoparticulate drug delivery vehicle with significant bioadhesive properties and sustained release profiles that are easily re-suspended in an aqueous matrix. The nanoparticles appear to have a hydrophobic inner-core surrounded by a hydrophilic coating that exhibits a significant positive charge. This positive surface charge aids in the bio-adhesive properties of the drug delivery system to adhere to the carbohydrates/glycoconjugate sites over-expressed on cancerous cells. This further suggests that the formulation may have a preference for the over-expressed mucopolysaccharides on the cell surface of cancerous cells. However, further investigation is needed to determine the exact nature and mechanism of the bioadhesive forces observed in these studies. In addition, these studies have shown that nanoparticulate systems consisting of chitosan and GMO are non-toxic to SCID mice *in vivo* and MDA-231 cells *in vitro*. Furthermore, the bioadhesive properties have significantly increased the effectiveness of paclitaxel *in vitro* and *in vivo* by increasing the amount of the drug at the therapeutic site, and prolonging the therapeutic duration. These advantages allow lower doses of PTX to achieve an efficacious therapeutic window, and thus, minimizing the adverse side effects associated with chemotherapeutics like PTX.

Even though the drug delivery system demonstrates significant therapeutic efficacy *in vivo* and *in vitro*, there appears to be some resistance to the treatment, and further investigation is required to
determine the exact nature of the resistance to therapy. The resistance demonstrated by the MDA-MB-231 cells in vivo may be related to multi-drug resistance efflux transport proteins associated with cancerous cells. This entices several questions like: 1) is there a sub-population of cells that differ in the expression of these efflux proteins, 2) would increasing the drug loading totally eradicate these resistance cells in the animal model, 3) does the expression of these proteins change in the in vivo conditions compared to the in vitro population, or 4) would a drug delivery system co-formulated with drug efflux transport modulators be more effective in totally eradicating the cancerous tumors?

REFERENCES: List all references pertinent to the report using a standard journal format (i.e., format used in Science, Military Medicine, etc.).
APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.