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Inverse-micelle synthesis of doxorubicin-loaded alginate/chitosan nanoparticles and *in vitro* assessment of breast cancer cytotoxicity

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Abstract

Naturally-derived polysaccharides, such as alginate and chitosan, can be assembled to form nanocarriers for the delivery of therapeutic agents. Here we exploit the electrostatic complexation of alginate/chitosan in a water-in-oil (w/o) emulsion process to produce doxorubicin (DOX)-loaded nanoparticles (~80 nm) with exceptional spherical morphology and uniformity. This robust synthetic route utilizes an aqueous phase dispersed in a cyclohexane/dodecylamine organic phase and is capable of encapsulating DOX in the nanoparticle solution. The uptake and efficacy of this novel formulation was evaluated in a murine breast cancer cell line, 4T1, with comparable 72 h IC₅₀ values of the nanoparticle solution (0.15 µg/mL) and free DOX (0.13 µg/mL). Overall, the favorable performance, physiochemical properties, and their facile production support these nanocarriers as promising platform for the delivery of aqueous soluble drugs.

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Authors' Contributions

JR and CS conceived and designed the study. JR, HW, and AD carried out the experiments. All authors analyzed the data. JR and CS wrote the manuscript. GS and CS revised the manuscript. All authors have given approval to the final version of the manuscript.

Competing Interests

The authors declare that they have no competing interests.

Declarations

Availability of data and materials

All datasets on which the conclusions of the manuscript rely are presented in the main paper.

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Keywords

alginate; chitosan; doxorubicin; breast cancer; cell viability

Introduction

Naturally derived carbohydrate polymers have been examined extensively for biomedical applications due to their favorable biocompatibility and material properties. Chitosan, the deacetylated form of chitin, is a biopolymer typically isolated from the shells of crustaceans and has been examined for drug delivery applications as nanoparticles [1–3]. Chitosan is often combined with alginate, a naturally derived anionic polysaccharide, in order to induce a charge interaction between its positive charges and the negative charges of alginate. This allows for complexation of the two biopolymers, and can lead to the formation of various therapeutic carriers [4, 5].

Polymer nanocarriers can be formed in a variety of synthetic techniques [6], including nanoprecipitation [7, 8], emulsification [9, 10], and layer-by-layer methods [11, 12]. The synthetic process can influence the structure and morphology of the resulting nanocarrier, as well as the integration and loading capacity of the encapsulated therapeutic [6]. Water-in-oil emulsification is a common method employed in the fabrication of these polysaccharide-based carriers [13–15], which involves the introduction of a small aqueous phase into a larger oil phase to disperse the aqueous phase into droplets. These droplets contain the polymer and can be modified to form a solid polymeric particle or hydrogel. The resulting particles can be extracted or put through another emulsification step to further augment the nanocarrier. Emulsification allows for formation of nanoparticles without large amounts of energy input from shearing or sonification [16]. Finding the correct ratio between polymer solutions, organic phase, and surfactant can allow for tuning of the size distribution of the resulting nanoparticle formation.

Alginate/chitosan complexes have been employed in the formulation of various therapeutic payloads including gatifloxacin for ocular delivery [17], temoporfin for photodynamic therapy [18], insulin for protein delivery [19], esculin for treatment of nephrolithiasis [10], and chemotherapeutics (doxorubicin/paclitaxel) for treatment of lung cancer [20]. Of interest to this work is doxorubicin (DOX), which is used in the treatment of various cancers and employed here as a model therapeutic with intrinsic fluorescence. Doxorubicin inhibits tumor growth by direct interaction with DNA and inhibition of topoisomerase II, leading to arrested replication [21]. It can also lead to the generation of reactive oxygen species, which can lead to apoptosis [22]. A significant clinical limitation for DOX are a number of well-characterized toxic side effects on the heart, brain, liver, and kidneys [23], increasing the need for carrier-mediated reduction of these off target effects through controlled release and/or targeting.

In this study, we employed a water-in-oil emulsification technique for the formation of alginate-chitosan therapeutic nanocarriers via the interaction between the biopolymers. In this unique process, the oil phase is a mixture of cyclohexane and dodecylamine as an added surfactant to aid in the formation of the nanodispersion; our water phase is water containing

the two polymers, as well as our chosen therapeutic DOX. Alginate is present in the inverse-micelle first, and chitosan is added after the emulsion has formed to complex with the alginate and produce the nanoparticles. In this process, DOX becomes entrapped in the nanocarrier and can be subsequently extracted. The resulting highly uniform nanoparticles were characterized and their efficacy *in vitro* assessed by cell-viability against a murine breast cancer cell line. Significantly, we present a robust synthetic process to generate alginate-chitosan nanocarriers, their physiochemical characterization showing sub-100 nm particles (~80 nm diameter), and therapeutic IC₅₀ value of 0.15 µg/mL against 4T1 murine breast cancer cells.

Methods

Materials

Sodium alginate (molecular weight 12,000 – 40,000), chitosan (molecular weight 50,000 – 190,000, 75 – 85 % deacetylated), and dodecylamine (98 %) were purchased from Sigma-Aldrich (St. Louis, MO), and used without modification. Doxorubicin hydrochloride was procured from MedChem Express (Monmouth, NJ). Cyclohexane and hydrochloric acid were purchased from Fisher Scientific (Fair Lawn, NJ). 18 MΩ Milli-Q water was used to dissolve the alginate, chitosan, and doxorubicin hydrochloride prior to their use in the formulation.

Nanoparticle Formulation

60 mg of alginate powder was measured into a glass scintillation vial, and solubilized using 4 mL of Milli-Q water. A stir bar was added to the vial, which was placed on a stir plate. Gentle mixing was allowed for 20–30 min. 80 mg of chitosan powder was measured into a separate scintillation vial. 3 mL of Milli-Q water and 1 mL of 1 M HCl were used to solubilize the chitosan powder. A stir bar was added to this vial, the vial was placed on a stir plate, and allowed 20–30 min for dissolution of the chitosan (acidic environment is necessary for dissolution of the chitosan). Doxorubicin HCl, purchased in a 50 mg/mL aliquot, was completely dissolved in 10 mL of Milli-Q water. The concentrations of the prepared stock solutions detailed here were 15 mg/mL for alginate, 20 mg/mL for chitosan, and a 5 mg/mL for doxorubicin. 100 mL of cyclohexane was added to a 100 mL PYREX reusable media storage bottle in a fume hood. The amber glass container with dodecylamine was gently warmed for 1 min under warm tap water to liquify the dodecylamine, which is a solid at room temperature. 2 mL of the liquid dodecylamine were pipetted into the 100 mL cyclohexane, now referred to as the organic phase. A stir bar was added to the glass bottle containing the organic phase, the bottle was placed on a stir plate, and the stir plate was set to 700 rpm. 100 µL of the 15 mg/mL alginate solution were pipetted directly into the organic phase, and 15 min of mixing were allowed.

A solution of chitosan and the “loading” material was prepared while the alginate mixed in the organic phase. For therapeutic loading, 80 µL of the 20 mg/mL chitosan solution was mixed with 20 µL of the 5 mg/mL DOX solution. The 100 µL of this mixture was pipetted into the organic phase after the 15 min of mixing with alginate had completed. A change in turbidity was observed as the chitosan mixture entered the organic phase. For empty

nanoparticles, 20 μ L of Milli-Q water was added to the 80 μ L of chitosan solution before addition to the organic phase, in place of the 5 mg/mL doxorubicin solution. 15 min of stirring was allowed once the chitosan mixture was added.

To extract the formed nanoparticles from the organic phase, 10 mL of Milli-Q water was added to the glass bottle, and allowed to mix for 5 min. The aqueous layer containing the nanoparticles and latent contaminants separated to the bottom of the glass bottle. A serological pipette was used to collect the 10 mL volume of the aqueous phase, and the aqueous phase was added to the top filter compartment of a 100 kDa centrifugal filter unit (Pall). The 100 kDa centrifugal filter unit was spun in a centrifuge at $3200 \times g$ for 20 min, or until all retentate volume had passed through the filter. This step removed large aggregates from the nanoparticle solution. The filtrate from the 100 kDa filter unit was transferred to a 10 kDa centrifugal filter unit (Millipore). The 10 kDa filter unit was spun at $3200 \times g$ for 10 min, filled with Milli-Q water, and spun again at $3200 \times g$ for approximately 7 min, or until the retentate volume had reached ~ 1 mL. The filtrate (latent small components from the synthesis) was discarded. The retentate, the purified nanoparticle solution, could then be used for characterization or experimentation.

Nanoparticle Characterization

Size and zeta potential characterization was carried out using a Malvern Zetasizer ZSP (Malvern Instruments, UK). Measurements were performed in triplicate, and values stated are the mean $\pm$ standard deviation of the three measurements. Transmission electron microscopy (TEM) was performed to further confirm size measurements, as well as gain insight into morphology of the nanoparticles. TEM images were acquired using a Technai F-20 transmission electron microscope operating at 4200 eV. Preparation of the TEM grids involved dropwise deposition of 10 μ L of post-filtered nanoparticle solution onto the copper surface of the formavar/carbon backed TEM grid (Ted Pella). Wet TEM grids were placed covered in a desiccator overnight prior to imaging to ensure proper drying.

To determine DOX concentration in nanoparticle solution, the fluorescence of the nanoparticle solution (containing DOX, a molecule with inherent fluorescence) was compared to a standard curve of the drug solutions. The samples were scanned for fluorescent signal at 470/550 nm using a M200 Infinite plate reader (Tecan). To determine the encapsulation efficiency, the ratio of the DOX in the final nanoparticle solution was compared to the DOX introduced into the formulation during the synthesis. To determine the release curve of the drug leaving the nanoparticle solution over time, the purified nanoparticle solution was divided into 100 μ L aliquots and placed into Slide-A-Lyzer MINI (ThermoFisher) dialysis devices with a molecular weight cutoff of 2 kDa. The devices were placed into 20 mL volumes of PBS under gentle agitation, and fluorescence of retentate samples were evaluated at various timepoints to determine the amount of DOX lost. Seven timepoints after initial reading were measured in triplicate (1, 2, 4, 8, 24, 48, 72 h).

Infrared spectroscopy was performed using a Nicolet iS5 Infrared Spectrophotometer (ThermoFisher) to gain insight into the species present in the final nanoparticle. Dry samples were placed over the diamond filament and compressed prior to the total internal reflectance

reading. Spectra for alginate, chitosan, DOX, and lyophilized nanoparticles were taken at high resolution from wavelengths of 4000 to 400 cm^{-1} .

Cell Culture

4T1 murine breast cancer cells (PerkinElmer, Waltham, MA) were maintained in RPMI 1640 culture medium supplemented with 10 % by volume fetal bovine serum (FBS) and 1 % by volume penicillin/streptomycin. The cells were brought to 90% confluency, passaged, and split at least twice prior to use in viability assays.

On days when plating occurred, cells were passaged, counted and diluted to an appropriate concentration to plate a seeding density of 2000 cells per well in a black walled, clear bottom 96-well plate (Corning). Counting of the concentrated cell suspension prior to plating was performed using a Countess II FL Automated Cell Counter (ThermoFisher). Dilute cell suspensions were pipetted into each well using an INTEGRA ASSIST automated pipetting robot (Integra), using a standard pipetting/mixing protocol to pipette 100 μL volume into each well. The cells were allowed to settle overnight before dosing on the next day.

On days when dosing occurred, nanoparticle and/or free drug solutions were passed through a 0.22 μm filter under sterile conditions. A dosing plate was prepared under sterile conditions to prepare solutions of media containing the nanoparticles and/or free drug solutions. Positive cell kill control wells contained 10 % by volume DMSO, and untreated wells contained the RPMI medium mentioned previously. The outer wells of the plate were not used (to reduce inconsistencies due to evaporation along outer wells of plate). The plating growth media was aspirated from each well, and the wells were dosed as follows, all to a final volume of 100 μL . In columns 2–9, rows B–D, half-fold dilutions of one of the prepared nanoparticle solutions were pipetted. In columns 2–9, rows E–G, half-fold dilutions of either empty nanoparticle solutions or free doxorubicin were pipetted. The positive control was pipetted into columns 10–11, rows B–D, and the negative control was pipetted into columns 10–11, rows E–G. A 100 μL volume of phosphate-buffered saline (PBS) was pipetted into the diamond spaces between the wells to help maintain liquid levels across the plate. The plate was then returned to the incubator until the timepoint used for a desired assay.

Alamar Blue Viability Assay

72 h after dosing, the media from the wells was aspirated, washed three times with PBS, and 100 μL of new media was added to each well. 10 μL of Alamar Blue cell viability reagent (ThermoFisher) was added to each well. Cells were allowed to incubate with the viability reagent for at least an hour before a fluorescence measurement at 560/590 excitation/emission wavelength was taken on an M200 Infinite M200 plate reader (Tecan). Background signal could be removed, as it was indicated by the average of the positive control wells. The values from all of the dosed wells could then be compared to the average of the negative control wells to give a % viability:

$$\% \text{ cell viability} = \frac{\text{signal from dosed well}}{\text{signal from negative control wells}}$$

LIVE/DEAD Viability Assay

72 h after dosing, the media from the wells was aspirated, washed three times with PBS, and new media containing 2 drops/mL of media NucBlue live cell reagent and NucGreen dead cell reagent (ThermoFisher) was added to each well. The cells were allowed to incubate with the reagents for 30 min, and then washed three more times with PBS prior to fixation with formalin. The cells were allowed 30 min for fixation in the formalin to occur, and then washed three more times with PBS. 100 μ L of PBS was added to each well, and the cells were imaged using the DAPI and GFP filter sets on an EVOS FL Auto Imaging System (ThermoFisher) equipped with a 20 X objective.

Results and Discussions

Nanoparticle Synthesis and Characterization

Alginate-chitosan nanoparticles containing DOX were formed through the interaction of negative carboxylic acid groups on alginate strands with positive amine groups on chitosan polymers. The complexation that forms the nanoparticles was carried out in an organic bath, forming the dispersed aqueous phase. Cyclohexane and dodecylamine composed the organic phase, while water with the alginate and chitosan were in the aqueous phase. After formation and extraction, the nanoparticles were passed through a low-pass 100 kDa filter to remove large aggregates, and a high-pass 10 kDa filter to remove small impurities. The particles were washed and passed through a 0.22 μ m syringe filter for sterilization prior to use with *in vitro* assays. A schematic of this process is shown in Figure 1.

The size distribution of the nanoparticles is shown in Figure 2A. The average size of the particles was 80.6 ± 4.9 nm, and the zeta potential was found to be -27.4 ± 1.2 mV. This indicates a negative surface charge imparted by the presence of carboxylic acids on the alginate chains. Spherical morphology and size confirmation of the nanoparticles can be observed in the transmission electron micrograph shown in Figure 2B. Infrared spectroscopy was used to generate spectra for alginate, chitosan, doxorubicin, and the nanoparticle product, and can be viewed in Figure 2C. There are many overlapping peaks in the spectra from 1000 to 1800 cm^{-1} , likely were the carboxylic acid peaks from alginate are, as well as peaks contributed by the primary amines from chitosan, possibly indicating the presence of both alginate and chitosan in the nanoparticle product. The broad peak at ~ 3600 cm^{-1} is likely imparted from the presence of water still present in the lyophilized nanoparticle product. The small peak next to it could be present due to the amines from chitosan, as that peak is present only on the chitosan.

The release of DOX from the alginate-chitosan nanocarriers was evaluated using dialysis units. The nanoparticle solution was divided up into 21 aliquots of 100 μ L, and dialyzed against 20 mL of pH 7.4 PBS. At each time point (1, 2, 4, 8, 24, 48, 72 h), three volumes were collected, and their fluorescence was compared to a standard curve for doxorubicin. The amount of DOX missing relative to the initial amount at each time point is plotted in

Figure 3. Initial fluorescent readings showed a concentration of $\sim 8.3 \mu\text{g/mL}$, giving an encapsulation efficiency of 8.3% (0.1 mg DOX put into system). The greatest amount of DOX released occurs in the first 24 h, but a large amount of the drug is still present ($\sim 20\%$) at that time. At physiological pH, roughly 60% of the DOX is still present in the nanoparticle solution at 8 h, showing a steady release profile over the time course of the experiment. This indicates that at normal pH, the doxorubicin does not show a burst release profile, but instead controlled release over the time it would be administered *in vivo*. In contrast, at pH 5.5, roughly 70% of the DOX has been released by the 8 h time point, with 90% of the drug released by 24 h. This release is much faster, and demonstrates that in an acidic environment, the particles release the drug much more readily. This was not part of the design for the nanoparticle, but faster release in slight to moderate acidic environments such as the tumor microenvironment ($\sim \text{pH } 6.8$ [24]) or in the endosome ($\text{pH } 5 - 6$ [25]) is desired for maximizing therapeutic efficacy.

Cell Uptake and Toxicity

In Figure 4, the cellular uptake of the alginate-chitosan nanoparticles by 4T1 murine breast cancer cells at 72 h is shown. The top two images, from left to right, show untreated cells and cells treated with empty alginate/chitosan nanoparticles. The bottom images, from left to right, show cells treated with DOX (left), and alginate-chitosan nanoparticles containing DOX (right). In blue, the nucleus of the cells is stained with DAPI. In red, the intrinsic DOX (detected with red fluorescent protein filter set) is shown near the periphery of the nucleus after fixation. Cell proliferation has been restricted in comparison to the no-treatment well due to the presence of the DOX, which inhibits the growth of the 4T1 cells. The red channel is empty in the untreated sample and the sample treated with empty nanoparticles due to the lack of DOX in those wells. The red signal of the DOX colocalized with the nuclei in the samples containing DOX indicates that the DOX is able to get into the cell, in both unencapsulated and encapsulated forms. This is preferable, as the drug needs to be inside the cell to be efficacious, through any of the mechanisms summarized earlier. The encapsulated form, with better controlled release in physiological pH, is more ideal for translation *in vivo* due to its lowered release of the DOX, and its higher circulation time, increasing the probability that the therapeutic could get to the tumor environment. Concentrations do not perfectly match ($0.21 \mu\text{g/mL}$ DOX for encapsulated vs. $0.16 \mu\text{g/mL}$ for free DOX) due to the starting concentration of DOX in the nanoparticles being lower than the starting concentration of DOX used for the dose-response curve. As described in the Methods section, the cell uptake images were taken from a duplicate plate that received the same treatment as the dose-response plate.

To demonstrate efficacy of the alginate-chitosan nanoparticle against a cancer cell line, empty nanoparticles, free DOX, and alginate/chitosan nanoparticles with encapsulated DOX were administered to 4T1 cells. Cell viability after 72 h exposure to the treatment was determined using Alamar Blue reagent. Figure 5A shows the percent cell viability of cells exposed to indicated concentrations of free DOX and alginate-chitosan nanoparticles containing DOX after 72 h. The concentration indicates the DOX concentration in both cases. The IC_{50} values were found to be $0.13 \mu\text{g/mL}$ for free DOX and $0.15 \mu\text{g/mL}$ for encapsulated DOX. The free DOX value is similar to that found at 72 h against 4T1 cells by

Du et al [26]. The dose-response curves for the free drug and the encapsulated drug are similar, as are the IC_{50} values, indicating similar therapeutic response. In general, the encapsulated therapeutic should have similar if not higher IC_{50} value due to the impeded release of the drug, assuming that the carrier has no inherent toxicity. To check for toxicity of the carrier, dose-response was determined for the empty alginate/chitosan carrier. This data is shown in Figure 5B. The toxicity associated with the unloaded nanoparticle is minimal, and likely not leading to any significant cell death. To further assess cell death, duplicate plates treated identically to the viability plates were assessed for cell viability using a LIVE/DEAD cell assay. In this assay, all nuclei are stained blue, and dead cells show a green signal overlapping with the nuclei. The results of this assay are shown in Figure 6, where the no treatment (A) and empty nanoparticle (B) images show few cells with green signal, indicating a low number of dead cells in these wells. Cells treated with DOX (C) and alginate/chitosan nanoparticles with DOX (D). Multiple cells shown in these wells have strong green signal around their nuclei, indicating that a large percentage are dead. These wells show a lower number of cells overall due to the administration of DOX, which inhibits growth of the cells in the well. The combination of the qualitative assessment of the toxicity with the LIVE/DEAD assay and the quantitative assessment with the Alamar Blue assay indicates that the DOX in the nanoparticles is effective at killing the 4T1 cells.

Conclusion

We present a quick and robust method of producing alginate/chitosan nanoparticles that can entrap aqueous therapeutic payloads; in this study, we chose doxorubicin as a model therapeutic. The nanoparticle solutions produced have high enough concentration to induce a therapeutic effect when used against the 4T1 murine breast cancer cell line *in vitro*. The nanoparticles are of the appropriate size (to not be quickly cleared by the reticuloendothelial system) to translate to preclinical testing in mice for *in vivo* efficacy testing in future work. While similar carriers have been made with alginate/chitosan for a variety of therapeutics, the synthesis platform presented here will hopefully enable iteration and translation into preclinical work, as well as augmentation for increased efficacy and targeting.

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List of Abbreviations

DOX	doxorubicin
TEM	transmission electron microscopy
DMSO	dimethyl sulfoxide

PBS	phosphate-buffered saline
DAPI	4',6-diamidino-2-phenylindole

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Highlights

- A robust and reproducible process for encapsulation of aqueous therapeutic in alginatechitosan nanoparticles was developed.
- Sub-100 nm polymeric nanoparticles were produced and characterized via dynamic light scattering, transmission electron microscopy, and IR spectrometry.
- Release of doxorubicin from the nanoparticle formulation shows steady, controlled release in neutral pH, and accelerated release in acidic pH.
- 4T1 murine breast cancer cells show uptake of the drug in free and encapsulated form, and show loss in viability when exposed to formulation after 72 h.

Figure 1.

Schematic depicting formulation of alginate-chitosan nanoparticles. Aqueous alginate, chitosan, and doxorubicin are combined in an organic bath composed of cyclohexane and dodecylamine, causing complexation of the alginate with the chitosan. Extracted nanoparticles are passed through two sets of filters for purification, removing any latent polymer and large aggregates.

Figure 2.

Characterization of alginate-chitosan nanoparticles. (A) Size distribution of nanoparticles determined by dynamic light scattering. (B) Transmission electron microscopy shows roughly spherical, less than 100 nm particles. Lower magnification scale bar indicates 500 nm, inset scale bar indicates 10 nm. (C) Infrared spectra of the three main components of the nanoparticles, as well as the lyophilized nanoparticle product.

Figure 3.

Release curves for alginate-chitosan nanoparticles in different environments. Citrate buffer at pH 5.5 showed faster release of the doxorubicin than PBS buffer at pH 7.4.

Figure 4. Fluorescence images showing DOX uptake after 72 h exposure in 4T1 cells. Treatments are: (A) No treatment, (B) 0.21 $\mu\text{g/mL}$ (DOX equivalent nanoparticle number) empty alginate-chitosan nanoparticle treatment, (C) 0.16 $\mu\text{g/mL}$ free DOX, (D) 0.21 $\mu\text{g/mL}$ DOX-loaded alginate-chitosan nanoparticles. Blue indicates cell nuclei; red indicates presence of DOX. Scale bars indicate 100 μm .

Figure 5.

Dose-response of 4T1 cells to various treatments. (A) Cell viability after 72 h exposure to free/loaded DOX shows similar dose-response. (B) Cell viability after 72 h exposure to empty alginate-chitosan nanoparticles shows minimal toxicity associated with the drug carrier.

Figure 6.

Fluorescent images of LIVE/DEAD assay. Blue indicates all cell nuclei; green indicates dead cells. Treatments are: (A) No treatment, (B) 0.21 $\mu\text{g/mL}$ (DOX equivalent nanoparticle number) empty alginate-chitosan nanoparticle treatment, (C) 0.16 $\mu\text{g/mL}$ free DOX, (D) 0.21 $\mu\text{g/mL}$ DOX-loaded alginate-chitosan nanoparticles. Lower number of cells in DOX treatment images due to presence of therapeutic inhibiting cell proliferation. Scale bars indicate 100 μm .