WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 5, 444-454. Research Article ISSN 2277– 7105

TARGETED AND CONTROLLED DELIVERY OF ALGINATE-BASED ENCAPSULATION OF DOXORUBICIN IN EGFR –RELATED BREAST CARCINOMA

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Article Received on
11 March 2015,

Revised on 02 April 2015,
Accepted on 23 April 2015

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ABSTRACT

Doxorubicin hydrochloride (DOX) is used in treating ovarian, lung, and breast cancers. Its use in cancer chemotherapy is limited due to short plasma circulation, long elimination half-life, and non-specific cytotoxicity that may affect the normal cells. Thus, we have designed a drug delivery system, using a naturally occurring anionic polymer that is used for drug delivery due to its inert nature, high degree of biocompatibility, low toxicity, inexpensive and mild gelation with divalent cations such as calcium in forming particles. In this research, calcium alginate was used to encapsulate doxorubicin and assessed its compatibility with calcium alginate as drug delivery system. pH compatibility of alginate particle formation were assessed relative to the affinity of DOX. Alginate efficiency to encapsulate DOX was also tested and characterized through morphological analysis of the particles with the use of phase contrast microscope and scanning electron microscope (SEM). Fourier transform infrared spectrophotometer (FTIR) was used to confirm if the DOX was able to encapsulate within the alginate particles. Results showed that alginate encapsulation was compatible with DOX at neutral pH and encapsulation efficiency was achieved at 99.30%±0.0045 for 10ppm of DOX and 95.16%±0.011 for the 1.25ppm of DOX. An observable DOX-alginate particles with size average of 100-300µm using phase contrast microscope (40-60X magnification) and scanning electron microscope (200-300X magnification). DOX release in phosphate buffered saline (pH 7.2) confirmed that the DOX was successfully encapsulated based in FTIR data with comparable differences in the functional groups of DOX, alginate, and DOX released from

the particles.

KEYWORDS: Calcium Alginate, Doxorubicin, Encapsulation, Ionotropic Gelation.

INTRODUCTION

Adriamycin (doxorubicin hydrochloride) DOX is an antineoplastic agent effective against a wide range of solid tumors but its long term use is limited due to its cardiac toxicity and dose-dependent cardiomyopathy. It is known to bind to DNA-associated enzymes, intercalate with DNA base pairs, and target multiple molecular targets to produce a range of cytotoxic effects.[1] It induces apoptosis and necrosis in healthy tissues causing toxicity in the brain, liver, kidney and heart. Its cardiotoxicity was due to preferential accumulation of iron inside the mitochondria following doxorubicin treatment.[2] Over the years, various delivery systems have been designed to devise an effective drug delivery system that would eliminate these adverse effects including liposomes, hydrogel and Nano particulate systems.[3,4]

Our objectives were to formulate calcium-alginate beads containing doxorubicin by ionotropic gelation method, characterize the resulting microparticles and evaluate the release characteristics of doxorubicin loaded calcium-alginate beads to enhance its delivery against cancer cells with minimal toxicity to normal healthy cells at physiological pH.

MATERIALS AND METHODS

Materials

Sodium alginate, calcium chloride dihydrate, thymol blue, bromcresol purple, methyl orange, hydrochloric acid, and sodium hydroxide were analytical grade and were purchased from Sigma (St. Louis, MO). Phosphate buffered saline (PBS) pH 7.2 was purchased from Gibco® Life Technologies (CA, USA). Doxorubicin hydrochloride (DOX) (Adrosal®) was purchased from Naprod Life Sciences, Pvt.Ltd. (Thane, India).

Cell lines for toxicity study

Cell lines used in this study were cancer cells and normal cells. Cancer cells are consists of Breast Adenocarcinoma with HER-2/neu marker (ATCC CRL-3127) and Breast Adenocarcinoma with EGFR marker (ATCC CRL-3180) which were purchased from American Type Culture Collection. Normal cell used in this study was Fibroblast which was purchased from Invitrogen (Life Technologies, USA).

Preparation of encapsulated doxorubicin in alginate particle by ionic gelation method

Alginate particle encapsulation was based on the study of Lotfipour *et al.*[5] with some modifications. Alginate solution (0.1% w/v) was prepared using sodium alginate and calcium chloride (100 mM) was prepared using calcium chloride dihydrate. Calcium alginate particles containing Doxorubicin hydrochloride (DOX) was prepared by mixing desired concentration of DOX into alginate solution and sprayed to calcium chloride at a distance of 5 cm. The calcium alginate particles were left in the gelling medium for 15 minutes to give ample time for the alginate particles to complete its crosslinking. The particle was then separated from the gelling solution through centrifugation at 3000 rpm for 4 minutes and washed with sterilized distilled water.

Assessment of pH compatibility of doxorubicin with alginate-based particle

To test compatibility of alginate capsules at different pH, calcium chloride solution (100 mM) and alginate solution (0.1% w/v) were prepared by dissolving appropriate amounts of calcium chloride dihydrate and sodium alginate in ultrapure water. This is based on the study of Khemani *et al.*[3] and Lotfipour *et al.*[5] but with some modifications. Alginate solution (0.1% w/v) was adjusted to three different pH; 4.2, 7.2 and 11.2, and added with few drops of thymol blue (basic), bromcresol purple (neutral), and methyl orange (acidic) to determine its affinity to alginate-based particles. The calcium–alginate particles were prepared by adding alginate mixtures at different pH conditions using the mentioned pH indicators to calcium chloride solution using a glass sprayer via ionic gelation method as previously mentioned.

Particle size analysis of alginate particles

Particle size and mean of alginate particles were analyzed using phase contrast light microscope (Optika B-353, Italy) and upright type light microscope (Nikon Eclipse 90i, USA). Surface morphology of alginate particles was analyzed using scanning electron microscope (SEM Hitachi TM3000, Shimadzu, Japan).

Encapsulation Efficiency of Encapsulated DOX

Encapsulation efficiency of calcium alginate particles was analyzed by measuring the released DOX in PBS pH 7.2 which served as the releasing medium and incubated for 37^{o}C for 24 hours. Released DOX was measured at an absorbance of 301nm which served as the optimum wavelength for concentrated DOX as analyzed by the microplate reader (Corona Microplate Reader Hitachi SH-1000, Japan). Encapsulation Efficiency (%) was calculated as:

% encapsulation = ((Abs standard – Abs released DOX) / (Abs standard)) x 100

Efficiency of alginate as an encapsulating agent and as a drug delivery system was further confirmed using a Fourier transform infrared spectrophotometer (FTIR Shimadzu IRPrestige-21, Japan) with the use of universal ATR as an internal reflection accessory. FTIR analysis was performed in concentrated DOX, alginate solution and released DOX that were constituted with sterilized distilled water. The samples were in an ATR chamber for analysis. Results were analyzed with the use of IRSolution software (IR version 1.60, Shimadzu Corporation).

Estimation of in vitro release of doxorubicin from alginate-based particles

Encapsulated DOX in alginate particles were prepared using phosphate buffer saline as disintegrating medium at different pH (pH 4.2, 7.2, and 11.2) and were incubated at 37±0.1°C. At different time intervals, samples of 1mL were collected from the release medium, placed in a microcentrifuge tube and stored in refrigerator until further reading. The concentrations of DOX released in the solution were determined with the use of a standard curve of known concentrations ranging from 1.25 to 10 ppm with correlation coefficient R^2 = 0.9838. The amount of DOX released per time was used to obtain the release profile of the encapsulated doxorubicin in alginate-based particles. This was done in order to determine and monitor the amount of DOX released from the alginate-based particles at different time intervals and to determine the appropriate pH condition for optimum release of DOX within a certain period of time.

***In vitro* cytotoxicity assay using MTT**

Efficiency of the encapsulated DOX and free DOX to kill cells were assessed by *in vitro* cytotoxicity assay using human breast cancer cell lines (ATCC® CRL-3127 and CRL-3180), and human normal cell line (Fibroblasts) purchased from American Type Culture Collection (ATCC) and Invitrogen respectively. The two breast cancer cell lines were known to express EGFR receptors. Effect of drug concentration on the apoptosis of breast cancer cells and fibroblasts were assessed by using different concentrations of free DOX which was added into culture medium. Breast cancer cells and fibroblasts (~1×10^6) were seeded into 96 well tissue culture plate (BD Falcon, New York) in a culture medium consisting of 20% (v/v) fetal bovine serum (FBS) and 80% DMEM 1X (GIBCO) supplemented antibiotic and antimycotic solution. It was incubated with humidified air containing 5% CO_2 at 37°C for 24 hours. After cultivation for 24 hours, 100µL of culture medium in the 96-well plate was removed and 100µL of DMEM (phenol red free) containing different concentrations of free DOX and

encapsulated DOX were added into each well and incubated under the same conditions for 24 hours. The control group was cultured with the normal fetal bovine serum–DMEM culture medium (phenol red free) supplemented with antibiotic and antimycotic solution. After incubation with test samples, 100µL of media was removed and the retained cells and test sample were subjected to MTT assay.

Cell proliferation or cytotoxicity assay was measured by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. A 20µL of MTT solution (0.5mg/mL) was added into each well and were observed under microscope. After incubation, 100µL of DMSO was added into each well, place in a dark condition at room temperature for 30 minutes before reading the absorbance. The absorbance was read at 570 nm using a microplate reader (Corona Microplate Reader Hitachi SH-1000, Japan). The measurements were re-read after 24 hours for 3 days and the data were expressed as mean ± standard deviation.

RESULTS

pH Compatibility of Encapsulated DOX in Alginate Particle

After encapsulating the alginate at different pH, it was observed that at neutral pH (Figure 1), alginate particles were formed in greater number and with better particle morphology compared to basic and acidic conditions. While at acidic and basic pH, particles were lesser in terms of number, smaller than the reported particle size and disintegrated with no observable color of pH indicator.

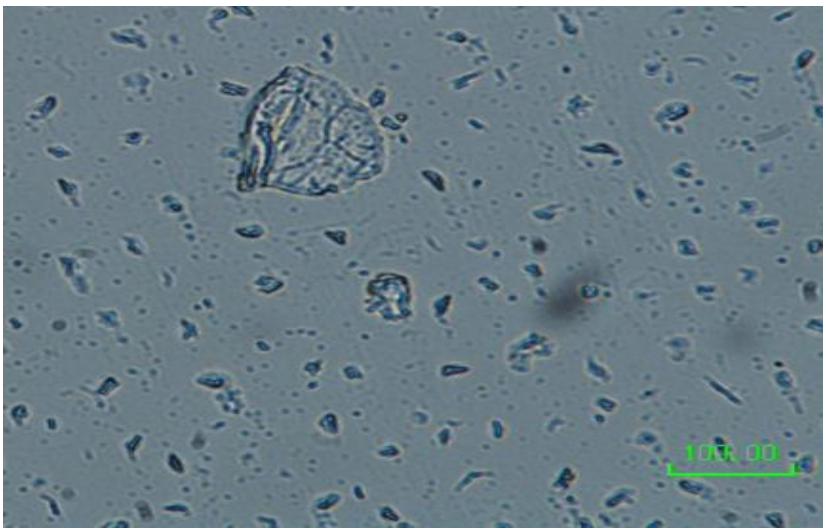

Fig. 1: pH compatibility of alginate solution stained with bromcresol purple (neutral pH) viewed under compound light microscope (20X).

Particle Size and Morphology of Encapsulated DOX

Doxorubicin hydrochloride (DOX) at concentrations ranging from 1.25ppm to 10ppm was encapsulated using 0.1% sodium alginate and cross-linked with 100mM calcium chloride. Encapsulation efficiency of the calcium alginate was evaluated by measuring the DOX released at 301 nm based on the optimum absorbance set by the microplate reader on concentrated DOX. A standard curve for DOX concentration was made with a linear equation of y=0.0039x+0.7246 with a linearity of R^2=0.9838. The standard curve was used in the entire experiment.

Particle size of calcium alginate particles were viewed under a phase contrast light microscope and upright light microscope. Mean particle size was measured and computed from the total of 27 particles. Based from the results, mean particle size from 300-400µm was computed from plain calcium alginate particle and 100-300µm was computed from the encapsulated DOX in alginate particle.

Actual particle size analysis under microscope was presented in Fig. 2 showing plain calcium alginate (fig. 2A) and encapsulated DOX (fig. 2B) based on scale bar measurements. SEM analysis of dried particles of calcium alginate showed that the dried calcium alginate was very brittle (fig. 2C) and it was also formed into crystal that was very rough and has many pores on its surface (fig. 2D).

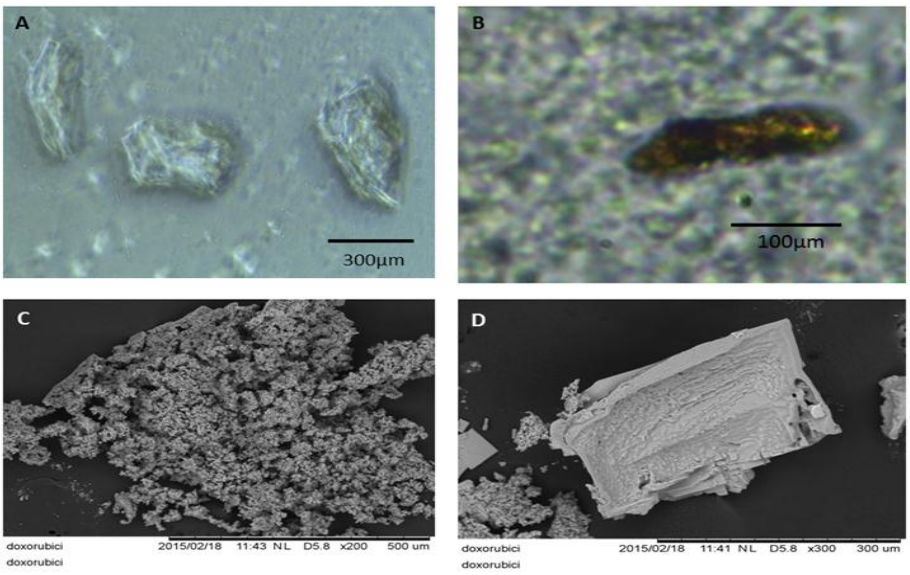

Fig. 2: Calcium alginate particles under phase contrast microscope (A) without DOX (40X) and (B) with 0.5mg/mL DOX (60X). Calcium alginate particle viewed under scanning electron microscope in (C) Disrupted form under 200X magnification (15kV) and in (D) Intact form containing DOX under 300X magnification (15kV).

Efficiency of Encapsulated DOX

The sodium alginate, DOX, and DOX encapsulated within a calcium alginate was analyzed using FTIR to confirm if DOX could penetrate the pores of the calcium alginate particles which is a characteristic indicative of a good drug delivery system (fig. 3). Spectrum on the FTIR result of sodium alginate (fig. 3A) showed peaks at 3250 cm^{-1}, 1400 to 1600 cm^{-1}, 1000 to 1100 cm^{-1}, and 1000 to 1400 cm^{-1}. Free DOX (fig. 3B) showed strong stretch on 1000 to 1100 cm^{-1}, hydroxyl stretch on 1050-1150 cm^{-1} for alcohol groups, 1400 to 1600 cm^{-1} for aromatic compounds, medium stretch at 1600 cm^{-1} indicates for amines, stretch on 1600 to 1800 cm^{-1} for alkene and carbonyl groups, 2800 to 2900 cm^{-1} stretch for alkanes, 3000 to 3500 cm^{-1} for alcohol groups, and 3300 to 3500 cm^{-1} for primary amines. In encapsulated DOX (fig. 3C), 1550 to 1650 cm^{-1}stretch was observed where the asymmetric bond of carboxylate ion shifted into lower frequency while the hydroxyl bond of the alginate was shifted to 3250 to 3500 cm^{-1} broad stretch. Shifting on the frequencies of the encapsulated DOX from the frequencies of sodium alginate was affected by the cross linking of the alginate with calcium ions present. Series of peaks observed from 1000 to 1100 cm^{-1} were due to the presence of α-L-guluronic acid which was part of the polymer of alginate.

Drug Release Profile of Encapsulated DOX

As shown in Fig. 4, doxorubicin has sustained released from the alginate particles at 8 to 20 hours of incubation in PBS at 37^{o}C with 99% encapsulation efficiency in 10ppm. Fast release of DOX was noticed at 4 to 8 hours at alkaline pH while sustained release of DOX in both neutral and acidic pH at 8 to 20 hours though only 12 to 14% doxorubicin was released in the medium. Its formulation has significantly inhibited at least 15 to 23% of ATCC-CRL 3180 breast cancer cells and normal fibroblasts with efficient slow release of DOX at normal physiological pH 7.2 and faster release rate in basic environment at pH 11.2.

***In Vitro* Cytotoxicity of Free and Encapsulated DOX**

Cell proliferative assay using MTT in normal fibroblasts and breast cancer cells (ATCC CRL-3180) with free and encapsulated DOX were shown in Fig. 5. Encapsulated DOX showed high inhibition on both cell lines compared to free DOX. Maximum inhibition was observed at 15 to 23% for both encapsulated and free DOX. Drug delivery was not specific as observed by the percent inhibition of the two cell lines were not significantly different from each other. Protection of normal fibroblast was not achieved because of the possibility that the calcium alginate particles incur further increase in acidity of medium used.

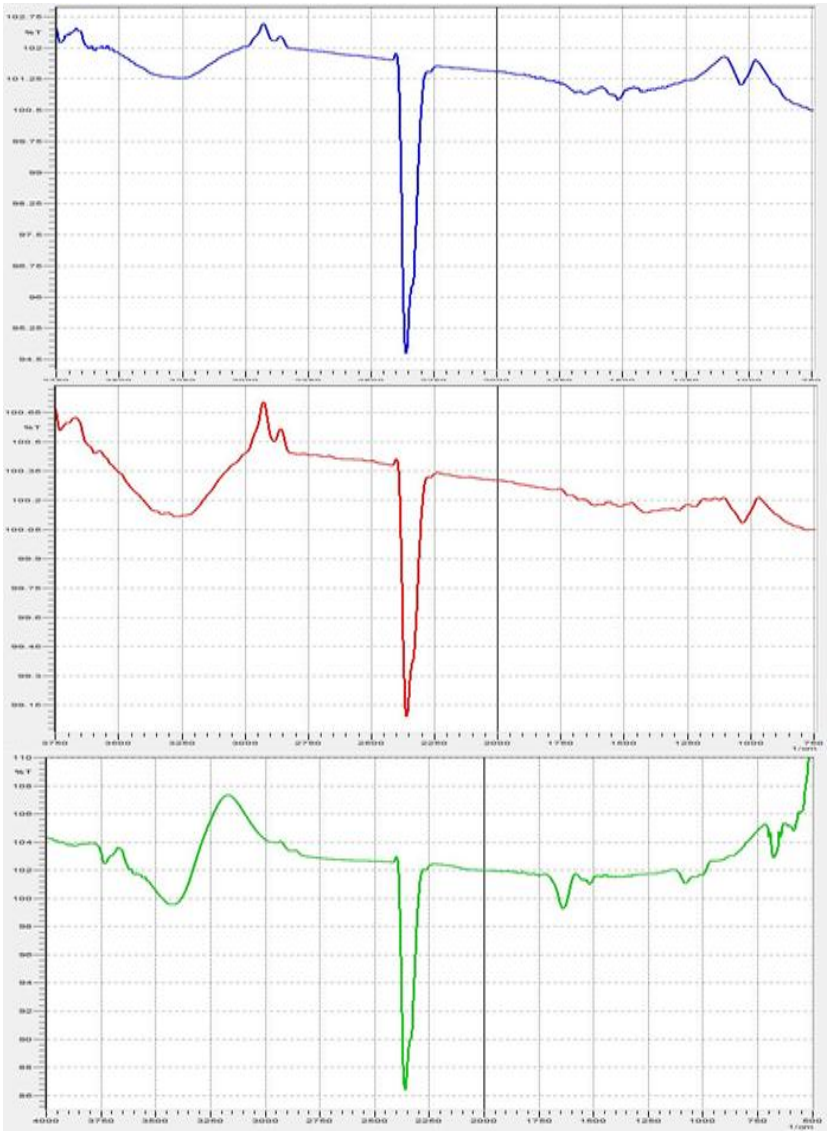

Fig. 3: FTIR Spectra of (A) sodium alginate, (B) Doxorubicin, and (C) encapsulated Doxorubicin showing the different peaks of corresponding functional group present.

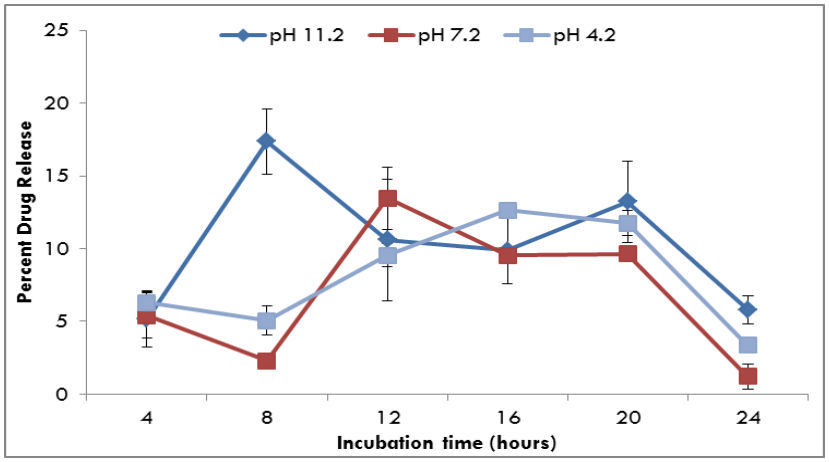

Fig. 4: Drug release profile of encapsulated DOX under different pH of releasing medium (1X PBS) ($p<0.05$).

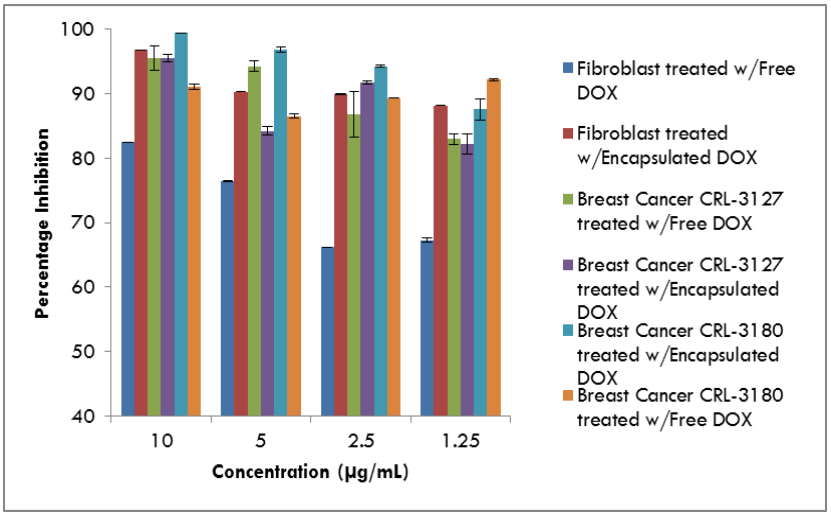

Fig. 5: Cytotoxicity assay using MTT of free and encapsulated DOX against human normal fibroblast, human breast cancer cells (ATCC® CRL-3127 and CRL-3180) ($p<0.05$).

DISCUSSION

In summary, we obtained doxorubicin calcium-alginate beads by ionotropic gelation with a particle size of approximately 100 µm and a rough surface. Encapsulation efficiency of alginate with DOX was found to be concentration dependent which is evident in the increase in percent encapsulation efficiency from 1.25ppm to 10ppm resulting to at most 99.30% at 10ppm. Upon reaching 100% encapsulation efficiency, there will be an observable excess DOX in the gelling medium which was calcium alginate. Encapsulation efficiency can be affected by different environmental factors such as time of agitation, speed of agitation, gelling time, concentration of alginate solution, and concentration of gelling medium.[6] It was observed from the microscopic analysis that upon entrapment of DOX in an alginate particle, the particle size became smaller than the usual alginate particle that has no entrapped drug. DOX may also affect the decrease on particle size due to the reaction of DOX with alginate and its cross linking with the help of calcium chloride.[7] According to Charles-Navarro *et al.*[6], average mean particle size of calcium alginate particle loaded with their test drug, Glibenclamide, was from 150 to 200 µm while Ramesh-Babu *et al.*[7] and Rodriguez-Llimos *et al.*[8] reported that calcium alginate particle loaded with their target drug was estimated with an average mean particle size of up to 300 µm. While Haeberle *et al.*[9] reported that mean size of calcium alginate particle size was observed from 150 to 300 µm even if they used micro nozzle and tested at different frequencies, particle size of the calcium alginate particle remains within the range. Lotfipour *et al.*[5] also reported average mean particle size of calcium alginate was range from 100 to 170 µm. Differences on the reported particles sizes were very little but they may vary because of the different variables and

parameters set during each experiments. Particle sizes that coincide within the range of acceptable values reported from different journals are very important due to their effect on drug release, kinetics and delivery. Characteristics of being crystal in nature and has rough edges and surface indicates that the calcium alginate was in its pure particle form and could carry drug even on its dry form which indicates a good drug delivery vehicle system.[1,10] While porous surface on the calcium alginate particle also suggest that it is a good drug delivery vehicle due to its strong capability to load drugs regardless of surface charge and hydrophilicity.[1] The presence of pores also contributes on better drug release, pharmacokinetics and pH and time-dependent drug release.[1,10]

Encapsulation efficiency of alginate with DOX was assessed by using FTIR and spectra showed sodium alginate peaks with OH^- stretching, COO^- symmetric and asymmetric stretching, C-O-C stretching, and presence of carboxyl and carboxylate which was also confirmed based on the result of Khemani *et al.*[3] Free DOX showed strong stretch that corresponds to the presence of ether, hydroxyl stretch for alcohol groups, C=C stretch for aromatic compounds, N-H groups for amines, C=C and C=O for alkene and carbonyl groups, alkanes, OH- broad stretch for alcohol groups, and N-H for primary amines which was also confirmed on the research of Khemani *et al.*[3] Encapsulated DOX was observed to have asymmetric bond of carboxylate ion while the hydroxyl bond of the alginate showed broad stretch. Shifting on the frequencies was affected by the cross linking of the alginate with calcium ions present. Presence of α-L-guluronic acid which was part of the polymer of alginate was observed by the presence of series of peaks.[3]

Its controlled release profile was satisfactorily achieved at 7% in PBS pH 7.4 at 37 ±1°C. The encapsulated DOX in alginate particle maybe a potent drug delivery vehicle specifically designed to treat targeted EGFR-related cancers. Its biocompatibility at neutral conditions and high encapsulation efficiency allows the drug to remain unaltered and maintain its potency at physiological pH. With this, it is recommend to use a consortia of alginate with other polysaccharide polymers to reduce the acidity of the alginate and vary the concentration of alginate and the calcium chloride that will further increase the efficiency and better sustained release of doxorubicin with reduced or no toxicity to normal cells.

ACKNOWLEDGEMENT

This work was supported in part by the University of Santo Tomas (UST) Research Center for Natural and Applied Sciences and the UST faculty research grant.

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