FR 1685

Final Report

Development of basic techniques for Nano-encapsulation of bioactive compounds.

NSF Grant : RG/2010/NANO/04

Principal Investigator: Prof. DN Karunaratne

Co-Investigator: Prof. V Karunaratne

Department of Chemistry
University of Peradeniya
Peradeniya

Section 1

Information regarding Project/Project Personnel:

i) Grant Number RG/2010/NANO/04
ii) Title of the Project - Development of basic techniques for Nano-encapsulation of bioactive compounds
iii) Principal Investigator Prof. DN Karunaratne
iv) Co-Investigators Prof. V Karunaratne
v) Institute(s) where research was being carried out Dept. of Chemistry, University of Peradeniya.
vi) Date of award 06/07/2010 (project commenced 01/01/2011)
vii) Date of completion of Project 11/12/2013
viii) Total allocation of funds (Rs) 2,516,258.00
ix) Total spent (Rs) 2,093,536.25
x) Number of Research Students employed One post graduate (with stipend), two final year undergraduates (no stipend).
xi) Post graduate degree completed with dates –thesis submitted on 11/12/2013
xii) Number of Technical Assistants and/or labourers employed and period of service none
xiii) Publications/Communications arising from the project during the reporting period.

1. W.M.T.N.B. Wanninayake, N.L.V.V. Karunaratne and D N Karunaratne. Preparation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanoparticles for sustained release of folic acid. *Proc. of Peradeniya University Research Sessions (PURSE),* Vol. 17, **2012**. (held in July 2013).

2. M. A. S. K. Menikarachchi, N. Karunaratne and V. Karunaratne. Nano-carrier system for controlled release of folic acid. *Proc. of Peradeniya University Research Sessions (PURSE),* Vol. 17, **2012**. (held in July 2013).

3. K.K.D.R.M. Wimalasinghe and D.N. Karunaratne. Formation of chitosan-alginate nanoparticles and encapsulation of curcumin. *Proc. of Peradeniya University Research Sessions (PURSE),* Vol. 17, **2012**. (held in July 2013).

4. M. A. S. K. Menikarachchi, D. N. Karunaratne, V. Karunaratne, V. Thevanesam & A. Ekanayake. Chitosan based Drug-Carrier System for Controlled Release of Amoxicillin, *International Conference on Chemical Sciences Proceedings,* **2012.**

5. M. A. S. K. Menikarachchi, D. N. Karunaratne, & V. Karunaratne. Chitosan based Nano-Carrier System for Controlled Release of Ascorbic Acid. *Poster presented at the Global Forum of Sri Lankan Scientists held at the Hotel Galadari,* **Dec 2011.**

6. M.A.S.K. Menikarchchi, N. Karunaratne and V. Karunaratne. Chitosan based nano-carrier system for controlled release of ascorbic acid. *Proc. of Peradeniya University Research Sessions (PURSE),* Vol. 16, **2011**.

Section 2

Executive Summary of the Project:

This should be limited to 200-250 words and include the scientific background and objectives, methodology and major findings

Encapsulation both at nano and microscale is a means of imparting improved properties to small molecules of interest. Enhanced solubility, bioavalability, reduced toxicity and targetability are some of the advantages of encapsulation. This project was based on the premise that procedures for encapsulation though known, were not established at a National level. Thus an attempt was made to use a variety of carriers and encapsulate drugs at the nanoparticle level.

Chitosan and alginate having biocompatibility, biodegradability and non toxicity were chosen as carrier material. Another lesser known polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) was also selected as a carrier. The methodologies for encapsulation were ionotropic gellation, precipitaion coacervation and for PHBV, double emulsion technique with spraying. Liposomes were also tested in the free form as well as coated with chitosan. Known bioactive compounds- ascorbic acid, folic acid, amoxicillin and curcumin were encapsulated.The techniques all enabled the formation of nanoparticles which were characterized by FTIR, SEM, particle size analysis and zetapotential measurements. The drug loading capacities measured indicated that in the case of hydrophobic molecules such as curcumin, loading was very low.

The release of drugs were monitored in vitro using dialysis membranes. The release profiles indicated that liposomes, liposomes coated with chitosan and chitosan nanopartilces all released nearly 60 -70% of their cargo in 7 h. However, the chitosan-alginate composite NPs showed longer release with 60% release in 24 h. The PHBV nanoparticles released folic acid at pH 7.4 over a time frame of over 10 d but at pH 1.0 the cargo was unloaded within 1 h. These findings are of relevance for delivery applications.

Section 3

Report in detail: should contain the following (not less than 2000 words excluding Tables and Figures)

i) Introduction/background

Although many drugs and bioactive compounds are known and are in use in various applications, the full benefit of the compound may not be obtained due to factors such as poor solubility, reduced uptake, sensitivity to external media and degradation of compound. To overcome these difficulties, compounds have been encased in carrier matrices to improve drug properties. Drug companies have performed extensive research for encapsulation techniques using polymers and liposomes. Each drug requires its own method of encapsulation to produce optimum benefit and needs to be treated individually. Microencapsulation is widespread in the pharmaceutical arena, however, nanoencapsulation is more advantageous due to improved delivery, penetration and absorption.

Nanocarriers can increase the solubility of a drug and improve its bioavailability. They have the added advantage of being able to penetrate tumours and can be used as targeted delivery systems in cancer treatment as well as to reduce toxicity and side effects.

Nanoparticles of polymeric matrix systems in which the drug is uniformly dispersed in the former, or which encapsulates or surrounds the drug dissolved or entrapped in an oil or aqueous core in the latter have been formulated by several researchers. The matrix

system is a biodegradable and biocompatible polymer. Polylactic acid (PLA), polyglycolic acid (PGA), poly-lactic-glycolic acid (PLGA) and polymethylmethacrylate (PMMA) are synthetic polymer matrices which have been used to encapsulate proteins, genes and DNA and deliver them effectively to target cells. Natural polymers have the added advantage of being water soluble as well as biodegradable. Chitosan, gelatin, albumin and sodium alginate are natural polymers. Natural polymer nanoparticles are superior at delivering drugs, reducing side effects, have increased absorption and improve and prolong therapeutic effects compared to synthetic polymer matrices.

Research towards the development of nanoencapsulation techniques was undertaken for the purpose of applications of these techniques in the pharmaceutical industry, cosmetics and textile industries. Before embarking on the production of encapsulated bio-active compounds, it is necessary to perfect the technique of encapsulation. The carriers or encapsulants selected were the natural polymers chitosan and alginate and a biopolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). Since these are of biological origin they are non toxic and biodegradable polymers and possess biocompatibility. The bioactive materials/drugs selected were amoxicillin, folic acid, ascorbic acid, and curcumin. Amoxicillin is an antibacterial drug which has a circulation time of approximately 2 hours. Therefore a slow release formulation which could keep the drug in circulation for a longer time would reduce the need for multiple administration.

The most important aspect will be to monitor the effectiveness of the product through in vitro as well as in vivo bioassays. In our project, the in vitro release from all the encapsulants was monitored to determine the time of release. The PHBV system showed promise as a slow release matrix in comparison to the Chitosan and alginate systems.

ii) Scientific scope of the project (overall and specific objectives)

Effective drug delivery ensures that the drug is in a form that is easily and rapidly absorbed into the system for fast action at the target site. Some drugs or therapeutic agents, which show promise in laboratory trials, fail to produce the same effect in the human body. The degradation of drug before it reaches the target site (poor bioavailability or inability to cross the blood brain barrier) or poor solubility of drug in the delivery medium results in decreased efficacy. The main thrust has been to perfect the technique of nanoencapsulation using the polymer matrices and to determine the drug release properties.

Our objectives in summary are:

1. To develop a method for nanoencapsulation using casienate/alginate/chitosan as well as clay polymers. Later we will look at synthetic polymers of the type PLGL etc.
2. To encapsulate known drugs (amoxicillin, Vitamin C and E etc.) and determine the optimum loading capacity, release properties and changes in solubility. Later the study will be extended to bioactive compounds.
3. To establish a practical and economical method for compound encapsulation. This will be useful for commercial companies interested in encapsulating various compounds.

iii) Materials and methods (including statistical methods)

Nanoencapsulation of ascorbic acid:

Extraction of Egg yolk lecithin (Phosphatidylcholine)
Fresh egg yolk was mixed with 95% ethanol (100 ml) and stirred until egg yolk completely dispersed and centrifuged at 1900g for 5 minutes. The precipitate was extracted twice with pet ether (50 ml) and washed twice with 90% ethanol (50ml). The ethanol and pet ether fractions were collected into a separatory funnel and allowed to separate into layers. Ethanol phase was evaporated using a Rotary Evaporator (Heidolph LABOROTA 4000) and dispersed in Hexane (35 ml). Chilled Acetone (150 ml) and the mixture centrifuged at 2000 g for 5 minutes (Sigma 3K30, Germany) and the precipitate (Polar Lipid Fraction) was collected.

Preparation of Ascorbic acid loaded liposome suspension
Phosphatidylcholine and cholesterol were dissolved in chloroform and solvent evaporated using a rotary evaporator (Heidolph LABOROTA 4000). Next, de-ionized water containing 1.4%, W/V Ascorbic acid was poured and solution was kept under mechanical shaking for 1¾ hour at 45 °C. The resulting mixture was kept overnight at 4 °C to obtain liposome suspension by self- assembly.

Coating process using sodium sulphate cross-linked Chitosan
Chitosan coated liposomes were prepared by the use of precipitation/ coacervation method. Chitosan was dissolved at a concentration of 0.25% (w/v) in a solution with 2% (v/v) of acetic acid and 1% (w/v) of Tween 80 and dropped into liposome suspension solution at a rate of 1 ml/min with mechanical shaking and continuous Nitrogen purging. Sodium sulfate solution (10%, w/v) was added to the solution at a rate of 1 ml/min under mild agitation (<60 rpm) and continuous sonication (Branson sonicator; model 2510) and centrifuged for 30 min at 10000 rpm (Sigma 3K30, Germany). The particles were re-suspended in de-ionized water, frozen and freeze-dried overnight using a freeze-dryer (Edwards K4, Britain).

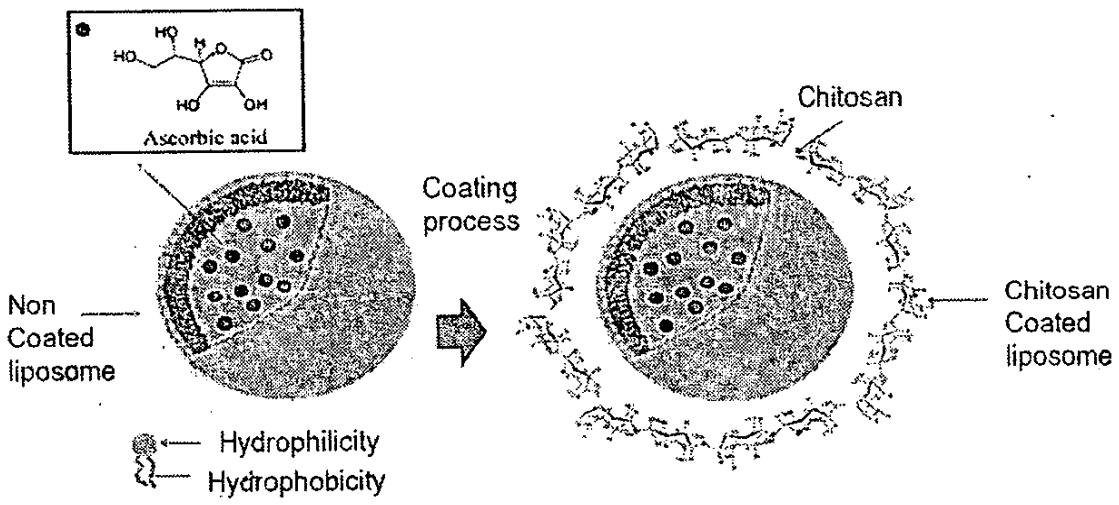


Fig. 3.1 A schematic of non-coated and a coated liposome

Nanoencapsulation of amoxicillin:

Preparation of Amoxicillin loaded liposome suspension
Same procedure as for ascorbic encapsulation was followed using 0.01%, W/V Amoxicillin instead of 1.4%, W/V Ascorbic acid.

Coating process using STTP cross-linked Chitosan
The chitosan solution (0.25% (w/v) in a solution with 2% (v/v) of acetic acid and 1% (w/v) of Tween® 80) was flush mixed with an equal volume of STPP (Sodium tripolyphosphate 0.5 mg/ml) solution and the crosslinking of chitosan-STPP particles began spontaneously via the ionic gelation mechanism. Next, the amoxicillin loaded liposome suspension solution was added into the prepared chitosan-STPP solution with mechanical shaking and continuous sonication (Branson sonicator; model 2510). The suspension was kept overnight at 4 °C and centrifuged for 30 min at 15000 rpm (Sigma 3-30KS, Germany). Supernatant was kept for further analysis of non-bound Amoxicillin. The particles were re-suspended in de-ionized water, frozen with 2% (w/v) of sucrose and freeze-dried overnight using a freeze-dryer (Edwards K4, Britain).

Nanoencapsulation of folic acid:

Precipitation/ Coacervation (PC) method for encapsulation of Folic acid
Chitosan was dissolved at a concentration of 0.25% (w/v) in a solution with 2% (v/v) of acetic acid and 1% (w/v) of Tween 80. The formation of the particles was achieved after the addition of sodium sulfate solution (10%, w/v). The addition was made at a rate of 1 ml/min under mild agitation (<60 rpm) and continuous sonication (Branson sonicator; model 2510). The suspension was centrifuged for 30 min at 3000 rpm (Sigma 3K30, Germany). The particles were re-suspended in de-ionized water, frozen and freeze-dried overnight using a freeze-dryer (Edwards K4, Britain), and kept frozen until further analysis. Next, freeze-dried chitosan particles were placed in PBS (pH 7.4) and sonicated for 30 min. Then, folic acid was added for incubation and the suspension was centrifuged for 30 min at 3000 rpm (Sigma 3K30, Germany). The particles were re-suspended in de-ionized water, frozen and freeze-dried using a freeze-dryer ((Edwards K4, Britain).

Ionotropic gelation (IG) method for encapsulation of Folic acid
Liposomes were prepared using phosphatidylcholine (lecithin, egg yolk) and cholesterol as described in the *Preparation of Ascorbic acid loaded liposome suspension* section using de-ionized water containing folic acid instead of ascorbic acid.
Same procedure as for *Coating process using STTP cross-linked Chitosan* was followed using the folic acid encapsulated liposomes instead of the amoxicillin loaded liposomes. The suspension was kept overnight at 4°C and centrifuged for 20 min at 25000 rpm (Sigma 3-30KS, Germany). The particles were re-suspended in de-ionized water, frozen with 2% (w/v) of sucrose and freeze-dried overnight using a freeze-dryer (Edwards K4, Britain).

Preparation of PHBV/Folic acid nanoparticles
PHBV/Folic acid nanoparticles were prepared by following modified simultaneous double emulsion (water-in-oil-in-water) solvent evaporation technique. At room temperature powder of PHBV 0.0500 g were solubilized in 4 ml of DCM and 1 ml of ACE during two hours, containing Tween-80 (100 microliters) as an emulsifier. Afterwards, 1 ml of folic acid was

added in the PHBV solution in ACE & DCM while continuously being homogenized at about 200 rpm using mechanical stirrer during 30 minutes, and then it was kept in sonication bath about 10 minutes while stirring with mechanical stirrer. Then this primary W/O emulsion was further added to 20 ml of external water with homogenization for 3 minutes to achieve the stable double emulsion (W/O/W). The resulting emulsion was very slowly poured (instilled for 15 min) into 100 ml of aqueous PVA solution (0.05% w/w) while continuously stirring at 1200 rpm to make the nanoparticles to solidify. The residual organic solvents were evaporated under negative pressure and the nanoparticles suspending in emulsion were collected by ultracentrifugation at 12000 rpm and washed with distilled water three times. Finally the products were dried by freeze drying technique and stored at -78 °C for further tests. The supernatant solution was dried and diluted up to 25.00 ml and stored for the analysis of folic acid content using spectrophotometry.

Identification of most suitable particle preparation method

In the above addition of the folic acid on to the oil phase (PHBV+DCM+ACE) was done by different techniques to obtain uniform, small in size, less agglomerating particles.

1. Folic acid addition – without sonication step.
2. Folic acid addition – with sonication step.
3. Folic acid addition – using hand spray technique, followed by sonication.
4. Folic acid addition – using atomization sprayer, followed by sonication.

In all the cases particles obtained at the final step were observed using light microscope to gain a general idea about the prepared samples. By observing those, method which gives a small, non-agglomerating & uniform set of particles was identified and further studies were carried out by chosen method.

Variation of PHBV/Folic acid ratio.

The folic acid concentration in water was varied in order to obtain nanoparticles with different ratio of PHBV and folic acid. (PHBV/folic acid 95/5%wt, PHBV/folic acid 90/10%wt, PHBV/folic acid 85/15%wt, PHBV/folic acid 80/20%wt). Accordingly, the weights of folic acid in 1 ml of water were 2.640 mg,5.56 mg, 8.82 mg, 12.5 mg. During the solvation of the folic acid, sodium hydrogen carbonate was added to water until a slightly alkaline environment was reached, all in order to increase the solubility of the folic acid.

Nanoencapsulation of curcumin:

Isolation of curcumin

Sun dried and well ground Turmeric rhizomes were extracted with dichloromethane to obtain about 2.18 % yield of Curcumin.(Anderson, A.M., et al. 2000).

Preparation of curcumin loaded chitosan-alginate nanoparticles

A curcumin incorporated $CaCl_2$ solution was added to a 0.063% sodium alginate solution at pH 4.9 dropwise under constant stirring. To this was added a 0.005% w/v solution of chitosan in 1% w/v acetic acid at pH 4.6. The resulting pregel was stirred for 45 min and the nanoparticles were separated by centrifugation at 12000 rpm, 4 °C for 40 min. The pellet obtained was freeze dried.

Determination of encapsulation efficiency and loading capacity

The drug loading capacity (LC) and loading efficacy (LE) were calculated using the following equations:

LC (%) = [(Drug (total) – Drug (supernatant))/ CL Drug] ×100 (1)

LE (%) = [(Drug (total) – Drug (supernatant))/ Drug (total)] ×100 (2)

(CL Drug = Chitosan coated Liposome, with Drug).
Note: for the PC method CL Drug = Drug loaded Chitosan nanoparticle
For curcumin method CL drug = curcumin loaded nanoparticles
For the polymer method CL drug = folate loaded PHBV

Characterization

For the characterization purposes, Scanning Electron Microscope (SEM) images were taken (SU6600, Hitachi) to observe the morphology of the particles. Optical microscope was used to observe the approximate size range. FTIR were obtained by using an IRPrestige-21, SHIMADZU spectrometer. The particle size and zeta potentials were obtained using a particle size/ zeta potential analyzer (Malvern zetasizer, Malvern Instruments Ltd.)

Release studies

Ascorbic acid

Nanoparticles were suspended in Phosphate buffer solution (pH 6) and inserted in to dialysis tubing (cellulose membrane), ends sealed and placed in a beaker containing Phosphate buffer solution (pH 6). Constant stirring was applied using a magnetic stirrer. 3 ml of aliquots were taken from the beaker and tested for Ascorbic acid at 266 nm UV absorbance until the observation of constant values.

Amoxicillin

To check the release properties, nanoparticles suspended in Phosphate buffer solution (pH 7.4) were dialyzed (cellulose membrane) against a Phosphate buffer solution (pH 7.4). Constant stirring was applied using a magnetic stirrer. 1 ml of aliquots were taken from the beaker and tested for Amoxicillin using C18 HPLC column (3 × 150 mm, Agilent Corp.). Mobile phase (50% acetonitrile/50% water) was pumped at 0.5 mL/minute resulting in typical retention times of 2.4 minutes. Concentrations were determined from peak absorbance measured at 229 nm until the observation of constant values.

Folic acid from Chitosan

Nanoparticles were suspended in Phosphate buffer solution (pH 7.4) and dialyzed against Phosphate buffer solution (pH 7.4). 3.0 ml aliquots were taken from the beaker under constant stirring and absorbance at 351 nm was measured to detect folic acid.

Folic acid from PHBV

Known mass of the prepared particles and 5 ml of buffer solution were kept inside a Dialysis Membrane Tubing which has a molecular weight cutoff of 1000 Daltons. And it was kept in a 20 ml PBS solution. The drug concentration in the medium was calculated using a calibration curve of the drug in the corresponding release medium at various concentrations. The nanoparticle dispersions in closed centrifuge tubes were kept at 25.0 °C and stored in the absence of light. At different time points, the supernatant was taken and analyzed.
The same procedure was followed using 0.1 mol dm^{-3} HCl as the release medium.

Curcumin

Nanoparticles were suspended in Phosphate buffer solution (pH 7.4) and dialyzed against Phosphate buffer solution (pH 7.4) with mild stirring. 2.0 ml aliquots were taken at relevant time intervals and absorbance at 452 nm was measured.

Antimicrobial activity of Amoxicillin-loaded chitosan nanoparticles

Chitosan coated Amoxicillin particles suspended in Phosphate buffer solution (pH 7.4) were dialyzed with stirring in a beaker containing Phosphate buffer solution (pH 7.4). After 1hr, 1 ml aliquot from PBS solution (dialyzate) was taken and subjected to two

fold serial dilution in BHI. Separate tube sets were prepared with 1ml each of double strength 3.45% (w/v) Brain Heart Infusion (BHI) agar medium). 10 µl drop of 10% diluted 0.5 MF (No. of colonies 1.5 X 10^6 cfu/ ml) *Staphylococcus aureus* (NCTC-6571 strain) was added to each serial dilution and incubated at 37 °C. Test was repeated for other tube sets at specified time intervals. Turbidity of the solutions was observed after 18-24 hour incubation. Tubes with less or no turbidity were streaked in blood agar plates for further clarification of existence of bacteria. A similar experiment was performed with amoxicillin alone (without encapsulation) in buffer inside the dialysis bag. After the incubation, clear solutions were streaked in blood agar plates to confirm the test results.

Statistical analysis

Standard deviations and the averages were calculated by using Microsoft Excel-2010 And all the graphs were drawn and corresponding statistical analysis done by using Origin-8.5 Pro software.

iv) Results/outputs

Results and Discussion

Part 1 Chitosan carrier system for ascorbic acid.

Characterization of the nanoparticles.

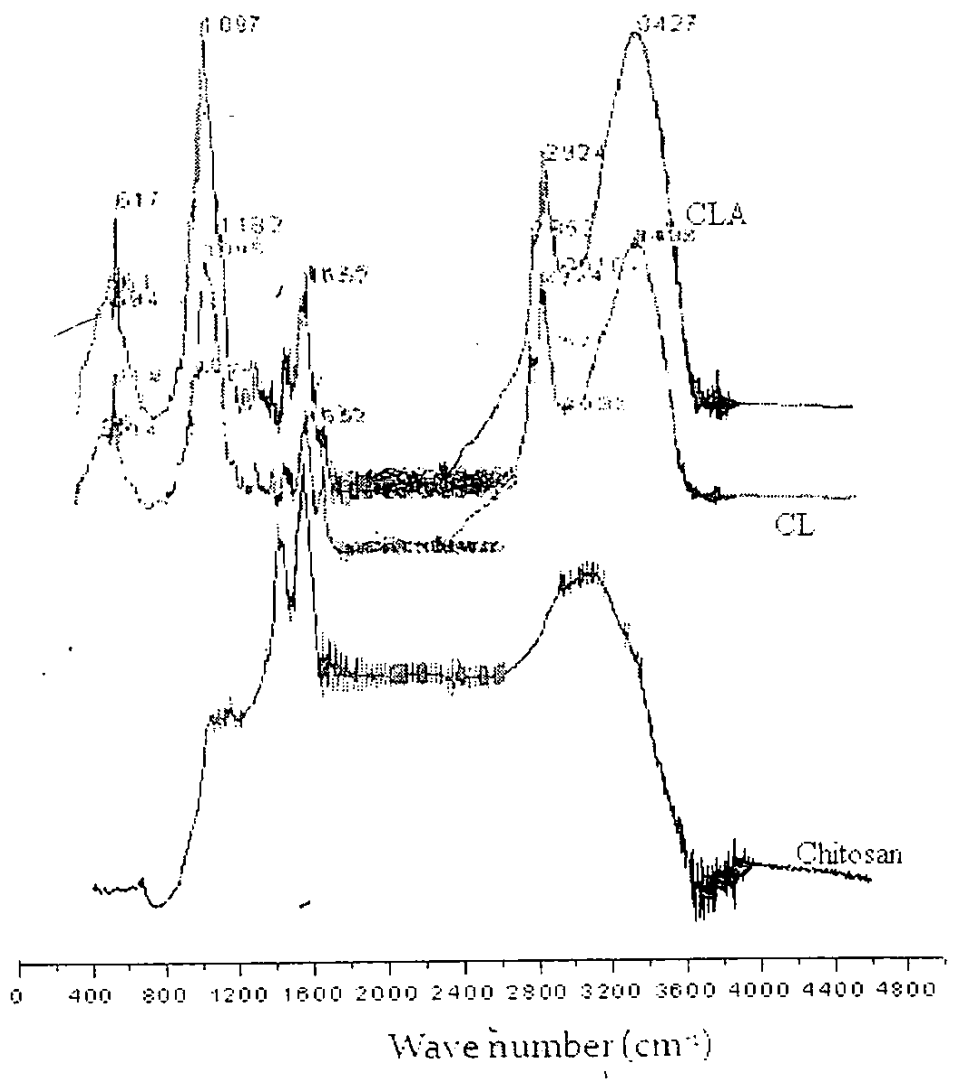
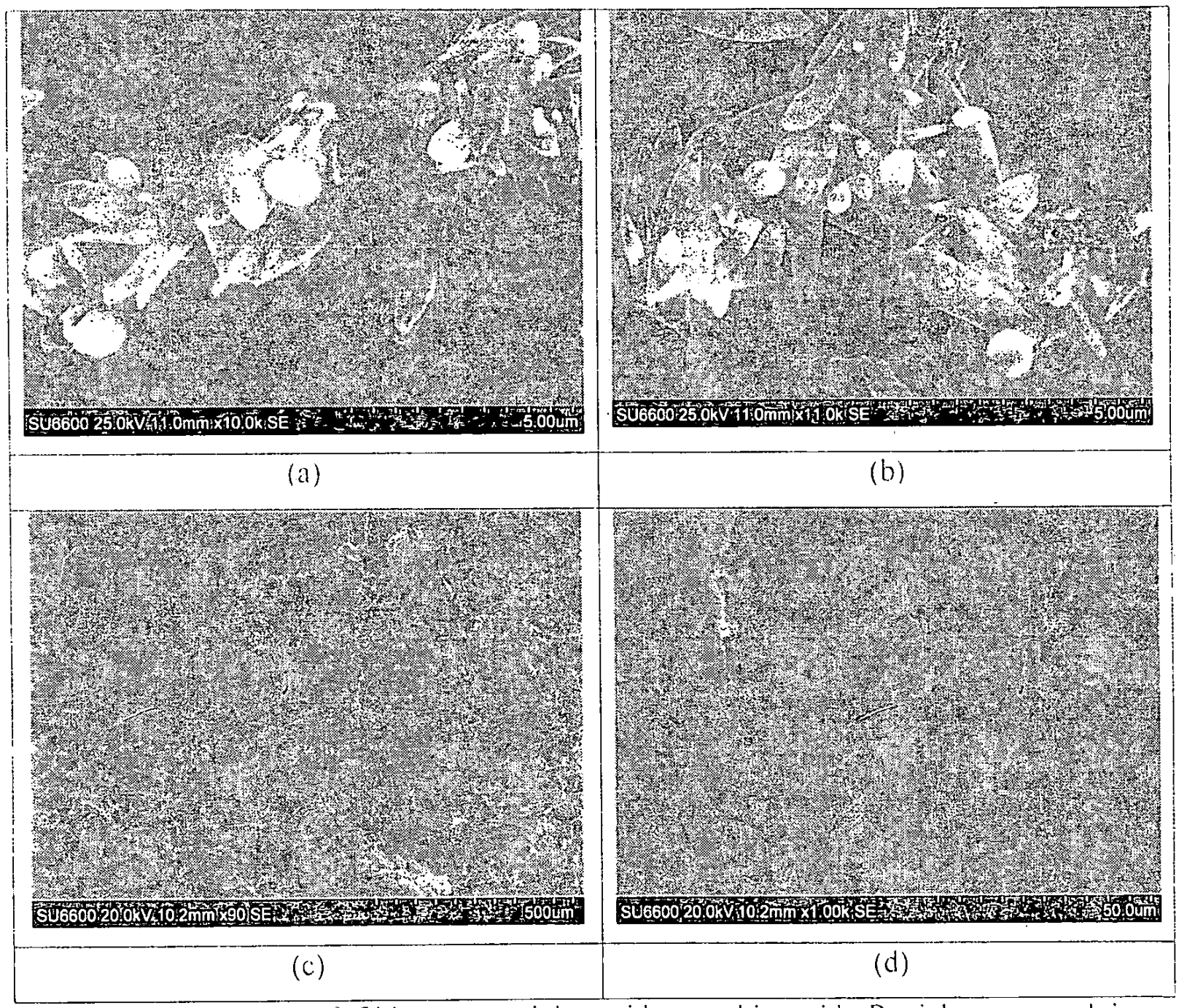


Fig 1 FTIR spectra of chitosan, chitosan coated liposomes (CL) and ascorbic acid encapsulated liposomes with chitosan coating (CLA).

FTIR comparison of loaded and unloaded nanoparticles were done. The FTIR spectra of chitosan, CL and CLA particles are shown in Fig. 1. The spectra of Chitosan coated

Liposomes (CL) and CLA indicate that the ascorbic acid is encapsulated within the chitosan coated liposomes. The strong and broad peaks in the 3400–3200 cm^{-1} ranges correspond to combined peaks of O-H stretching and intermolecular hydrogen bonding. The C=O stretching (amide) peak is observed near 1633 cm^{-1}.
According to these observations it is evident that the formation of cross-linked chitosan particles has taken place.

The SEM images of the ascorbic encapsulated liposome coated with chitosan was obtained. The SEM images were taken for the samples with and without nitrogen environment (Fig. 2).



(a) (b) (c) (d)

Fig. 2 SEM images of Chitosan particles with ascorbic acid. Particles prepared in nitrogen environment (a) and (b). Particles prepared without nitrogen environment (c) and (d).

Micro sized particles were obtained when the particles were prepared in nitrogenated environment (Fig 2 a and b). Preliminary tests without nitrogen environment showed early degradation of ascorbic acid (Fig 2 c and d). Therefore sample preparation was performed in nitrogen environment.
Loading capacity:

The drug loading capacity (LC) and loading efficacy (LE) obtained are shown in Table 1. The loading capacity was found to be very low but loading efficiency was moderate.

Table 1 The drug loading capacity (LC) and loading efficacy (LE)

	LC (%)	LE (%)
	3.36	52.38

Release of ascorbic acid:

According to Fig 3, when ascorbic acid is encapsulated in the chitosan coated liposome, sustained release over 8 h is observed. Free ascorbic acid in solution was dialyzed as a control and was found to be released within 1-2 h.

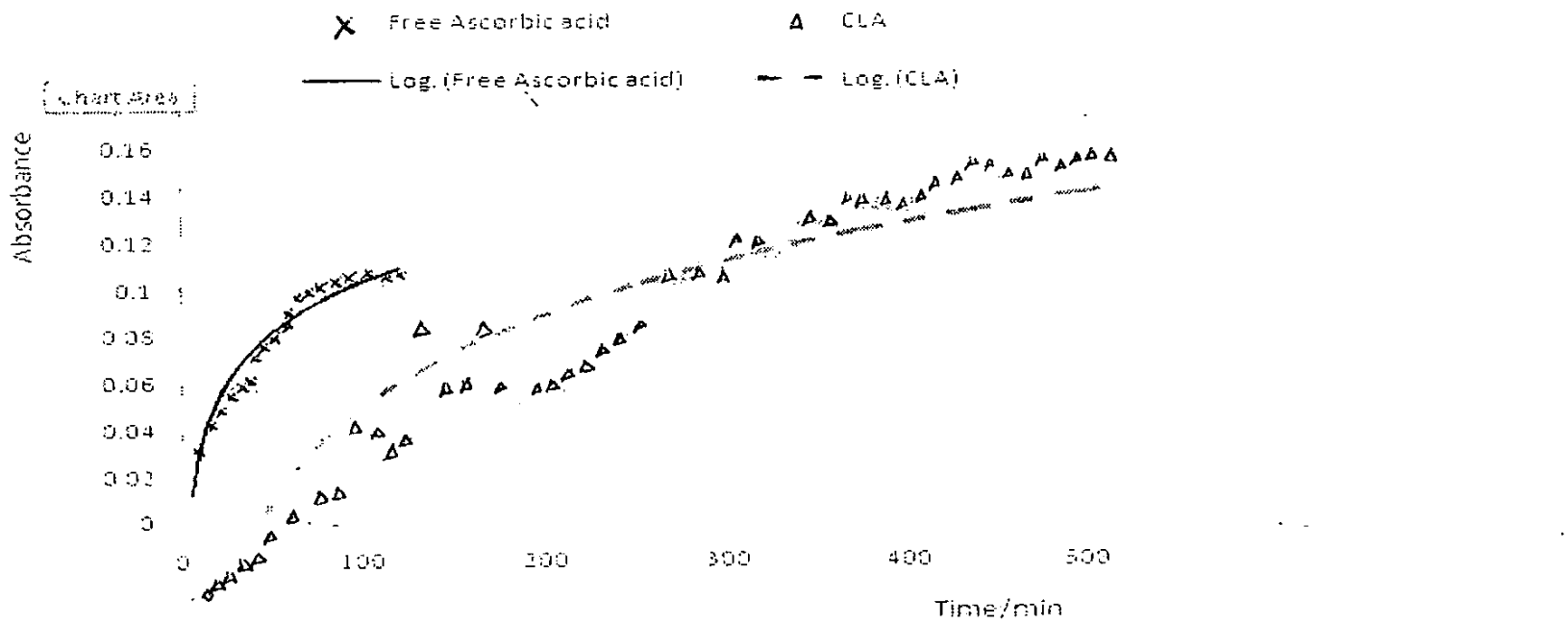


Fig. 3 Release behavior of free Ascorbic acid and Ascorbic acid from Chitosan-coated particles in Phosphate buffer solution (pH 6).

Conclusion

The objective was to find a method for encapsulating ascorbic acid in liposomes with chitosan coating. Ascorbic acid is sensitive to heat and alkali and requires careful handling during the encapsulation process. Particles prepared in the nitrogen environment does effect the morphology and stability of ascorbic acid. The carrier system was prepared using sodium sulphate as the cross linker for chitosan and showed formation of micro range particles. The release of Ascorbic acid from this carrier system lasted over an 8 hr. time period indicating sustained release. The drawback of this method is the poor loading capacity.

Part 2 Chitosan carrier system for amoxicillin.

The carrier system developed for ascorbic acid in Part 1 had drawbacks that the particle size was large and the loading capacity was low. In order to maximize the loading efficiency and loading capacity of the delivery system ionotropic gelation method with sodium tripolyphosphate as the cross linker was used along with amoxicillin as the model drug. Sucrose was added as a lyoprotectant prior to the freeze drying of the sample.

Amoxicillin is an antibiotic with a circulation time of 1.5 h. It is usually administered 8 hourly in three doses. The objective of this study was to modify the carrier to obtain a

slow release such that administration could be minimized to once a day or twice daily. In addition to monitoring the release profile of amoxicillin, the activity of the amoxicillin as a drug was confirmed with *Staphylococcus aureus* (NCTC-6571 strain).

Characterization

The chitosan coated liposomes loaded with amoxicillin showed very similar FTIR spectra (Fig.4 as with the ascorbic encapsulated particles (Fig 1). According to these observations it is evident that the formation of cross-linked chitosan particles has taken place. Therefore the change from sodium sulphate to STTP for crosslinking has not affected the nanoparticle composition.

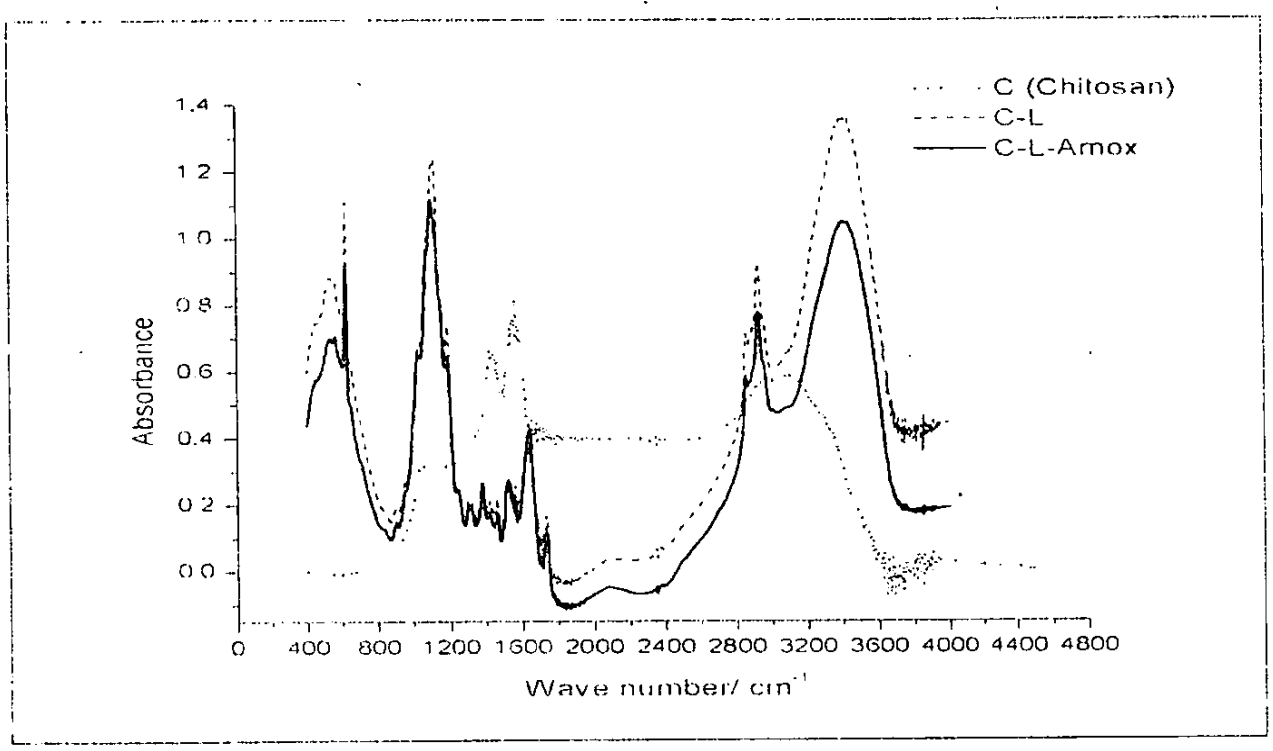


Fig. 4 FTIR data for Chitosan (C), Chitosan coated Liposome without Amoxicillin (C-L) and Chitosan coated Liposome with Amoxicillin (C-L-Amox)

SEM images were taken for the sample with sucrose as the lyoprotectant (Fig. 5).

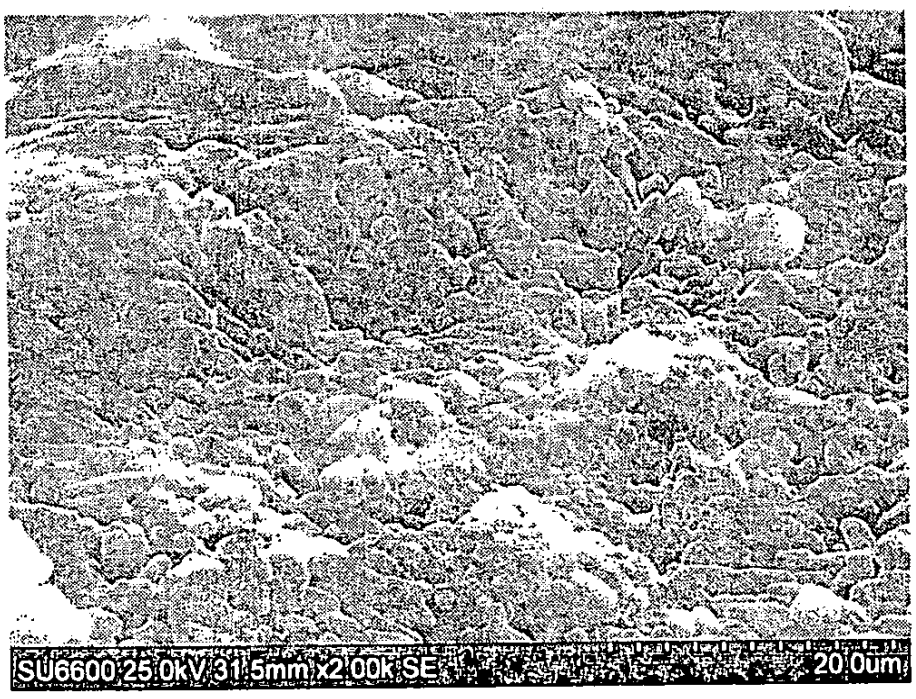


Fig. 5 SEM image of chitosan particles crosslinked with STPP in sucrose medium.

NATIONAL SCIENCE FOUNDATION

INTERNAL MEMO

FROM:- Mrs. Eranji Chathurika (SO)

TO:- Head / NSLRC Through + Head / RD

DATE:- 10/03/2015

10/3/15

SUBJECT:- The Final Report submission to NSLRC

The Final Report of the (with soft copy) RG/2010/NANO/04 by Prof. D.N. Karunarathna, Department of Chemistry, University of Peradeniya is hereby submit to the NSLRC.

Good.

Not in the system
Details need to be entered

Pujitha
11/03/15

Morphology of the particles was in micro range crystalline form with the sucrose. But size of the delivery vehicle measured in the particle size analyzer shows 18 nm mean diameter in solution. Mean value for the zeta potential is indicated as -12.6 mV ensuring that the particles have negatively charged surface. However, the low negative value is indicative of low stability and greater tendency for aggregation. The large particle size observed in the SEM images may be due to particle aggregation, while the particle size measurement in solution recorded a small size.

Loading capacity:
The drug loading capacity improved to 6.08% but the loading efficacy (LE) remained almost the same at 51.61%. However, the loading capacity is still low and either sodium sulphate or STTP as crosslinker has not made any improvements to the LC.

Release of amoxicillin.
In order to study the effect of the crosslinker on the release of amoxicillin, the release profile was observed by HPLC (See Fig. 6). Since it was known from other studies in our laboratory that liposomes release drugs within a time period of 6-8h, the release profiles of both liposome encapsulated amoxicillin and chitosan coated liposomal amoxicillin were subjected to the same release conditions. The percentage of amoxicillin released in the first 6 hours amounts to approximately 70%. Liposomal amoxicillin and chitosan coated liposomal amoxicillin both show nearly similar release profiles. Therefore there was no advantage in having the chitosan coating for prolonging the release of the drug.
In addition when compared to the release observed with the sodium sulphate crosslinked chitosan in Part 1, there is no significant change in the time of release.

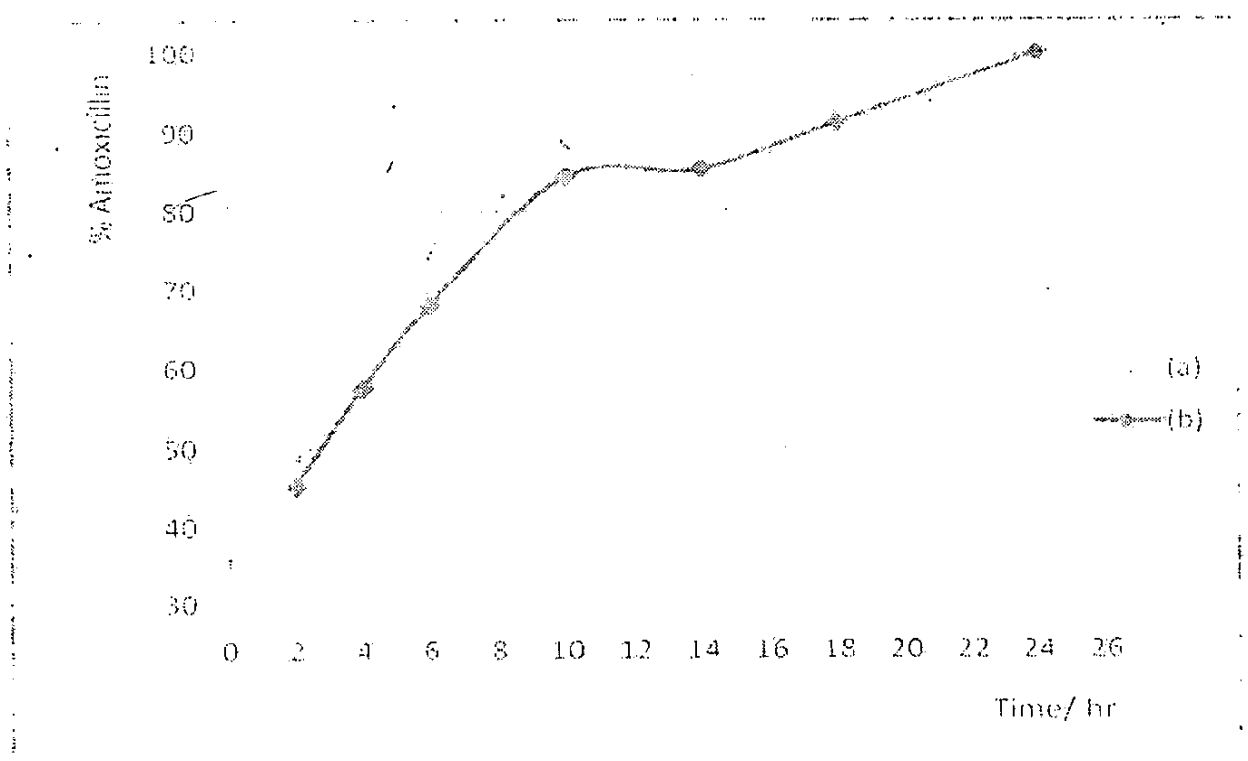


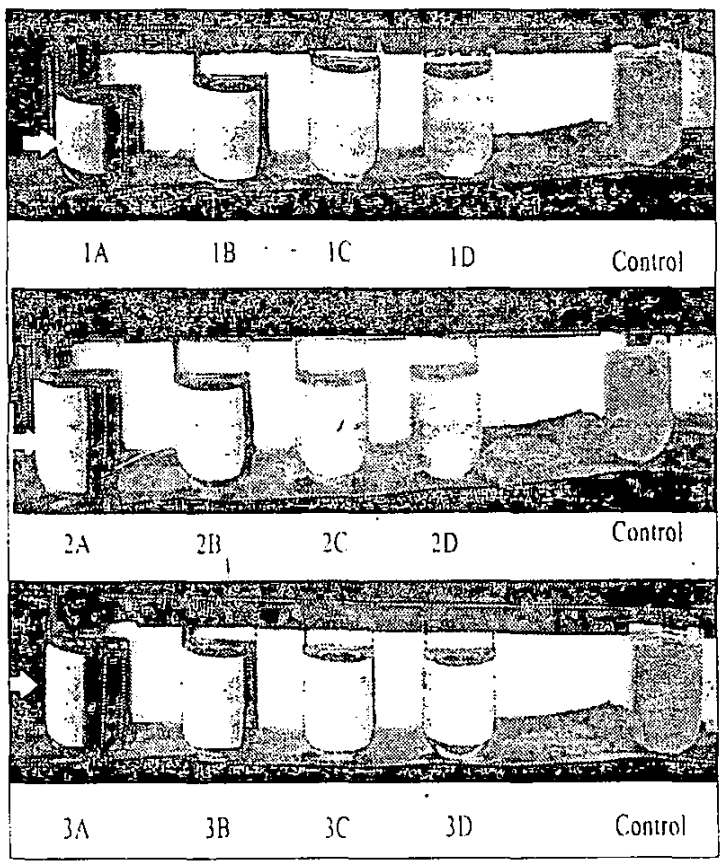
Fig. 6 Release properties of Amoxicillin at 229 nm detection in C18 HPLC column. (a) Release behavior of Amoxicillin in liposome (b) Release behavior of Amoxicillin in chitosan coated liposome.

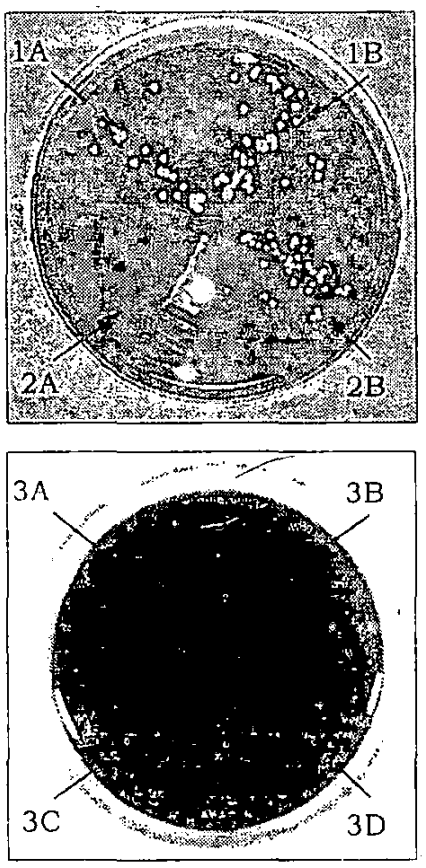
In vitro testing of amoxicillin release.
The amoxicillin released from the chitosan coated liposomes was tested by the ability of the drug to prevent the growth of *Staphylococcus aureus* (NCTC-6571 strain). Release of Amoxicillin from the drug-carrier system was over a period of 8 h. Amoxicillin solution (without drug-carrier system) eluted from the dialysis bag within an hour and half. Both eluants were tested for antibacterial activity.

The Amoxicillin without the drug carrier showed turbidity in the dilutions in the first hour of release. However, after an hour and a half the concentration of drug released was high enough to clear all the dilutions which are indicative of all of the amoxicillin permeating through the dialysis bag in one and a half hours (see Table 2). The clearing of the tubes is shown in Fig 7.

Table 2 Turbidity observation for each time interval (ABCD – BHI with two fold serial dilution of Amoxicillin (without drug-carrier system), Control-BHI without Amoxicillin; after incubation at 37 °C for 18-24 hr., (-) refers as no turbidity and (+) refers as turbidity)

No	Time Interval/ hr	Tube Label				
		A	B	C	D	Control
1	0.5	-	-	+	+	+
2	1.0	-	-	+	+	+
3.	1.5	-	-	-	-	+





(a) (b)

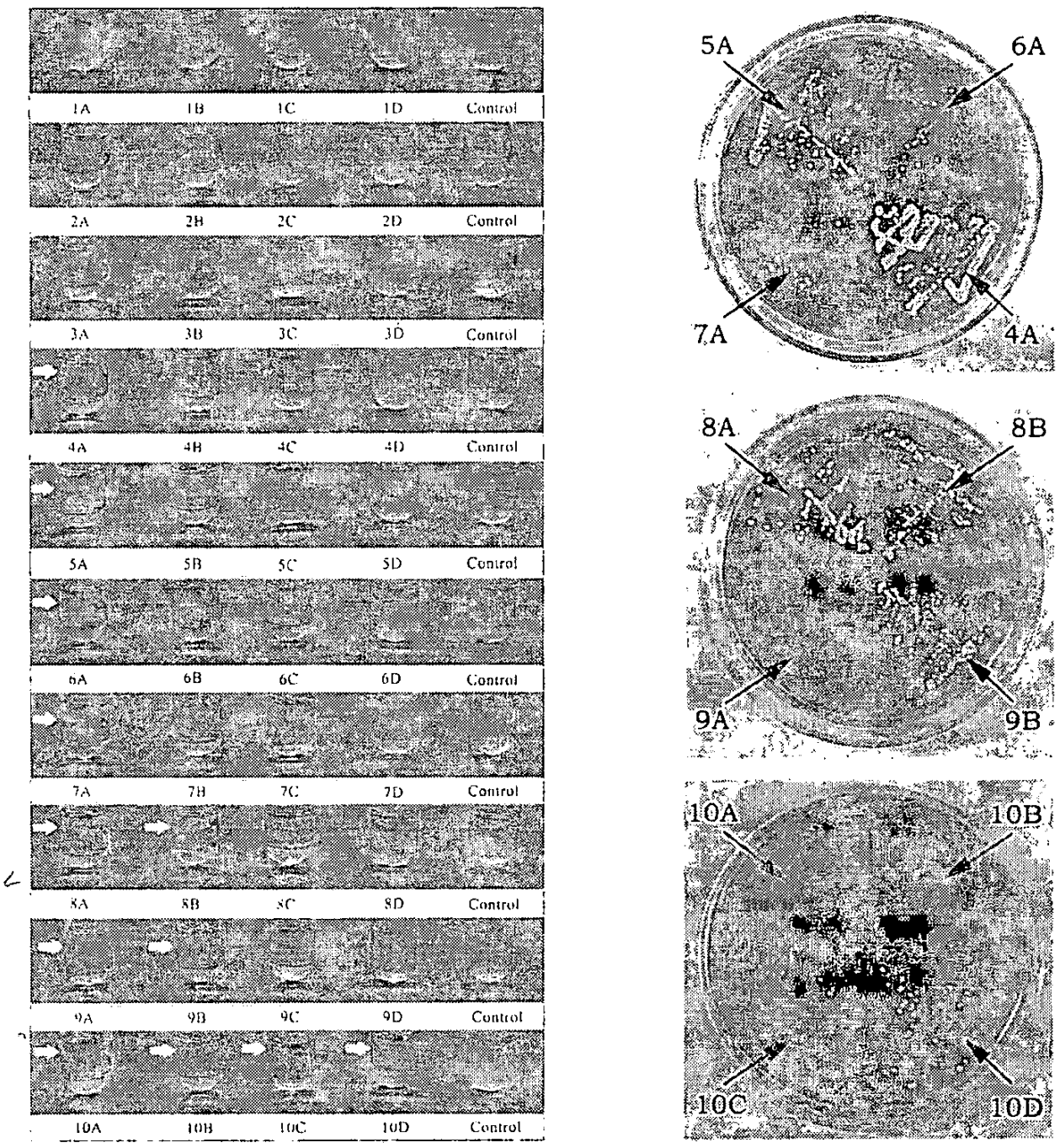
Fig. 7 (a) Appearances of mixtures containing serial dilution of Amoxicillin (without drug-carrier system) in BHI broth and control (BHI without Amoxicillin) after incubation at 37 °C for 18-24 hr. (solutions with less or no turbidity are indicated with an arrow); (b) Streak plates for tubes with less or no turbidity, in blood agar plates.

The activity of the amoxicillin released from the chitosan coated liposomes when tested in a similar manner is shown in Table 3.

Table 3 Turbidity observation for each time interval (ABCD – BHI with two fold serial dilution of Amoxicillin (with drug-carrier system). Control- BHI without Amoxicillin; after incubation at 37 °C for 18-24 hr., (-) refers as no turbidity and (+) refers as turbidity)

No	Time Interval/ hr	Tube				
		A	B	C	D	Control
1	1.0	+	+	+	+	+
2	2.0	+	+	+	+	+
3	3.0	+	+	+	+	+
4	4.0	-	+	+	+	+
5	5.0	-	+	+	+	+
6	6.0	-	+	+	+	+
7	6.5	-	+	+	+	+
8	7.0	-	-	+	+	+
9	7.5	-	-	+	+	+
10	8.0	-	-	-	-	+

Initial release of Amoxicillin was noted after 4h and a steady increase in Amoxicillin concentration was detected until 8 hours. The dilution series (tube 9A, 10A) at 7.5 and 8h respectively, did not show any bacterial colonies when streaked on blood agar plates (Fig. 8). The next dilution (9B and 10B) shows the increased concentration of released drug and there were no colonies in 10B compared to 9B.



(a) (b)

Fig. 8 (a) Appearances of mixtures containing serial dilution of Amoxicillin (released from CLAmox sample) in BHI broth and control after incubation at 37 °C for 18-24 hr. (solutions with less or no turbidity are indicated with an arrow); (b) Streak plates for tubes with less or no turbidity, in blood agar plates.

Conclusion

The morphology of the particles appeared better than the cross linking using sodium sulphate. The LC and EE did not show an improvement with STTP. Drug release from the encapsulated system was sustained over an 8h period as opposed to free drug which permeates within 1.5 h. In this study it could be concluded that a steady concentration in the system could be maintained over a 4 h period (effective from 4 h after administration). Such a sustained release is of advantage considering that Amoxicillin has an elimination half life of 1 hour. Thus the activity of the drug can be prolonged over a longer period compared to administration of free drug. The effect on drug release from liposomes coated with chitosan was monitored and compared with uncoated liposomes. The release was measured using HPLC. The release profiles for both systems were similar implying that the chitosan coating did not affect the release of drug from the uncoated liposomes.

Part 3 Chitosan carrier system for folic acid.

In the previous experiments the chitosan polymer coating was cross linked with two different cross linkers; sodium sulphate and sodium tripolyphosphate. Here folic acid was used as the active compound and encapsulated to determine the best carrier system for release of folic acid. Preliminary tests with Ascorbic acid prepared in Precipitation/ Coacervation (PC) method (Part 1-ascorbic acid) resulted micro range particles and drawbacks with loading of the active compound. Therefore Folic acid was entrapped in PC method without using the liposomal system. Ionotropic Gelation (IG) method was used same as in Part 2- amoxicillin, but with higher centrifugal force. Folic acid entrapment is important in delivery of folic acid and also as a targeting moiety which is specific to the folate receptors.

Characterization

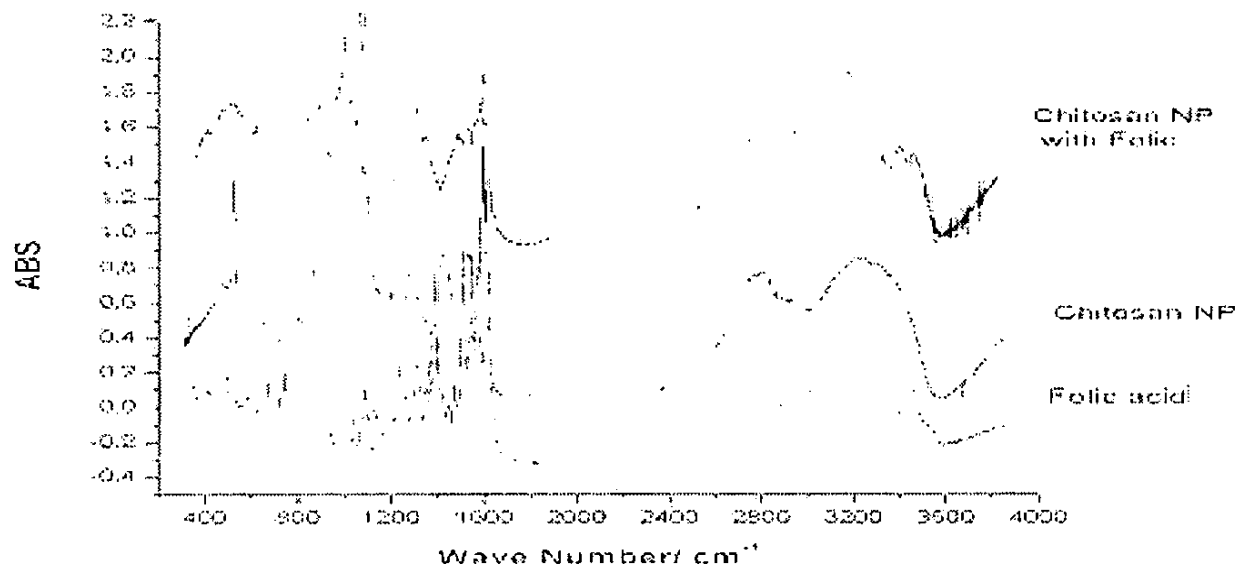


Fig. 9 FTIR spectrum of Folic acid, Plain Chitosan Particles using ionotropic gelation method and of Folic acid loaded chitosan Particles using ionotropic gelation method

The FTIR spectra of Plain Chitosan Particles, Folic acid and Folic acid loaded chitosan for precipitation/ coacervation method were obtained and were of similar nature as reported in the previous Part 1. The FTIR spectra obtained from the ionotropic gelation method are shown in Fig 9.

Table 4 Size and Zeta potential values for the particles with and without folic acid, obtained from precipitation/ coacervation method and ionotropic gelation method

Method		Size/ nm	Zeta Potential/ mV
Precipitation/ Coacervation	Without folic	585.9	+17.5
	With folic	487.9	-31.8
Ionotropic Gelation	Without folic	37.49	+30.9
	With folic	30.89	+21.3

The particle size measurements indicated that the PC method yielded larger sizes than the IG method (see table 4). The zeta potential decreased on encapsulation of folic acid. However, from the zeta potential values obtained only the PC method gave a negative zeta potential on encapsulation which was within the stability range for the particles.

Loading capacity:
The loading capacity was in the same range as previous tests, but LE has shown a significant increase for both the PC and IG methods as shown in table 5.

Table 5 The loading capacity (LC) and loading efficacy (LE) of folic acid using PC and IG methods.

Method	LC (%)	LE (%)
Precipitation/ Coacervation	4.58	91.61
Ionotropic Gelation	4.92	89.56

Increase in loading efficacy may be due to entrapment and conjugation effect of Folic acid with chitosan [Kim, S. and Lee, J., 2011]. As Kim, S. and Lee, J. suggests –COOH in Folic acid can bind with protonated amine groups of chitosan. The IG method also indicates an increase in Folic acid entrapment. So the free Folic acid conjugates with chitosan.

Release of folic acid.
Release properties were observed in Phosphate buffer solution (pH 7.4) at 351 nm UV absorbance.

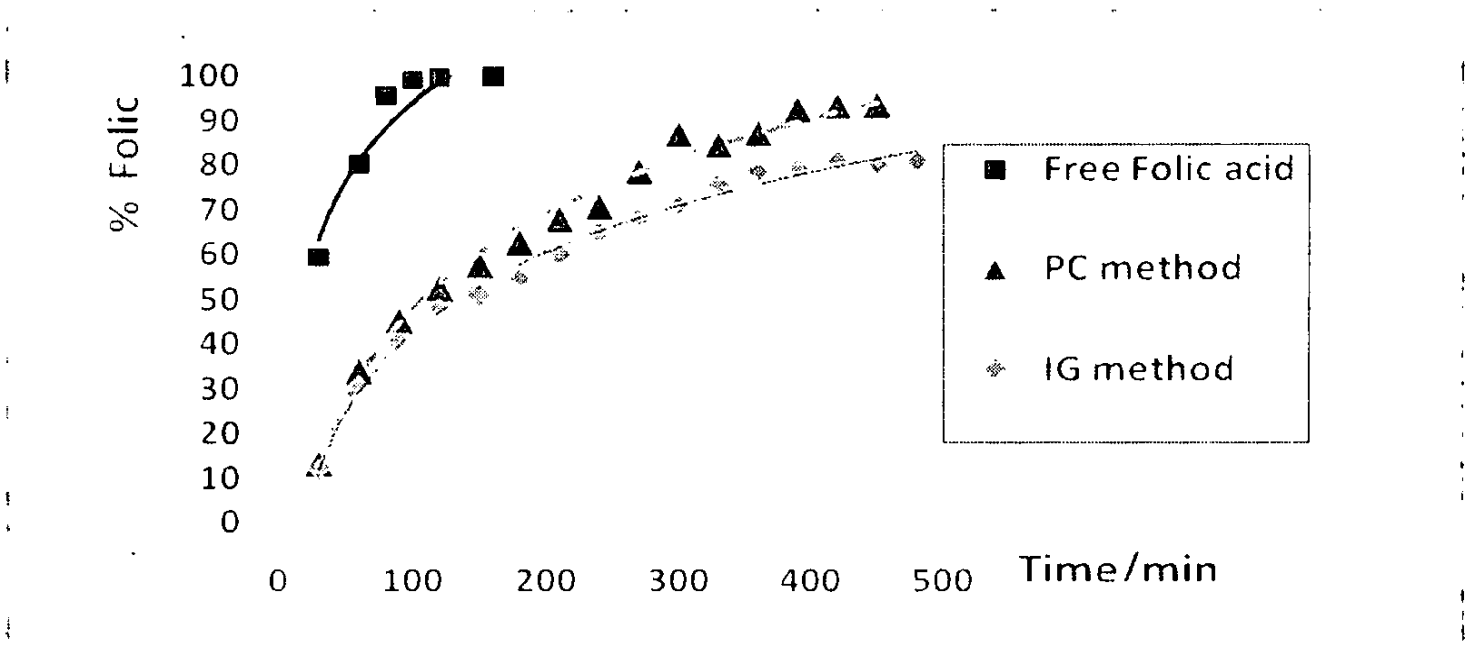


Fig. 10 Release behavior of free Folic acid and chitosan bound Folic acid in Phosphate buffer solution (pH 7.4) at 351 nm UV absorbance, using precipitation/ coacervation (PC) method and Ionotropic Gelation (IG) method.

Release studies were performed as before where release of Folic acid encapsulated and unencapsulated was over a period of 8 h and an hour and half respectively.

Conclusion.

Loading capacity of the model compounds remained within the same order whereas loading efficacy showed significant improvement when using Folic acid as the model compound, suggesting that the structure of the active compound also affects the efficiency of loading. Particles were obtained in much smaller sizes when the delivery systems were prepared in ionotropic gelation method. When considering the particle sizes and the morphology of the particles, ionotropic gelation method is advantageous than the precipitation/ coacervation method.

Part 4 Alginate Carrier system for curcumin.

Curcumin is a well known anticancer drug and coloring agent. Since it is water insoluble, handling of Curcumin is extremely hard in biological systems. Therefore Chitosan-Alginate nanoparticles have used as the mediator in drug delivery systems and other applications as they are water soluble. Nanoparticles were obtained by modifying a two step method described by Rajanavory et.al.,2003 using Chitosan as the Cross linking agent. Polymers were blended with Curcumin to examine the effect on in-vitro release by the amount of drug initially loaded. All the release studies were done in pH 7.4 Phosphate Saline Buffer and analyzed spectrophotometrically. The structural and morphological characterizations of nanoparticles were done using IR spectroscopy and Polarized Light Microscopy.

Characterization

The average yield of Curcumin was about 2.18 % by the weight of the root Turmeric. Extracted Curcumin was chromatographed on TLC in 3.0% Methanol and 97.0% Dichloromethane solvent system. Three bands were observed with Rf factors 0.35, 0.50 and 0.80.

The FTIR spectra of the starting materials- chitosan, alginate, and the Curcumin loaded (Fig 12) and unloaded (Fig 11) nanoparticles were obtained.

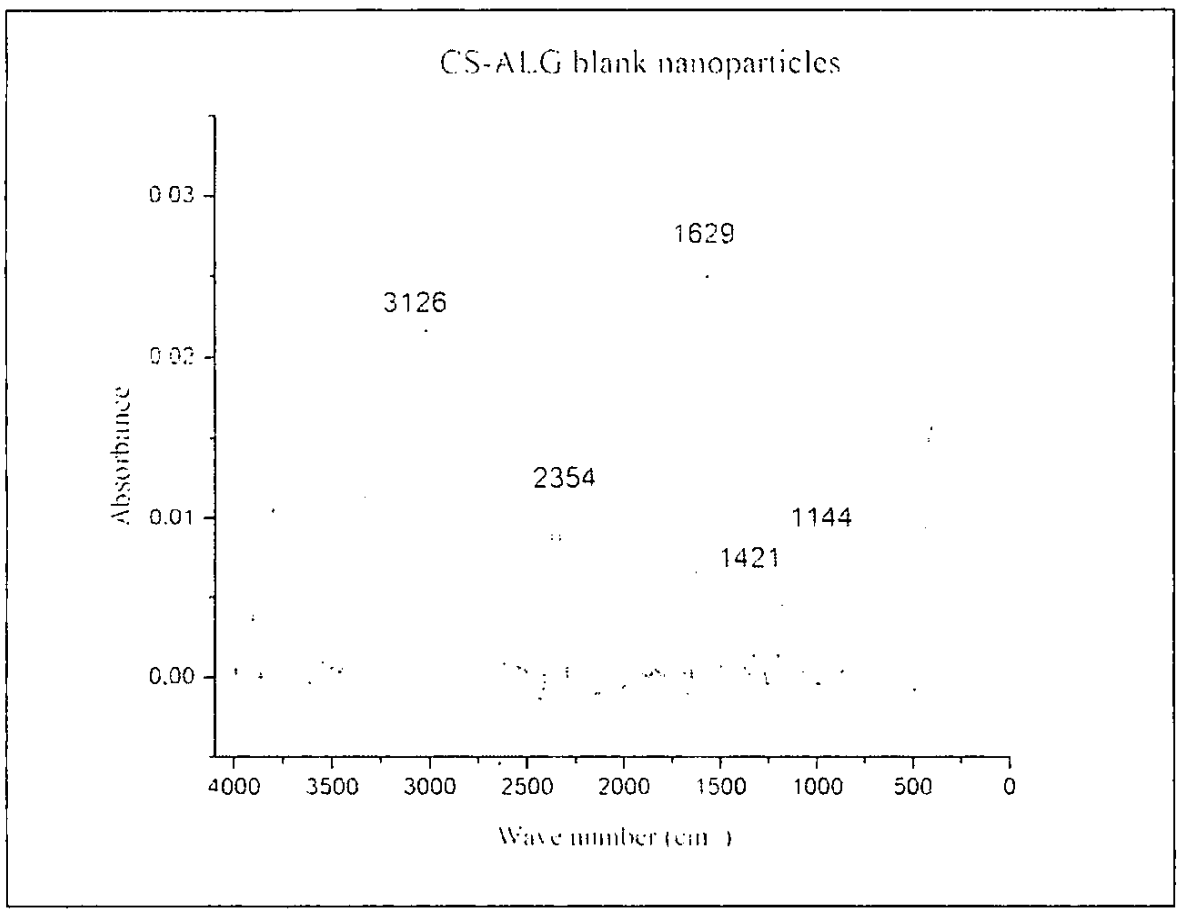


Figure 11 FTIR spectrum of the unloaded CS-ALG nanoparticles

In the FTIR spectrum of CS-ALG nanoparticles (Fig 11) several peak shifts can be observed from The IR spectra of chitosan or alginate alone. When ALG and CS form the complex. peaks at 1612 cm^{-1} and 1414 cm^{-1} shift into a little higher value but for hydroxyl groups it shifts to a lower value leading to reduce the broadness of the peak. Peak at 1414 cm^{-1} of Alginate and peak at 1402 cm^{-1} of Chitosan shift to higher values giving a peak at 1421 cm^{-1} in blank CS-ALG nanoparticles FTIR spectrum. A peak at 1629 cm^{-1} of blank nanoparticles is for the shift of peaks 1616 cm^{-1} in ALG and 1612 cm^{-1} in CS. Similar shifts can be observed in Curcumin loaded CS-ALG nanoparticles (Fig 12).
From the optical microscope an approximate size of 600nm was obtained for the NPs.

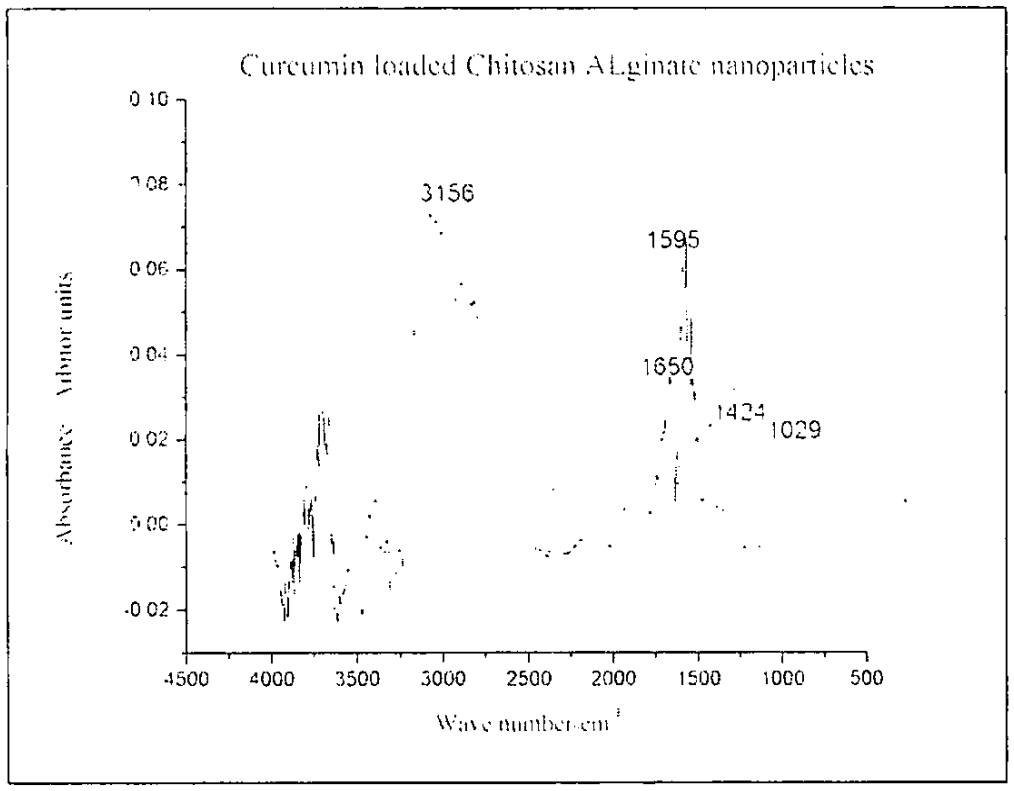


Figure 12: FTIR spectrum of Curcumin loaded CS-ALG nanoparticles

Loading capacity:
Though an encapsulating efficiency of 34% was achieved, the loading capacity was very low (0.3 %). Due to its high hydrophobic nature, at high Curcumin concentration it tends to precipitate out of the reaction mixture rather than encapsulate within the nanoparticles. Three separate attempts at encapsulation were made where the higher yield of NP gave lower EE (table 5).

Sample number	Loading Capacity (LC) / %		Encapsulation Efficiency (%)	Percentage yield (%)
	Theoretical	Experimental		
01	0.24	0.16	21.93	27.01
02	0.24	0.35	34.02	23.87
03	0.95	0.07	7.91	37.23

Table 5. Percentage yield of curcumin loaded NP and their EE and LC.

In-vitro release studies

The release of curcumin from the nanoparticles in pH 7.4 PBS was monitored spectrophotometrically at 425 nm. According to Fig 13. approximately 10% release was observed in the first 6 hours of release. After 24 hours 30% was released until at 96 h (8 d) 60 % release was achieved. Due to the very low EE, the measurement of curcumin release may not be very accurate. However, the encapsulation was carried out three times and similar release results were obtained for samples 1 and 2, but sample 3 only released 30 % of its cargo even after 96 h.

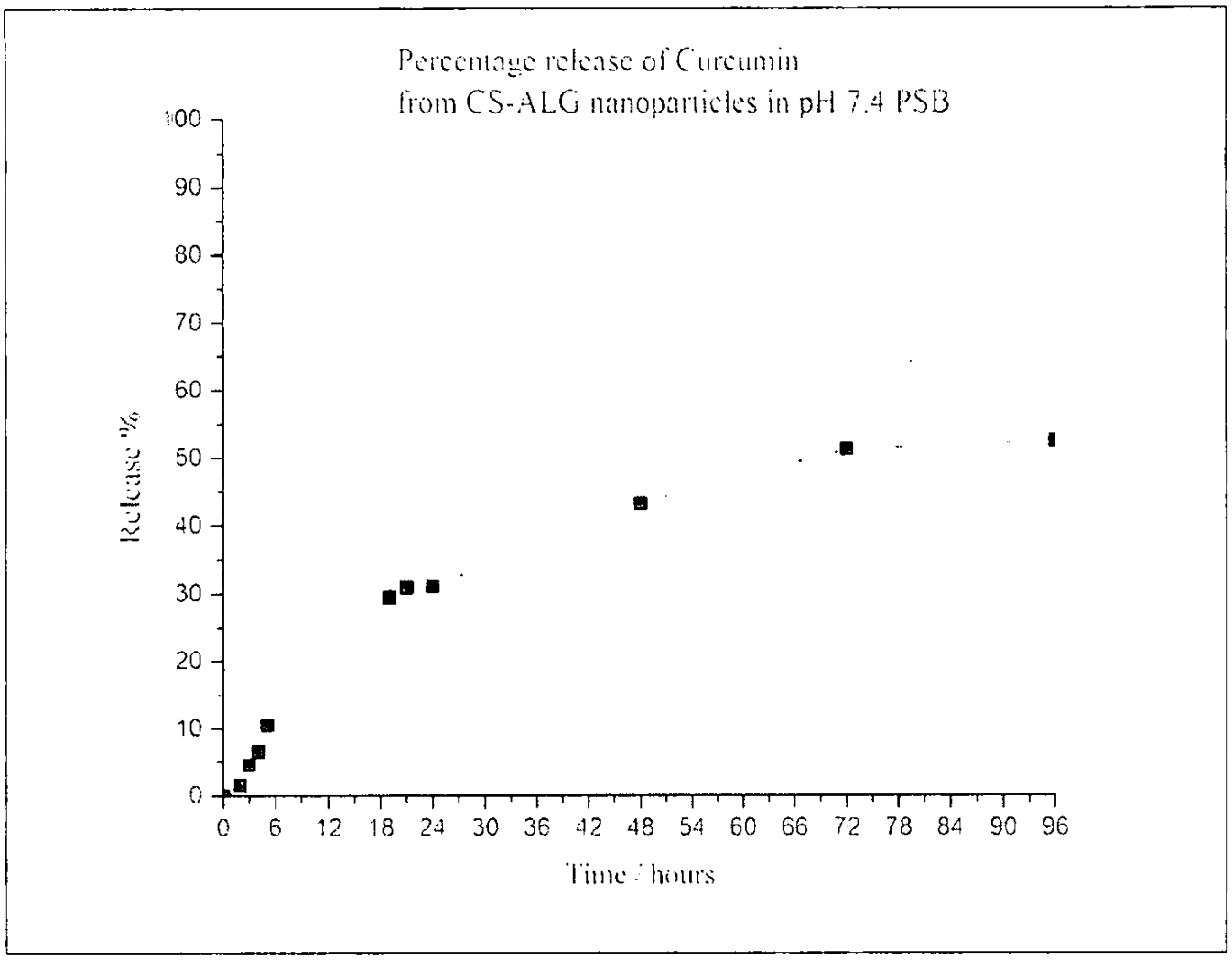


Fig 13 Curcumin release from alginate-chitosan nanoparticles.

Conclusion.

Since Curcumin is a highly hydrophobic compound Chitosan-Alginate nanoparticles formulation is the best method to prepare water soluble nano Curcumin for use as a drug and food coloring agent in aqueous medium. Chitosan and Alginate are bio compatible, bio degradable and non toxic and therefore suitable for food applications. But pH is an important factor in handling Chitosan, Alginate and specially Curcumin. Yield of the nanoparticles can be enhanced by increasing the amount of Curcumin used. But it decreases the Encapsulation efficiency and loading capacity. Slow release of the drug is enhanced successfully with the nanoparticles formulation. With the decrement of Encapsulation efficiency in-vitro release becomes much slower.

Part 5 PHBV Carrier system for folic acid

PHBV (poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (Fig 14) is a biodegradable. nontoxic. biocompatible copolymer produced naturally by bacteria.

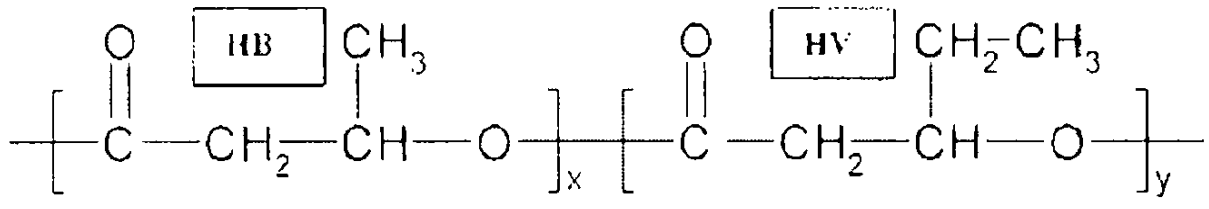


Fig 14 Chemical structure of PHBV.

It is composed of hydroxybutyrate (HB) units and hydroxyvalerate (HV) units and is a polyester. The property of the PHBV depends upon the ratio of the two monomers. An increase in the ratio of 3-hydroxybutanoic(HB) acid to 3-hydroxypentanoic(HV) acid results an increase in melting point and water permeability. It is non toxic and compatible with excipients and therapeutic agents and therefore was chosen as a candidate for drug encapsulation.

Characterization

Several techniques were tried out to synthesis PHBV /Folic acid particles in order to obtain particles in nano size range.

First technique used was based only on homogenization using mechanical motor, but absence of ultrasonic homogenization. In this technique the obtained particles had a size around 25 – 55 μm.(optical microscope)

Second technique used to see the influence of sonication on the size of the particles. The particle size is greatly reduced but some agglomerated particles were observed.

Third technique using a hand sprayer yielded particles that were uniform, non-agglomerated and with relative diameter about 1 micron.

The fourth method used an atomizer sprayer gave particles of diameter less than 1 micron.

Due to difficulty in handling the atomizer sprayer, the third technique was used in this study.

Next the Folic acid concentration in water was varied in order to obtain particles with different ratio of PHBV and folic acid. Percentage yield. Encapsulation efficiency and Loading capacity were calculated separately for each formulation (Table 6)

Table 6 Loading capacity, Encapsulation efficiency and percentage yields for particles of different PHBV/ folic acid ratios.

PHBV/folic acid %	percentage yields	Loading capacity	Encapsulation efficiency
95/5	51.14 ± 0.17	51.59 ± 1.74	5.13 ± 0.10
90/10	49.84 ± 0.27	49.65 ± 1.05	9.97 ± 0.20
85/15	49.74 ± 0.74	50.96 ± 1.42	15.38 ± 0.50
80/20	50.34 ± 0.74	52.11 ± 1.19	20.73 ± 0.74

Data given as mean ± SD (n = 3)

The IR spectra of PHBV/folic acid 95/5% nanoparticles are presented in Fig 15.

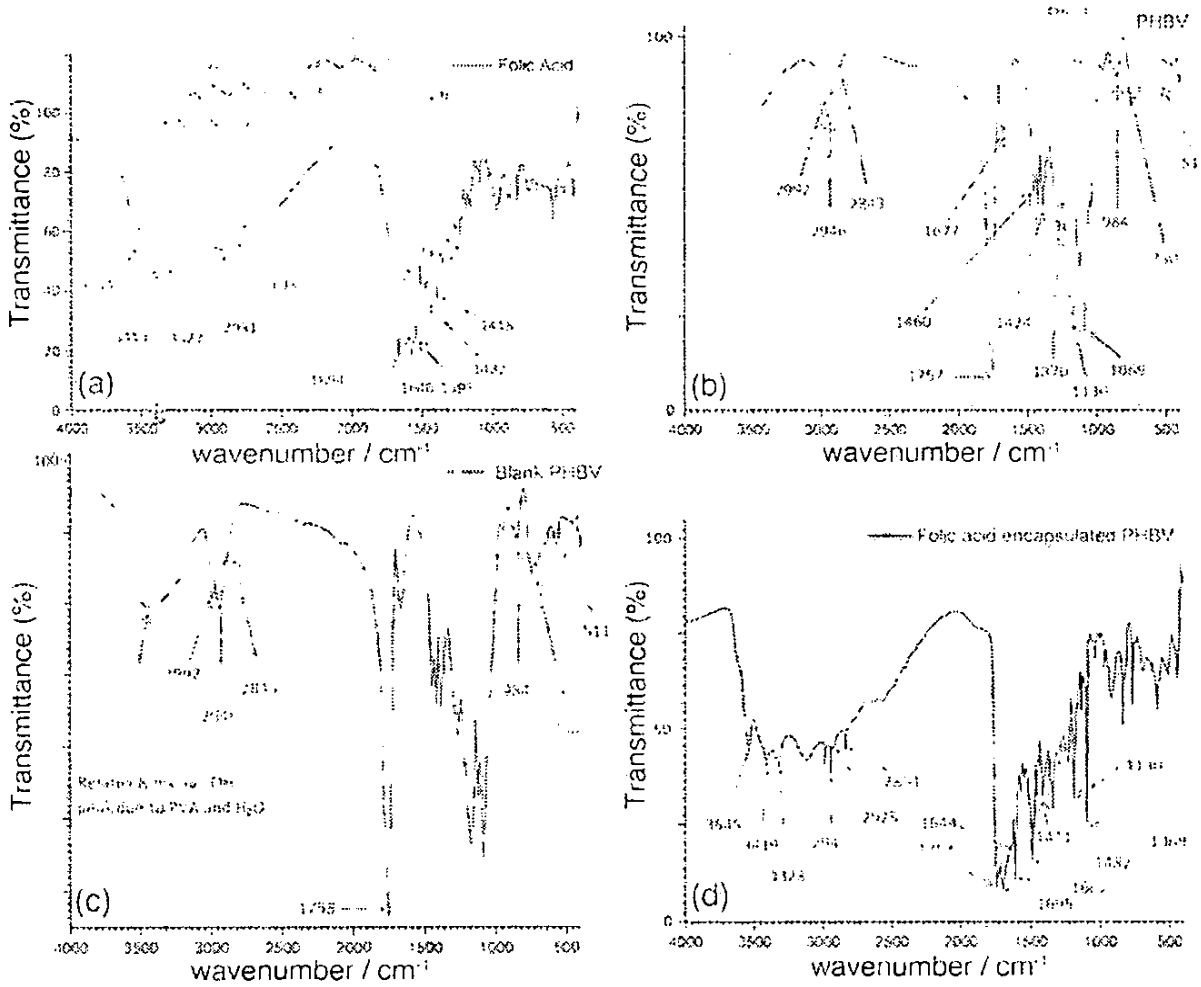


Fig 15 FTIR spectra of (a) Folic acid (b) PHBV (c) PHBV nanoparticles (d) Folic acid encapsulated PHBV NPs.

Besides the characteristic groups for PHBV, the spectra show all the characteristic groups for folic acid. The band at 3545 cm-1 belongs to the hydroxyl (O-H) stretching, while the bands at 3419 cm-1 and 3328 cm-1 are N-H stretching vibration bands. The bands at 2942, 2925 and 2851 cm-1 correspond to –C-H stretching vibrations, C=O bond stretching vibration of carboxyl group appears at 1695 cm-1, while the band at 1644 cm-1 belongs to C=O bond stretching vibration of $-CONH_2$ group. The band at 1602 cm-1 relates to the bending mode of N-H vibration. The band at 1482 cm-1 was attributed to the characteristic absorption band of phenyl ring. Characteristic bands from 800 to 975 cm-1 corresponded to symmetric –C-O-C- stretching vibration. Moreover, the antisymmetric –C-O-C- stretching leads to bands between 1060 and 1150 cm-1. And there are no other significant shifts in other bands, which suggests the encapsulation of Folic acid in to PHBV nanoparticles without strong chemical interactions.

In-vitro release studies

PHBV NPs released the drug over a period of more than 2 weeks in the pH-7.4 medium (Fig 16 a). This is a very different release profile from all the previous studies (parts 1-4) as it can be seen that about 60% of drug release takes nearly 6 days. In pH-1 medium, folic acid was released within 1 hour (Fig 16 b). This phenomenon is very useful for the mode of administration whether oral or intravenous as the gut pH can release the drug very fast.

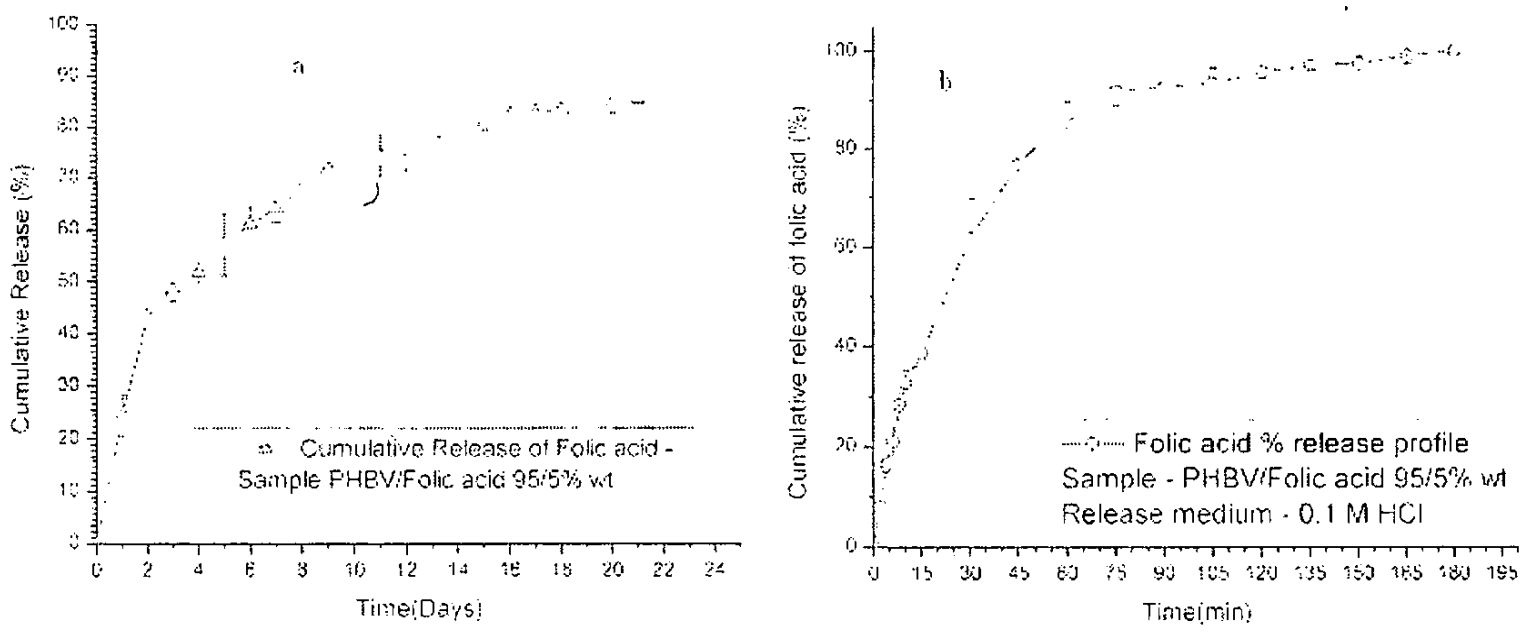


Fig 16 Release of folic acid fromPHBV nanoparticles a) at pH 7.4 and b) at pH 1

Conclusion.

The double emulsion technique results in formation of relatively small size particles (relative diameter ≤1 micron) using hand sprayer and atomized sprayer techniques with sonication bath and a mechanical stirrer. Further confirmation of the size by particle size analysis is necessary. FT-IR data suggest that Folic acid can be encapsulated in to PHBV polymer matrix without strong chemical interactions, meaning that structure of the folic acid is intact in the PHBV polymer matrix. All the samples have a similar percentage yields (about~50%), with loading capacity around 50 %. *In Vitro* drug release confirmed that PHBV release the drug over a period of more than 2 weeks in the neutral medium (pH-7.4) however, in acidic medium (pH-1.0) release occurred faster with nearly 90% of the drug released within 1 hour.

v) Discussion

The basic techniques used (precipitation coacervation, ionotropic gelation, double emulsion technique) with a variety of polymers (chitosan, alginate and PHBV) as well as use of liposomes have shown that liposomes encapsulated with chitosan or liposome themselves are able to release drugs within a time frame of 6 -7 h. The chitosan-alginate NP and chitosan NP systems also works in the same time frame. However, PHBV polymer has shown great promise due to its ability for prolonged release.
The nature of the drug loaded also contributes to loading capacity and encapsulation efficiency. Hydrophobic compounds like curcumin recorded very low loading capacities. Therefore each drug requires careful selection of a carrier for optimal loading and release. Another factor that affects the particle size was seen to be the method of particle formation. In the double emulsion method for PHBV, in addition to sonication, spraying of the solvent using a sprayer greatly enhanced the size of particles.

vi) Conclusions

The objective of this research was to find a method for encapsulating drugs and bioactive material for drug delivery. The encapsulation techniques tested were liposomes, chitosan

coated liposomes, chitosan NPs, chitosan-alginate NPs and PHBV nanoparticles. The drugs tested were (i) ascorbic acid encapsulated in liposomes with chitosan coating cross linked with sodium sulphate, (ii) amoxicillin encapsulated in liposomes with chitosan coating cross linked with STTP, (iii) folate encapsulated in (a) chitosan NPs (b) folate encapsulated in liposomes with chitosan coating cross linked with STTP and (c) folate encapsulated in PHBV NPs, and (iv) curcumin encapsulated in chitosan-alginate nanoparticles.

Encapsulation of ascorbic acid revealed that particles prepared in the nitrogen environment does effect the morphology and stability of ascorbic acid. The carrier system prepared using sodium sulphate as the cross linker for chitosan produced particles in the micro range. The poor loading capacity of the system and the susceptibility of ascorbic acid to air were the drawbacks of this system.

Using amoxicillin and cross linking chitosan with STTP resulted in particles with better morphology, however the LC and EE did not show an improvement. Amoxicillin encapsulated liposomes and amoxicillin encapsulated liposomes coated with chitosan had similar release profiles. Therefore it can be concluded that the chitosan coating did not slow down the release of drug from the uncoated liposomes as expected.

The third modification to the chitosan carrier system was to formulate plain chitosan nanoparticles and also repeat the STTP crosslinking method using a higher centrifugal force. Again there was no significant improvement to loading capacity with the drug tested (folic acid) as was with both ascorbic acid and amoxicillin. Thus we conclude that the structure of the active compound as well as the method of preparation affects the efficiency of loading. Particles were obtained in much smaller sizes when the delivery systems were prepared using the ionotropic gelation method. When considering the particle sizes and the morphology of the particles, ionotropic gelation method is advantageous than the precipitation/ coacervation method.

The next carrier for encapsulation selected was chitosan cross linked with alginate. Here the bioactive material curcumin was used for encapsulation. Curcumin is poorly water soluble and the yield of encapsulated material was decreased by the solubility effects. Release of curcumin was very slow with approximately 30% released in 24 h. The very low loading capacity may also contribute to this as the concentration released is low. However, it may be worthwhile to test release of hydrophilic molecules with this system as the possibility of sustained release is greater with this system.

The double emulsion technique for PHBV encapsulation of folic acid resulted in high yields as well as good loading capacity. The release of drug over a period of more than 2 weeks in the neutral medium (pH-7.4) was the key point of this system. In addition it was notable that in acidic medium (pH-1.0) release occurred faster with nearly 90% of the drug released within 1 hour. Therefore this study for encapsulation techniques for drugs indicates that polymers such as PHBV have superior release properties over other polymers such as chitosan and alginate.

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viii) Problems if any, encountered during the implementation of the project

Usual initial problems of postgraduate students getting accustomed to the project are inevitable. However, this student took a long time to familiarize himself and therefore some parts (part 4 and 5) were researched by two final year undergraduate students. At the start the lack of a high speed centrifuge hampered the project. Request to utilize the equipment allocation for purchase of centrifuge accessories was denied. Constrains of flow of funds hampered purchase of chemicals.

ix) Major findings and follow up activities.

From these studies we have found out that liposomes and chitosan nanoparticles have similar release profiles. The chitosan alginate composites showed release profiles for release over 24 h but since the curcumin is poorly water soluble, this needs to be verified with hydrophilic compounds. However, the fact that a poorly soluble compound like curcumin was encapsulated and released shows that nanoencapsulation helps to solubilize water insoluble compounds. This project was the basis for more work carried out in our laboratories because the

techniques for encapsulation were perfected. This is a major plus point for the research output of our laboratory group.
Follow up activities suggested are for using other polymers such as PLGL and neutral polysaccharides as carrier material. We have already completed some research on using protein as carrier material with good results and hope to begin on neutral polysaccharides soon.

Section 4

Impact of Research results:

i) Relevance of results achieved to scientific advancement
Scientific advancement in the fields of pharmaceuticals, nutrition and cosmetics is possible with these findings. Applications in these areas need sponsorships from industry without which it is difficult in the present set up. Results obtained need scaling up to pilot studies and clinical trials before implementation. Specially since these applications include- delivery of water insoluble drugs, inclusion of bioactive materials in cream formulations, and delivery of nutraceuticals.

ii) Relevance of results achieved to national/socio-economic development
Extremely relevant to National development if quality drugs and nutritional aspects are further developed. Problems encountered with nutritional deficiencies can be alleviated by using these techniques for nutrient encapsulation.

iii) Dissemination/application of research output
Research output application is solely dependant on the interests of a commercial party or industry. Knowledge transfer and further testing of delivery regimen is possible in such an event.

Section 5

Miscellaneous

i) List of major equipment acquired during the project period and their functionality
No major equipment was purchased. Only Accessories for FTIR

ii) List of publications/communications arising from the project and/or presentations made at seminars, workshops etc. (Please attach copies)

Note: copies of 1-4 and 6 are attached. 5 was a poster presentation.

1. W.M.T.N.B. Wanninayake, N.L.V.V. Karunaratne and **D N Karunaratne.** Preparation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanoparticles for sustained release of folic acid. *Proc. of Peradeniya University Research Sessions (PURSE),* Vol. 17, **2012.** (held in July 2013).
2. M. A. S. K. Menikarachchi, **N. Karunaratne** and V. Karunaratne. Nano-carrier system for controlled release of folic acid. *Proc. of Peradeniya University Research Sessions (PURSE),* Vol. 17, **2012**. (held in July 2013).
3. K.K.D.R.M. Wimalasinghe and **D.N. Karunaratne**. Formation of chitosan-alginate nanoparticles and encapsulation of curcumin. *Proc. of Peradeniya University Research Sessions (PURSE),* Vol. 17, **2012**. (held in July 2013).

4. M. A. S. K. Menikarachchi, **D. N. Karunaratne,** V. Karunaratne, V. Thevanesam & A. Ekanayake. Chitosan based Drug-Carrier System for Controlled Release of Amoxicillin, *International Conference on Chemical Sciences Proceedings*. **2012.**

5. M. A. S. K. Menikarachchi. **D. N. Karunaratne,** & V. Karunaratne. Chitosan based Nano-Carrier System for Controlled Release of Ascorbic Acid. *Poster presented at the Global Forum of Sri Lankan Scientists held at the Hotel Galadari,* **Dec 2011.**

6. M.A.S.K. Menikarchchi, **N. Karunaratne** and V. Karunaratne. Chitosan based nano-carrier system for controlled release of ascorbic acid. *Proc. of Peradeniya University Research Sessions (PURSE).* Vol. 16. **2011**.

Section 6

Summary Statement of Expenditure (indicate under Personnel, Equipment, Consumables, Travel and Subsistence and Miscellaneous)

See attached

Interim Financial Report

The financial position of grant No. **RG/2010/Nano/04** as at 31.03.2014 awarded to DR./Prof.D.N Karunarathne by National Science Foundation is as follows.

		Funds received By the Univ./ Institution	Total Expenditure Rs. Cts.	Balance available Rs. Cts.
Personal:	Research Student	997.000.00	1.047.428.00	(50.428.00)
	Technical Assistant	-	-	-
	Other (P. G Reg. Fees)			-
Equipment:	Foreign	-	-	-
	Local	550.000.00	438.200.00	111.800.00
Consumables	Foreign			
	Local	500.000.00	590.758.25	(90.758.25)
Travel & Subsistence:			-	-
Miscellaneous:		67.500.00	17.150.00	50.350.00
	TOTAL	2.114.500.00	2.093.536.25	20.963.75

Unspent balance of the funds received

Funds received	Rs.	2.114.500.00
Actual Expenditure	Rs.	2.093.536.25
Balance	Rs.	20.963.75
Cash advance	Rs.	
Balance as at 31.03.2014	Rs.	20.963.75

for Assistant Bursar/ Fac. of Science

31.03.2014
Date

Assistant Bursar
Faculty of Science
University of Peradeniya

Section 7

i) Grantees' signatures

D.N. Karunaratne

V. Karunaratne

ii) Comments of the Head of the Department/signature

Completed the project successfully.

Head
Department of Chemistry
University of Peradeniya
Peradeniya

Prof. A.D.L. C. Perera (Head/Dept of Chemistry)

iii) Head of the Institution's signature

Vice - Chancellor
University of Peradeniya
Peradeniya
Sri Lanka

Revised date: 30/07/2010

NS.SCI.18

PREPARATION OF poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) NANOPARTICLES FOR THE SUSTAINED RELEASE OF FOLIC ACID

W. M. T. N. B. Wanninayake, N. L. V. V. Karunaratne, D. N. Karunaratne

Department of Chemistry, Faculty of Science, University of Peradeniya

Biodegradable polymers are predominantly used in a variety of biomedical and food applications. Specially poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) co polymer particles have been studied as a food packing material and as a material for drug delivery with a controlled release both *In vivo* and *In vitro*.

In this project PHBV particles containing a water soluble model drug (Folic acid), were obtained by the double emulsion/solvent evaporation technique. Folic acid was encapsulated into PHBV polymer matrix by means of homogenization of aqueous and organic phases. Several samples were prepared in order to observe the effect of each composition on the encapsulation efficiency, and Drug loading. The concentration of folic acid in water was changed to obtain particles with different ratios of PHBV and Folic acid. These particles were obtained by using Polysorbate 80(tween80) as a surfactant and polyvinyl alcohol (PVA) as a film forming agent in primary and secondary emulsions respectively.

Particle production conditions were varied in order to investigate the influence of the mixing conditions on the particle size. The primary emulsion formation step was done using mechanical stirrer equipped with blade impellers. Using a hand sprayer and an atomized sprayer apparatus several sets of samples were prepared by varying the addition method of inner aqueous phase on to the oil phase. An ultrasonic bath was used to obtain smaller particles. In order to observe the resulting particles and to obtain an idea about the particle size distribution, all the samples were monitored using an optical microscope and were compared with standard min-u-sil (1~micron diameter) particles.

The obtained particles were non-agglomerated, uniform and with particle size in nanometer and micrometer range. Particles obtained by using atomized sprayer apparatus and ultrasonic bath with mechanical stirrer had a mean diameter of less than 1 micrometer, whereas the particles obtained by hand sprayer method had a mean diameter of 1 micrometer. The samples were further characterized using Infrared Spectroscopy (IR) and Ultraviolet Spectroscopy. IR spectroscopy results suggested that no chemical bond between the polymer and the drug was formed. *In vitro* drug release demonstrated the influence of PHBV on the dissolution profile of Folic acid. It was observed that in pH 7.4 buffer solution, folic acid was released over a period of 22 days.

NS.SCI.25

NANO-CARRIER SYSTEM FOR CONTROLLED RELEASE OF FOLIC ACID

M. A. S. K. Menikarachchi, N. Karunaratne, V. Karunaratne

Department of Chemistry, Faculty of Science, University of Peradeniya

Chitosan has gained much attention as a non-toxic, biocompatible and biodegradable polymer in targeted and controlled drug delivery. The primary amine groups and the hydroxyl groups of chitosan makes it suitable for the formation of a variety of delivery vehicles. Folic acid an essential nutrient of the vitamin B complex, and is composed of three chemical structures namely 6-methylpterin, *p*-aminobenzoic acid and glutamic acid. Many disorders like megaloblastic anemia, neurological disturbances; and neural tube defects are connected with deficiency of Folic acid. Folic acid can also be used as a targeting moiety, which is specific for the folate receptors.

The present study suggests a potential folic acid delivery system and a drug targeting system based on chitosan polymer. Chitosan nanoparticles were obtained using a precipitation/ coacervation method and incubated with Folic acid to obtain Folic bound Chitosan nanoparticles. Nanoparticles were characterised using FT-IR spectrum, particle size and zeta potential analysis. Release properties of the nanoparticles were checked by suspending in Phosphate buffer solution (pH 7.4) with the aid of dialysis tubing cellulose membrane bag and analysing the amount of Folic acid released at 351 nm UV absorbance.

FTIR spectrum of Folic acid loaded Chitosan shows a similar spectrum to Chitosan particles, other than shifting of amide peak, ensuring that most of the Folic acid moieties encapsulated are inside the Chitosan particles. Particle size distribution around 487.9 nm and Zeta potential with -31.8 mV ensures that the particles are nano sized and stable. Controlled release of Folic acid from encapsulated particles over seven hours shows a distinguishable enhancement comparable to free Folic acid release.

Funding: National Science Foundation, Research Grant RG/2010/NANO/04.

NS.SCI.19

FORMATION OF CHITOSAN-ALGINATE NANOPARTICLES AND ENCAPSULATION OF CURCUMIN

K. K. D. G. R. M. Wimalasinghe, D. N. Karunaratne

Department of Chemistry, Faculty of Science, University of Peradeniya

Curcumin is a well known anticancer drug and colouring agent which is a poly phenolic compound. It can be extracted from the *Curcuma longa* rhizome via a simple laboratory method. Since it is water insoluble, handling of Curcumin is extremely difficult in biological systems. The main aim of producing nano drug delivery systems is for targeting delivery and slow release. Natural polymers are widely used for this purpose because they are biocompatible, and ecologically safe. Chitosan and Alginate, are highly hydrophilic polysaccharides. Formation of drug loaded gel beads with Chitosan or Alginate only, has been reported. However the sizes of these gel beads which are in the micrometer range are not very effective for sustained and gradual release of the drug. The size of the gel beads is important o obtain a successful drug delivery and desired solubility. Therefore, Chitosan-Alginate nanoparticles have been used as the mediator in drug delivery systems and other applications as they are water soluble and this blend of polysaccharides gives the drug loaded gel beads in nanometer scale. Particulate dispersion of solid in size 10-100 nm is defined as nanoparticles.

Nanoparticles were obtained from the two step Rajaonarivony method with modifications. $CaCl_2$ was used to obtain Calcium Alginate smooth ionic pre gel. Since Alginate and Chitosan are oppositely charged polymers, cross linking enhances the gel strength and also the sustained release of Curcumin from nanoparticles via a diffusion control mechanism or a swelling control mechanism. Therefore nanoparticles were obtained from a spontaneous nanoparticles formulation method. The Polymers were blended with different amounts of Curcumin to examine whether the invitro release is affected by the amount of drug initially loaded. All the release studies were done in pH 7.4 Phosphate Saline Buffer to provide physiological conditions. The release of the drug was analysed spectrophotometrically. The structural and morphological characterizations of nanoparticles were done using IR spectroscopy and Polarized Light Microscopy.

Since Curcumin is highly hydrophobic Chitosan-Alginate nanoparticles formulation is the best method to prepare water soluble nanoparticles making Curcumin a drug with successfully enhanced slow release.

MT 8

Chitosan Based Drug-Carrier System for Controlled Release of Amoxicillin.

M. A. S. K. Menikarachchi[1], *V. Thevanesan*[2], *D. N. Karunaratne*[1], *V. Karunaratne*[1] *and A. Ekanayake*

[1]*Department of Chemistry, Faculty of Science, University of Peradeniya, Peradeniya*
[2]*Department of Microbiology, Faculty of Medicine, University of Peradeniya, Peradeniya*
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Development of the drug-carrier systems, for the prolonged availability of the drugs, has gained much attention in recent years. The aim of the present study was to produce Amoxicillin entrapped drug carrier system, test the release properties and antibiotic activity against *Staphylococcus aureus*. Chitosan acts as a fine encapsulation matrix for a drug, with its non-toxic, biodegradable and biocompatible polymeric properties. Amoxicillin trapped liposomes were coated with chitosan, with the aid of Tween 80 and sodium sulphate. The chemical structure was analyzed by FTIR.

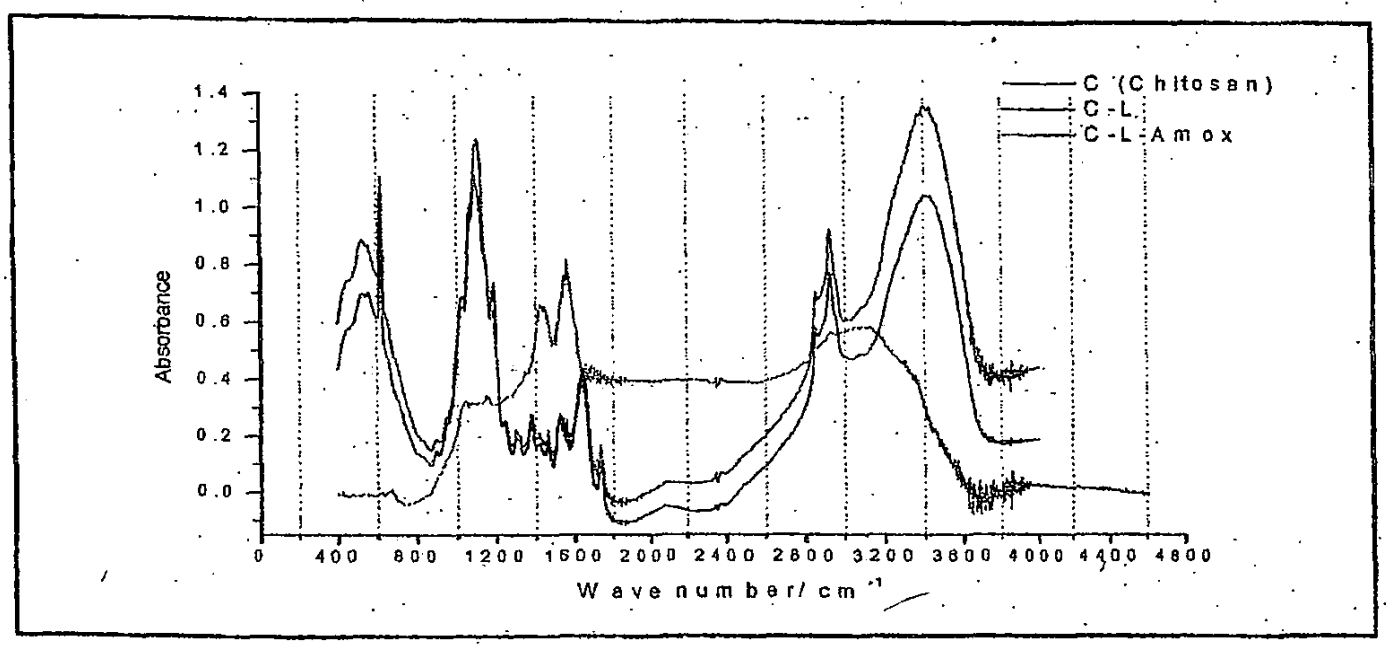

Figure 1. FTIR data for Chitosan (C), Chitosan coated Liposome without Amoxicillin (C-L) and Chitosan coated Liposome with Amoxicillin (C-L-Amox)

Activity of the antibiotic was tested against *Staphylococcus aureus* (NCTC-6571 strain) in BHI broth medium. Controlled release of Amoxicillin from encapsulated particles over five hours shows a distinguishable enhancement comparable to free Amoxicillin release.

Financial assistance by the National Science Foundation, Research Grant RG/2010/NANO/04 is acknowledged.

Proceedings of the Peradeniya University Research Sessions, Sri Lanka, Vol. 16, 24th November 2011

Chitosan based Nano-Carrier System for Controlled Release of Ascorbic Acid

M.A.S.K. Menikarachchi, N. Karunaratne and V. Karunaratne

Department of Chemistry, Faculty of Science, University of Peradeniya

There is an increasing interest in the development of new delivery systems for the controlled release of drugs and bioactive agents. Among these delivery systems, encapsulation of the drugs using a biodegradable matrix shows a promising pathway for the enhancement of the bioavailability of the drugs.

The aim of the present study was to produce a chitosan-based nano-carrier system and check the release properties of the entrapped drug comparatively to the release of the free drug, to ensure whether there is a controlled release property in the nano-carrier system.

Chitosan is a non-toxic, biodegradable and biocompatible polymer with interesting biological and chemical properties. Ascorbic acid (Vitamin C) was used as a model drug for the process.

Ascorbic acid trapped liposomes were coated with chitosan, with the aid of Tween 80 and sodium sulphate. The chemical structure was analyzed by FTIR and controlled release of ascorbic acid from encapsulated particles over seven hours shows a distinguishable enhancement comparable to free ascorbic acid release.

Financial assistance by the National Science Foundation, Research Grant RG/2010/NANO/04 is acknowledged.

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