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Adebisi, Adeola O. and Conway, Barbara R

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Lectin-conjugated microspheres for eradication of *Helicobacter-pylori* infection and interaction with mucus

Adeola O. Adebisi and Barbara R. Conway

Department of Pharmacy, School of Applied Sciences, University of Huddersfield, Queensgate,
Huddersfield, HD1 3DH, UK

***Corresponding author**

Barbara R. Conway

E-mail address, b.r.conway@hud.ac.uk

Tel # +44(0)1484 472347

Fax # +44(0)1484 472182

Abstract

Using second generation mucoadhesives may enhance targeting antibiotics for eradication of *Helicobacter pylori* from the stomach for the treatment of peptic ulcer. The aim of this research was to prepare and characterise ethylcellulose/chitosan microspheres containing clarithromycin with their surfaces functionalised with concanavalin A to produce a floating-mucoadhesive formulation. The microspheres were prepared using an emulsification-solvent evaporation method. Particle size, surface morphology, *in vitro* buoyancy profile, zeta potential, drug entrapment efficiency, *in vitro* drug release and release kinetics of the particles were determined. Lectin was conjugated to the microsphere surface using two-stage carbodiimide activation and confirmed using FTIR, fluorescence studies and zeta potential measurements. Conjugation ranged from 11 - 15 µg Con A / mg microspheres which represents over 56 % efficiency although there was some drug loss during the conjugation process. Conjugation did not have a significant effect on the buoyancy and release of drug from the microspheres using a mucus diffusion model with 53 % and 40 % of drug released from unconjugated and conjugated microspheres within 12 h. Conjugation improved mucoadhesion and interaction with porcine gastric mucin compared to unconjugated microspheres. The buoyancy and improved mucoadhesion of the microspheres provides potential for delivery of CMN and other drugs to the stomach.

Keywords: *Helicobacter pylori*, microspheres, ethylcellulose, chitosan, concanavalin A, mucoadhesive

1: Introduction

Helicobacter pylori (*H-pylori*) is implicated in the development of chronic active gastritis and an aetiological factor in the development of peptic ulcer, gastric mucosal-associated lymphoid tissue lymphoma and gastric carcinoma (Dunn et al., 1997; Peterson, 1991; Suerbaum and Michetti, 2002). Almost 0.5 million new cases a year of gastric cancer, about 55% of the total cases worldwide, have been linked to *H-pylori*. In addition, it was predicted that by 2020, it would feature in the top ten leading causes of death worldwide (Kawahara et al., 2005; Murray and Lopez, 1997). Infection is considerably higher in developing countries (80-90%) than in developed countries where the prevalence ranges between 10-50% of the total population (Rothenbacher and Brenner, 2003). *H-pylori* attach to the gastric epithelial cells and a major feature of its infection is that it causes progressive injury to the gastric mucosa and its function (Lehmann et al., 2002; Suzuki and Ishii, 2000). It is sensitive to many antibiotics *in vitro*; however no single agent is effective alone *in vivo* (Bazzoli et al., 2002), therefore a minimum of two antibiotics in combination with gastric acid inhibitors are used for eradication. After infection, the bacterium resides below the gastric mucus adherent to the gastric epithelium and access of drugs to this target site is rather limited. In addition, the bacteria can acquire resistance to commonly used antimicrobial drugs (Iijima et al., 2004) so a combination of two antibiotics such as clarithromycin (CMN), amoxicillin and metronidazole, along with a gastric acid inhibitor, is the currently recommended therapy for the eradication of *H-pylori* (Georgopoulos et al., 2012). Failures in treatment can result from the persistent rise in resistance of this bacterium to these antibiotics, the hostile environment of the stomach reducing antibiotic bioavailability at the site of action (Batchelor et al., 2007) and the formation of biofilms by *H-pylori* on the gastric mucosa epithelium which confer resistance to many antimicrobial agents (Cammarota et al., 2012). Such challenges have encouraged research into producing alternative therapies. Restricted gastric residence of current antibiotic formulations also limits contact time with the bacteria, therefore, gastroretentive drug delivery systems (GDDS) such as floating and mucoadhesive systems may be useful for improving exposure of the bacteria to higher local levels of antibiotics. GDDS are designed to be retained in the stomach for extended durations in order to prolong the residence time of dosage forms in the stomach, thereby leading to enhanced bioavailability and reduced dosing frequencies of

the drug (Park and Robinson, 1984) and in the case of antibiotics for *H-pylori* treatment, this would help in eradication from the target site.

Conventional drug delivery systems are not retained in the stomach for long periods, therefore it is difficult to achieve minimum inhibitory concentrations (MIC) in the gastric mucosa where the *H-pylori* reside. Gastroretentive systems in drug delivery have been discussed in some recent review articles (Adebisi and Conway, 2011; Pawar et al., 2011; Streubel et al., 2006; Talukder and Fassihi, 2004). In the treatment of peptic ulcer, there is a need for a more targeted drug delivery system to enhance the eradication of the bacteria by optimising the contact between the drug delivery system and the target biological surface. Mucoadhesive systems help to provide an intimate contact between the delivery system and the underlying target biological surface, thereby improving the therapeutic performance of the drug (Boddupalli et al., 2010) by plugging and sealing unto infected and inflamed mucosal cells in the gastrointestinal tract (Akiyama et al., 1998). Additionally, they can also release the drug in a sustained manner. Floatability will also provide buoyancy to the formulation during this period. These systems will maintain contact with the mucus layer and provide a controlled release of drugs such as antibiotics. In order to achieve mucoadhesion, there is a requirement for a highly expanded and hydrated polymer network, which could enhance contact between the microspheres and the mucus layer (Fundueanu et al, 2004) and the presence of a large volume of liquid helps with the retention of the buoyant microspheres. A combination of strategies using a mucoadhesive-floating system will ensure a site specific delivery and explore the synergy between the two systems, thereby overcoming the shortcomings associated with individual strategies (Ahuja et al., 1997).

Site specific targeting of drugs to a biological surface can also be achieved through conjugate-receptor interactions. Labelling the surface of polymeric drug carriers, such as microspheres, with appropriate conjugates enables targeting of surface receptors on selected cell types. When such systems contact the relevant surface receptor, they can interact potentially being retained at the cell surface. Delivery of drugs directly to the site of interest instead of systemically can result in a lower required dose leading to both cost savings and reduction of potential unwanted side effects. Lectins may provide a site specific targeted drug delivery to mucosal cells due to the fact that they bind to carbohydrate residues specifically and non-covalently (Chowdary and Rao, 2004; Naisbett and Woodley, 1995) and are resistant to protease degradation (Gabor et al., 1997;

Kilpatrick et al., 1985). Lectins have been described as ‘second generation mucoadhesives’ (Kompella and Lee, 1992) and are found in organisms ranging from viruses and plants to animals (Barondes et al., 1994; Ezpeleta et al., 1999). Concanavalin A (Con A) is a lectin extracted from the jack bean, *Canavalia ensiformis*. It binds specifically to certain structures found in various sugars, glycoproteins, and glycolipids, mainly internal and non-reducing terminal α -D-mannosyl and α -D-glucosyl groups (Goldstein and Poretz, 1986; Sumner et al., 1938). This helps in their role in biological-recognition events (Rini, 1995).

In this study, floating-mucoadhesive microparticles containing ethylcellulose (EC) and chitosan were loaded with clarithromycin (CMN) and characterized. The microspheres were conjugated with Con A to form a lectin-drug carrier complex to ensure both controlled and targeted delivery of drug to improve the eradication of *H-pylori*. Although a previous study has described conjugation of Con A on EC microspheres (Jain and Jangdey, 2009), this research further studies the effect of various variables such as grades of EC used, chitosan concentration and Con A added on CE. Also, the impact of conjugation on drug loading, drug entrapment, drug release and mucoadhesion was assessed. The impact of gastric environment should be considered when developing such systems (Noqieira et al., 2013) and in addition, robust *in vitro* mucoadhesion tests were performed which can be useful in cases where *in vivo* models are not readily available.

2: Materials and Methods

EC-10 (low viscosity EC), EC-46 (high viscosity EC), chitosan (high molecular weight), polyvinyl alcohol (PVA), CMN, Con A and FITC-Con A, N-hydroxysuccinimide (NHS) and 1-ethyl-3,3-(dimethylaminopropyl) carbodiimide (EDAC) were obtained from Sigma Aldrich (UK). All other chemicals used were of analytical grade and were used as received.

2.1: Chromatographic conditions for CMN assay

Chromatographic separation was performed on a Shimadzu HPLC equipped with a SPD-20 AV Prominence UV/VIS detector set at 210 nm, an LC 20 AT pump, and SIL-20A Prominence autosampler. The data acquisition was carried out on a LC solution software integrator. Separation was performed using a SphereClone 5 μ m ODS (2) column (150 x 4.6 μ m) (Phenomenex) and CMN was eluted with a mobile phase comprising of KH_2PO_4 : ACN, pH 4.6 (50:50 v/v) containing 5 mM 1-octanesulfonic acid sodium salt (1-OCTS) at

a flow rate of 1.5 ml/min determined at 50°C. The pH was adjusted with phosphoric acid. Injection volume was 50 µl with a total run time of 6 minutes.

2.2: Preformulation studies

2.2.1: Solubility profile of CMN

Solubility of CMN was determined in different buffers from pH 1.2 to 8 maintained at 37 °C. Excess CMN was added to 100 ml of each buffer and agitated continuously for 8 hours in a water bath. Samples were filtered, diluted and analysed by HPLC.

2.2.2: Stability profile of CMN and determination of degradation rate constant

CMN (50 mg) was placed in 100 ml buffers at pH 1.2, 2.0, 3.0, 5.0, 7.0 maintained at 37 ± 1 °C and stirred at 100 rpm. Samples were taken at different time intervals. The pH was adjusted to 5.0 with sodium hydroxide solution (for samples with pH below 5.0) to prevent further degradation of the drug during the analysis. The samples were analysed using HPLC and results compared to those obtained at time 0 h. The degradation of CMN in acidic media is assumed to follow pseudo-first order kinetics and the half-life ($t_{1/2}$) of CMN was determined from the pseudo-first-order degradation rate constant (k).

2.3: Preparation of floating microspheres

Microspheres were prepared using an emulsification/evaporation method (Jain and Jangdey, 2009; Zheng et al., 2006). The drug and the polymers (EC-10 or EC-46) and chitosan were dissolved in dichloromethane. An aqueous solution of surfactant (1 %w/v PVA) was prepared and the polymer-drug solution was added to the aqueous solution at the ratios defined in Table 1. The emulsion formed was stirred continuously at 2000 rpm for 1 hour using a mechanical stirrer. The microsphere suspension was heated to 40 °C under reduced pressure, recovered by filtration, washed and dried at 40 °C for 24 h.

2.4: Characterisation of floating microspheres

2.4.1: Morphology and micromeritics

The morphology and surface structure of the microspheres were examined by scanning electron microscopy (SEM) using a Cambridge Instruments Stereoscan 90 microscope.

Particle size was determined using a Malvern Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK) laser diffraction particle size analyser.

2.4.2: Yield, loading and drug entrapment efficiency (DEE)

Microspheres (20 mg) were agitated for 12 h in methanol to allow extraction of the drug from the polymer. The amount of drug extracted was diluted and analysed by HPLC. The experiment was performed in triplicate and the DEE and yield were calculated as follows:

$$\% \text{ Drug entrapment} = \frac{\text{actual drug content}}{\text{theoretical drug content}} \times 100$$

$$\% \text{ Yield} = \frac{\text{weight of microspheres}}{\text{weight of drug polymer and other non volatile solids}} \times 100$$

2.4.3: Moisture content: Samples (approximately 10 mg) were heated from 25 °C to 350 °C at a heating rate of 10 °C/min using a Thermobalance (Mettler TG50). All thermogravimetric analyses (TGA) were performed in an open pan with purge and protective nitrogen gas flow of 20 ml/min. The moisture content was determined as the loss of mass resulting from loss of water between 50 to 150 °C (Mai et al., 2012; Nassar et al., 2003).

2.4.4: Differential Scanning Calorimetry (DSC): Thermal analysis of the pure drug, polymers and microspheres were carried out using a DSC Mettler Toledo calibrated with an indium standard. Approximately 5-10 mg of the microspheres were weighed and sealed in the standard aluminium pans in the DSC. The samples were held at 25 °C for 1 minute and then heated from 25 °C to 300 °C at a rate of 10 °C / minute under nitrogen atmosphere.

2.4.5: FTIR: The samples were scanned from 400 - 4000 cm⁻¹ at ambient temperature using a Thermo Nicolet 380 FTIR with Diamond ATR. The characteristic peaks of IR transmission spectra were recorded from triplicate samples.

2.4.6: Powder X-ray diffraction analysis (P-XRD)

The effect of the encapsulation process on the crystallinity of CMN was investigated using P-XRD. X-ray diffractograms of CMN, EC, chitosan, physical mixtures of drug and polymers, blank and drug-loaded microspheres were recorded using a Bruker diffractometer (Bruker D2 Phase, United Kingdom). Samples were placed in a stainless steel holder and the surface of powder was levelled manually for analysis. The sample was exposed to X-ray radiation (Cu K α) with a wavelength of 1.5406 Å. The sample was scanned between 5 and 40° of 2 θ with a step size of 0.019° and a step time of 32.5 s.

2.4.7: *In vitro* buoyancy tests

Microspheres (500 mg) were placed in 900 ml of simulated gastric fluid (SGF) pH 2.0 and pH 5.0 buffer containing 0.02 %w/v Tween 80 in a USP dissolution apparatus II. This dispersion was stirred continuously at 100 rpm for 12 hours after which time buoyant microspheres and the settled microspheres were collected, dried and weighed. The buoyancy was determined by the weight ratio of buoyant microspheres to the sum of both the buoyant and settled particles:

$$\% \text{ Buoyancy} = \frac{W_f}{(W_f + W_s)} \times 100$$

where, W_f and W_s are the weights of the floating and settled microspheres, respectively. All the tests were carried out in triplicate.

2.4.8: *In vitro* release studies

Drug release from the microspheres was carried out using a USP II paddle dissolution apparatus. Microspheres, equivalent to 100 mg of CMN, were packed in hard gelatin capsules and immersed in 900 ml buffer (pH 2.0 and 5.0) containing 0.02 %w/v Tween 80. The solution was maintained at 37° ± 1°C and at a rotation speed of 100 rpm. Samples were analysed by HPLC with a correction applied for degradation using the degradation rate constant k (Chun et al., 2005). The *in vitro* release profiles were subjected to various mathematical models to study mechanisms of release (Najib and Suleiman, 1985, Higuchi, 1963, Korsmeyer et al., 1983). For spherical matrices, if $n = 0.43$, it indicates Fickian diffusion, $0.45 \leq n < 0.89$, indicates an anomalous non-Fickian transport; $n = 0.89$, a case II transport and $n \geq 0.89$, a supercase II transport drug release mechanism dominates.

2.5: Floating-mucoadhesive microspheres

2.5.1: Conjugation of Concanavalin A to microsphere surfaces

The activation of the carboxyl group of EC microspheres was carried out by the addition of 10 ml 0.1M EDAC and 10 ml of 0.11 M NHS in PBS (pH 5.8) (Damink et al., 1996; Irache et al., 1994). After incubation for about 3 hours, excess activating agents were removed by washing with PBS. The microspheres were re-suspended in a solution containing Con A in PBS. The suspension was left overnight and microspheres collected by centrifugation to remove any free lectin, washed three times with distilled water and stored at 4 °C until required. For drug-loaded microspheres, FITC-labelled Con A was used to determine the conjugation efficiency.

2.5.2: Determination of CE

The amount of bound Con A and CE was estimated using a Folin- Ciocalteu phenol assay. The reagents used include Reagent A (2 g Na₂CO₃ in 100 ml of 0.1N NaOH); Reagent B (0.5 g of CuSO₄ · 5H₂O in 100 ml, 1% sodium /potassium tartrate); Reagent C (50 ml of Reagent A with 1 ml of Reagent B) and Reagent D (1:1 dilution of Folin – Ciocalteu phenol reagent with water). 10 ml of Reagent C was added to a suspension containing 100 mg of conjugated microspheres, mixed thoroughly and allowed to stand for 30 minutes. 1 ml of Reagent D was added and mixed rapidly. After 30 minutes, the solution was filtered and absorbance was measured against a blank using a UV-Visible spectrophotometer (Jenway 6305 UV/VIS Spectrophotometer) at 750 nm. For drug-loaded microspheres, the difference between the fluorescence of the FITC-Con A solution before and after conjugation was measured using a Shimadzu Fluorescence Spectrophotometer. FITC-Con A was measured at excitation and emission wavelengths of 493 nm and 516 nm respectively and the CE determined from the concentration of free lectin in the supernatant (Ezpeleta et al., 1999). The effect of chitosan concentration on conjugation efficiency was also studied with varying concentrations of chitosan (0 - 2 %w/w). Also, varying concentrations of Con A (0.25 – 1 mg/ml) were incubated with the microspheres to determine its effect on conjugation efficiency.

2.5.3 Zeta potential (Zp)

The zeta potential of the microspheres was measured by electrophoresis using a Zetasizer Nano Z (Malvern Instruments Ltd., UK). The microspheres were dispersed in media at pH 7.0 by ultrasonication at a concentration of 2 %w/w for 30 minutes at 25°C.

2.5.4: *In vitro* interactions with PGM

The binding capacity of Con A–conjugated microspheres was determined by mixing 1 ml (1 mg/ml) of PGM suspension in PBS (0.05 M, pH 7.4) with equal volumes of the conjugated suspension. Following incubation at room temperature (approximately 20 °C) and at 37 °C, the samples were centrifuged at 10,000 rpm for 10 min. The amount of bound mucin was determined by UV spectroscopy at 251 nm (Agilent Cary 60 UV-Vis Spectrophotometer). The PGM binding efficiency of the microspheres was calculated using the equation

$$\% \text{ PGM binding} = \frac{C_0 - C_s}{C_0} \times 100$$

where, C_0 is the initial concentration of the PGM used for incubation and C_s is the concentration of free PGM in the supernatant. The reference consisted of the same amount of PGM present in the samples. The activity of unconjugated microspheres with PGM was used as a control. *In vitro* diffusion of CMN through 3 % mucin at pH 2 and pH 5 was studied using vertical Franz diffusion cells. The receptor compartment was filled with 30 ml buffer (at both pH) maintained at 37°C and agitated by stirring with a magnetic stirrer. Dialysis membrane (cut off MW 14,000) was mounted between the donor and receiver cells with the mucin dispersion representing an unstirred layer of mucus gel layer and 1 ml of receptor fluid was sampled, replaced and analysed by HPLC. All experiments were performed in triplicate.

2.5.4: *In vitro* wash off test

A 2 cm wide and 2 cm long piece of pig gastric mucosa was mounted onto a glass slide and accurately weighed microspheres were spread onto the wet surface of the tissue specimen, and incubated for 60 minutes in a humidity chamber to facilitate interaction between the microspheres and the mucosa. The slide was positioned at an angle of 45° and maintained at 37 ± 1°C. Buffers (pH 2.0 and 5.0), previously warmed to 37 ± 1 °C, were circulated over the tissue at a rate of 10 ml/min for 10 minutes supplied via a peristaltic pump (Watson Marlow model 202). The microspheres remaining on the tissue surface were removed after the test and the percentage of the remaining microspheres was calculated using the formula:

$$\% \text{ Mucoadhesion} = \frac{W_0 - W_1}{W_0} \times 100$$

where W_0 = weight of microspheres applied initially and W_1 = weight of microspheres rinsed off.

2.5.5: CMN stability in microspheres dispersed in SGF (pH 2.0 and 5.0)

Microspheres containing an equivalent of 100 mg CMN were dispersed in 100 ml of buffers at pH 2.0 and 5.0 and shaken at 100 rpm at 37 ± 0.5 °C in water-bath for between 0 - 360 minutes. The drug content of the filtrate (amount of CMN released from the microspheres) and the microspheres (amount of CMN retained in the microspheres) were determined separately by HPLC and the chromatogram observed for presence of the acidic degradation products of CMN.

2.5.6: Storage stability studies

The microspheres were sealed in capsules and stored at room temperature (approximately 20° C) and 4° C over a period of three months. The average diameters, zeta potential, DEE, mucoadhesion and *in vitro* drug release were determined by methods described previously.

2.6: Statistical analysis: The experimental data were expressed as mean \pm SD (standard deviation). Student t tests and one way analysis of variance (ANOVA) were used to compare different parameters on the mean particle size, DEE, drug release and to determine the statistical significance where appropriate. Probability values $p < 0.05$ was considered significant.

3: RESULTS AND DISCUSSION

CMN microspheres were prepared using an oil-in-water emulsion solvent evaporation method. The drug is encapsulated in a gastro-resistant polymer film of EC which can act as a physical barrier and prevent the degradation of CMN, which is unstable in the acidic pH of the stomach. EC, being a water insoluble polymer, helps to modify the release and prolong the action of the antibiotic in the acidic environment of the stomach. The physicochemical properties of the microspheres, conjugation with Con A, mucoadhesion and the *in vitro* release profile of drug was assessed using both low and high viscosity EC.

Mucoadhesion was further modified by inclusion of chitosan in the microsphere to further enhance the specific binding of the conjugated microspheres.

3.1: Preformulation studies

3.1.1: CMN HPLC assay

CMN was detected at 210 nm across a concentration range of 10 µg/ml to 50 µg/ml with a retention time of approximately 2.5 min. The limit of detection (LOD) and limit of quantification (LOQ) were 1.85 ± 0.01 µg/ml and 5.63 ± 0.05 µg/ml respectively. The intra-day and inter-day relative standard deviation (RSD) were less than 5% and recovery was more than 96 % over 80-120 %.

3.1.2: Solubility and stability profile of CMN in buffers

The solubility of CMN is pH dependent with a decrease in solubility with increasing pH and the highest solubility observed at the lowest pH measured (Figure 2a). The stability of CMN at different pH levels was assessed because of its known instability at acidic pH (Erah et al., 1997; Nakagawa et al., 1992) and without this, results obtained from the dissolution study can underestimate the amount of drug released (Rajinikanth and Mishra, 2008). The stability profiles are presented in Figure 2b. The degradation rate constants (k) at pH 1.2, 2.0, 3.0 and 5.0 were 1.45 ± 0.13 h⁻¹; 0.45 ± 0.01 h⁻¹, 0.055 ± 0.007 h⁻¹ and 0.0028 ± 0.0003 h⁻¹ respectively, with CMN being more stable as pH increased and the degradation half-lives ($t_{1/2}$) were 0.47 ± 0.04 h, 1.53 ± 0.005 h, 12.65 ± 1.72 h and over 100 h respectively. These results correspond with literature reports with calculated k and degradation half-life ($t_{1/2}$) at pH 2.0 at 37 °C being 0.472 h⁻¹ and 1.47 h (Chun et al., 2005).

3.2: Characterisation of microspheres

3.2.1: Micromeritics and morphology

The microspheres were smooth and spherical in shape as seen in Figure 3. The surface of the microspheres became smoother and less porous with an increase in EC concentration. There was no evidence of collapsed particles (with increasing polymer concentration), aggregation of particles or presence of free drug crystals. The conjugation process did not

affect the microsphere morphology or the structural integrity with the surface appearing similar to that of the unconjugated microspheres. The microspheres ranged in size from 71-183 μm (S1-10 series) and 66-190 μm (S-46 series) with the particle sizes not being significantly different ($p > 0.05$) despite the apparent difference in molecular weight of the EC polymers and thus viscosity of the droplets. The mean particle sizes of the floating microspheres significantly increased ($p < 0.05$) with increasing polymer concentration as shown in Table 2.

3.2.2: Yield, Drug loading and drug entrapment efficiency (DEE)

The yields ranged from 57 to 83 % with the DEE being between 50 % and 95 %. Both parameters increased with increasing viscosity and polymer concentration ($p < 0.05$) in the microspheres (Table 2).

3.2.3: TGA and DSC

Moisture content of the microspheres was less than 2 %w/w (results not shown). DSC for pure CMN showed a sharp symmetrical melting endotherm at 227.5 $^{\circ}\text{C}$ as expected (Figure 4a). There was a slight reduction in the melting endotherm of CMN and broadening of the CMN peak in the drug-loaded microspheres (Figure 4a and 4b) suggesting a less ordered crystal structure exists in the microspheres. This demonstrates that there was no significant interaction between the drug and any other components of the formulation. In the conjugated microspheres, the absence of any other extra peaks confirms that the conjugation did not alter the thermogram (Figure 4c).

3.2.4: FTIR

The IR spectra of CMN (Figure 5) showed the characteristic band of hydrogen bonds between $-\text{OH}$ groups vibration at 3479.1 cm^{-1} . The characteristic band $\text{C}=\text{O}$ vibration of the lactone group was at 1732.6 cm^{-1} and a strong absorption band at 1692.8 cm^{-1} belonging to the carbonyl ketone peak and for $\text{N}-\text{CH}_3$ stretching of aromatic ring at 1423.0 cm^{-1} . The IR spectra for EC shows a distinct peak at 3479.0 cm^{-1} which is due to the $-\text{OH}$ groups present on the closed ring structure of the polymer's repeating units and the intra- and intermolecular hydrogen bonding (Ravindra et al., 1999). The asymmetric peak observed between 2974.2-2870.6 cm^{-1} may be due to the $-\text{CH}$ stretching. The peak present at 1375.2 cm^{-1} is due to $-\text{CH}_3$ bending and the smaller peak at 1444 cm^{-1} is due to the $-\text{CH}_2$ bending. The broad peak at 1103.8 cm^{-1} may be due to the $\text{C}-\text{O}-\text{C}$ stretch in the cyclic ether

(Desai et al., 2006). The formulations showed characteristic peaks which were close to the principal IR peaks of the drug, confirming the presence of CMN in the microspheres indicating no strong interactions between the drug and the polymers used and the stability of the drug during the microencapsulation process. Conjugation of Con-A was through the formation of amide bonds between the $-NH_2$ groups of Con A and $-COOH$ on EC. This was confirmed with the presence of two amide bonds (I and II) with peaks at 1716.1, 1651.1 and 1538.6 cm^{-1} which are characteristics of amides mainly in proteins (Anande et al., 2008; Jain and Jangdey, 2009). These peaks were noticeably absent in the unconjugated microspheres.

3.2.5: P-XRD

EC and chitosan showed their amorphous nature while CMN was highly crystalline (Figure 6). The diffraction patterns of the physical mixture and the drug-loaded microspheres showed similar peaks of the CMN, however they were broader and had reduced intensities relative to that of the pure drug. This could be ascribed to the crystalline nature of the drug in the microspheres. The diffraction patterns of both the conjugated and unconjugated microspheres were similar, meaning the conjugation did not affect the crystallinity of the drug in the formulation.

3.2.6: *In vitro* buoyancy profile

Buoyancy was between 78 – 97 % after 12 h, increased with increasing polymer concentration and floating occurred immediately on contact with the dispersion media. Conjugation and pH of dispersion media had no significant effect on the buoyancy of the microspheres ($p > 0.05$) but the addition of drug decreased buoyancy (Table 2).

3.3.6: *In vitro* release studies

Drug release from microspheres was determined in SGF pH= 2.0 (Figure 7a) and buffer at pH = 5.0 (Figure 7c) to cover the pH range for when CMN is used alone or in combination with omeprazole (Gustavson et al., 1995) since omeprazole can increase gastric pH to 4.0-6.0. Also, this covers potential variations in stomach pH in the presence and absence of food. Tween 80 was added to the dissolution medium to increase the wetting and hydration of the polymers (El-Kamel et al, 2001). At pH 2.0, the dissolution of the unencapsulated drug was rapid and complete within 3 hours (Figure 7a). The release of drug from the

formulations at this pH was biphasic with an initial burst release followed by a second moderate release rate which is a characteristic feature of matrix diffusion kinetics. The burst release and release rates were reduced with increase in polymer concentration and increase in polymer viscosity (Table 3) ($p < 0.05$). The observed reduction in the burst release is due to a reduced amount of surface-associated drug, since there was an increase in DEE with increases in the polymer concentration and viscosity as evident from $t_{50\%}$ values (time required for the release of 50 % encapsulated drug) (Figure 7a,b). Also, an increase in polymer/drug ratio led to an increase in the particle size, decrease in surface/volume ratio, increase in polymer density and an increase in diffusion path length all of which contribute to prolonging drug release (Muramatsu and Kondo, 1995). EC has a low water permeability while both chitosan and CMN are relatively soluble at low pH, therefore formulations containing more drug will most likely have a higher initial burst release (S2-10 / S2-46) than those with less drug (S5-46/ S5-10) in acidic pH. Also, dissolution of chitosan may lead to increased permeability of the microspheres, especially at later times. At pH 5.0, solubility of CMN and chitosan is reduced and drug release was much slower from the microspheres. Drug release rate was also slower with an increase in polymer viscosity and polymer concentration (Figure 7b). The drug release rate from Con S3-46 was slower than the corresponding unconjugated S3-46 at pH 2.0, with no associated burst release as seen with S3-46. This may be due to the loss of the surface associated drug and the reaction at the surface of the microspheres rendering it slightly less permeable than unconjugated microspheres. Drug release at pH 2.0 from all formulations fitted well to Higuchi kinetics ($R^2 = 0.97$ to 0.99) indicating diffusion controlled release dominated. For the Korsmeyer–Peppas model ($R^2 = 0.97-0.99$), the release exponent 'n' of all the formulations was between 0.23 – 0.44, indicating Fickian diffusion-controlled release (Table 3) with the exception of only one formulation, Con S3-46. This supports the formation of a monolithic microspheres that release drug by Fickian diffusion. The 'n' value for Con S3-46 was 0.64 which indicates anomalous non-Fickian transport. This change in drug release kinetics may be due to the conjugation effects on the microsphere surface.

3.4: Concanavalin-A conjugated microspheres

The conjugation reaction involves the use of water soluble carbodiimide which reacts with the carboxyl group on EC, leading to the formation of an amine reactive O-acylisourea intermediate. The intermediate may react with an amine on the lectin forming a stable

amide bond. This intermediate is susceptible to some side reactions such as hydrolysis, therefore the solutions are stabilized with the addition of NHS by converting it to an amine reactive NHS ester, thus increasing the efficiency of the coupling reaction. The use of the carbodiimide method is better than other crosslinking agents such as glutaraldehyde because it minimises the risk of structural modification of the conjugated ligands at locations which may be critical to their binding activity.

3.4.1: Characterisation of conjugation microspheres

The particle size of the microspheres increased with conjugation with S1-46 increasing from about 65 μm to about 115 μm (ConS1-46). This may be due to the presence of a layer of Con A on the surface of the microspheres. The amount of Con-A bound onto the surface of the microspheres was between 11.2 ± 0.3 and 15.3 ± 1.9 μg Con A / mg microspheres with a CE of 56 - 77 % of the initial lectin concentration (1 mg/mL). Residual PVA present on the microsphere surface leading to surface -OH groups being present can act as a steric barrier to a higher conjugation (Scholes et al., 1999) and the presence of chitosan may also contribute to lower efficiency than expected. There was a reduction in CE with the addition of drug and this may be due to the presence of drug on the surface of microspheres rendering some of the -COOH unavailable for conjugation. The CE of Con A to the microsphere increased significantly ($p < 0.05$) with increasing lectin added (Figure 8a). This contrasts with WGA conjugation efficiency to PLGA nanospheres (Weissenbock et al., 2004) which was found to be independent of lectin concentration. Increasing the concentration of chitosan from 0 to 2 % w/w in the formulation led to about 32 % reduction in CE (Figure 8b). Using different grades of EC did not have a significant effect on the CE of blank microspheres as the CE of both Con S1-46 and Con S1-10 were similar, however for drug loaded microspheres there was an increase of about 16 % in CE with Con S3-46 when compared with Con S3-10. The coupling process and washing led to the loss of loosely encapsulated surface-associated drug with a reduction in drug loading of Con S3-46 by about 14 % compared to S3-46 (Table 2). There was more drug loss observed with S3-10 with a loss of about 30 % in Con S3-10 which was more than double the loss observed in Con S3-46. The DEE of these conjugated microspheres was above 49%.

3.4.2: Zeta potential

Zp for S1-46 and Con S1-46 were -10.8 ± 0.6 mV and $+29.4 \pm 1.07$ mV respectively while S3-46 had values of -13.4 ± 1.5 mV and Con S3-46 was $+18.3 \pm 1.5$ mV. The negative Zp of the unconjugated microspheres was due to the presence of uncapped end carboxyl groups present at the surface of EC as a result of deprotonation at pH 7.0. The conjugation of Con A on the microsphere surface normally causes a positive increase in the Zp of microspheres (Anande et al., 2008). The presence of drug had a negative effect on the Zp of the microspheres and the influence of drug on Zp of microspheres has been reported (Huang et al., 2003; Martinac et al., 2005). The conjugation was performed at a slightly acidic pH (pH=5.8), therefore Con A (isoelectric point = 4.5 - 5.5) would be positively charged under this condition and this may explain the increase in particle surface charge density by neutralizing the negative charge. Lectin coating on gliadin microparticles positively increased the Zp of the microparticles (Umamaheshwari and Jain, 2003). The positive Zp is important for effective electrostatic interaction of the microspheres with the negatively charged sialic acid of the gastric mucosa leading to prolonged gastroretention.

3.4.3: *In vitro* interactions with PGM

The conjugated microspheres have a higher affinity for PGM than unconjugated particles (Figure 9a). PGM binding to S1-10 / S1-46 and Con S1-10/Con S1-46 as a function of time is shown in Figure 9b. The amount of mucin bound to the ConS1-46 was about 81% more than that bound to S1-46. An increase in bound mucin of about 79 % was observed with Con S1-10 (Figure 9a). The equilibrium for the interaction was reached after incubation between 60 to 120 minutes (Figure 9b) and the adsorption process was not affected by the temperatures used ($p > 0.05$).

3.4.4: *In vitro* drug diffusion through PGM

The release of drug from the microspheres through the mucin solution was sustained over a period of 12 h at both pHs. There was no burst release and there was an initial lag time of approximately 120 minutes observed with Con S3-46 which was longer than that observed for the S3-46 (Figure 10). The absence of the burst release and the sustained release of drug from the formulation may be due to the loss of the surface associated drug or binding of the mucin to the surface of the microsphere, thus presenting a further barrier to diffusion. S3-46 did not also show an obvious burst release and the release rate was significantly higher than that of the Con S3-46. The flux of the drug through the mucin

dispersion and membrane was $145.0 \pm 5.73 \mu\text{g cm}^{-2} \text{h}^{-1}$ (ConS3-46) and $243.9 \pm 8.85 \mu\text{g cm}^{-2} \text{h}^{-1}$ (S3-46) compared with $576.5 \pm 10.52 \mu\text{g cm}^{-2} \text{h}^{-1}$ from a saturated solution at pH 2.0. This represents a reduction of about 40% in drug flux with the ConS3-46 compared with S3-46 due to reduced drug availability at the particle surface and an increased diffusional pathlength. At pH 5.0, the fluxes were significantly lower than those at pH 2.0 ($p < 0.05$) as expected with the decrease in drug solubility. In the presence of mucin, there was still a sustained and adequate release of drug from the conjugated microspheres and the presence of lectin on the surface of the microsphere did not hinder the release of drug from the microspheres in the presence of mucin. Mucus is a major barrier to diffusion for drugs and nutrients with a capacity for binding. The mucus layer of the stomach and the intestine are reported to be 50 - 600 μm and 15 - 450 μm respectively (Khanvilkar et al., 2001; Lee and Nicholls, 1987; Norris et al., 1998) and the target *H-pylori* reside in and below this. The rate of drug transport through mucus can be an important determinant of the efficacy of a formulation and better represents the *in vivo* environment.

3.4.5: Ex-vivo wash off test

Mucoadhesion of conjugated microspheres to porcine gastric mucosa at pH 2.0 was $64.0 \pm 3.58 \%$ to $78.7 \pm 2.87 \%$ compared with 9.9 ± 4.37 to $15.5 \pm 3.64 \%$ of the unconjugated microspheres, which corresponds to an average enhancement in mucoadhesion of 85 %. At pH 5.0, there was a similar increase with 90% increase in mucoadhesion with the conjugated microspheres. This is due to the lectin coating on the microsphere and this had a significant effect on mucoadhesion ($p < 0.05$) due to the affinity of Con A to the glycoproteins of the stomach mucus and mucosa. Increasing chitosan concentration reduced CE; however comparing mucoadhesion properties of conjugated microspheres with or without chitosan shows that although CE was decreased in the presence of chitosan, mucoadhesion was lower than those with both chitosan and lectin showing a synergistic improvement in mucoadhesion.

3.4.6: Stability of microspheres in SGF (pH= 2.0 and buffer pH= 5.0)

CMN degrades below pH 3 into 5-O-desosaminyl-6-O- methyl-erythronolide A, with the loss of the cladinose sugar (Mordi et al., 2000; Nakagawa et al., 1992) and this could be detected via a stability-indicating HPLC method used. During stability studies at pH 2.0,

this degradation product was present after the first hour; while at pH 5.0, there was no degradation over the duration of the study 6 h. The drug present in the microspheres at pH 2.0 and pH 5.0 was stable for the duration of the study and was protected from degradation.

3.4.7: Storage stability

After 3 months at 4 °C, there was no significant difference in the particle size, zeta potential, DEE, drug release and the mucoadhesion of the formulations as shown in Tables 4 and 5. The activity of the lectin was still maintained whilst stored at this temperature since there was no significant change in the proportion of microparticles adhered to the porcine gastric mucosa. However, storage at room temperature led to a slight change only in the mucoadhesion with all the other parameters being almost the same. Drug release from these microspheres was unaffected by storage but to preserve lectin efficiency, lower storage temperatures may need to be used (Figure 11).

4: Conclusion

CMN loaded ethylcellulose microspheres were prepared using the solvent evaporation method and the mucin binding lectin, Concanavalin A, was successfully attached to the microspheres up to a maximum of 15.3 µg Con A per mg microsphere. The inclusion of CMN did not significantly reduce the amount of Con A bound to the surface of the microspheres, however, conjugation led to a reduction in the DEE of the microspheres with more drug loss being observed with the S-10 series. *In vitro* mucohesion studies confirmed the enhancement of mucohesion due to the presence of lectin. The drug loading, DEE, buoyancy and *in vitro* drug release in both the dissolution media and mucin dispersion were not compromised by the conjugation process. A synergistic increase in adhesion was observed when chitosan was included in the formulations and a combination approach may facilitate enhanced delivery of antibiotics to the target site in the stomach.

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Figure 1: Schematic representation of the unconjugated and conjugated CMN microspheres

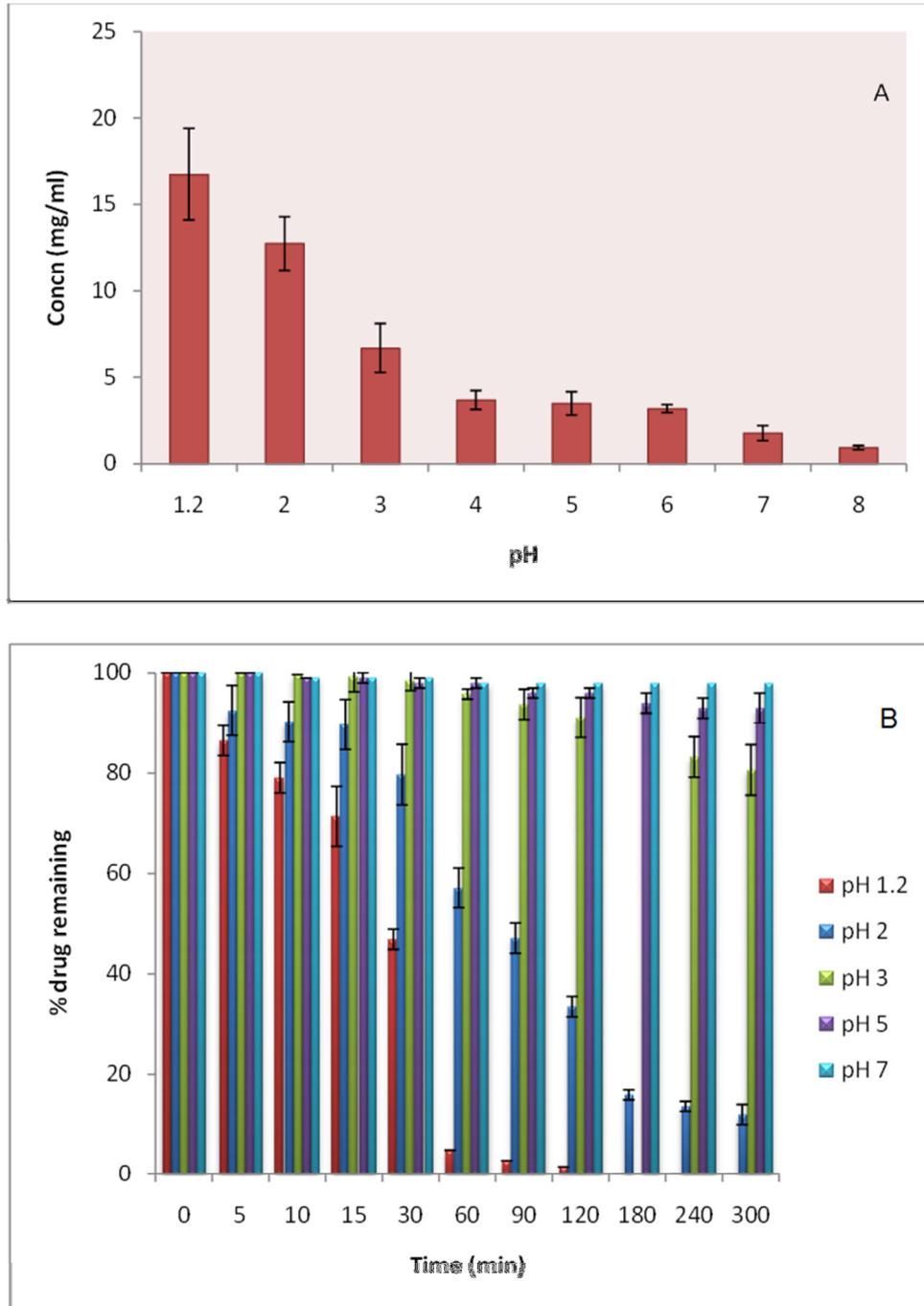


Figure 2: A) Solubility profile of CMN and B) Stability profile of CMN in different buffer pH at 37°C.
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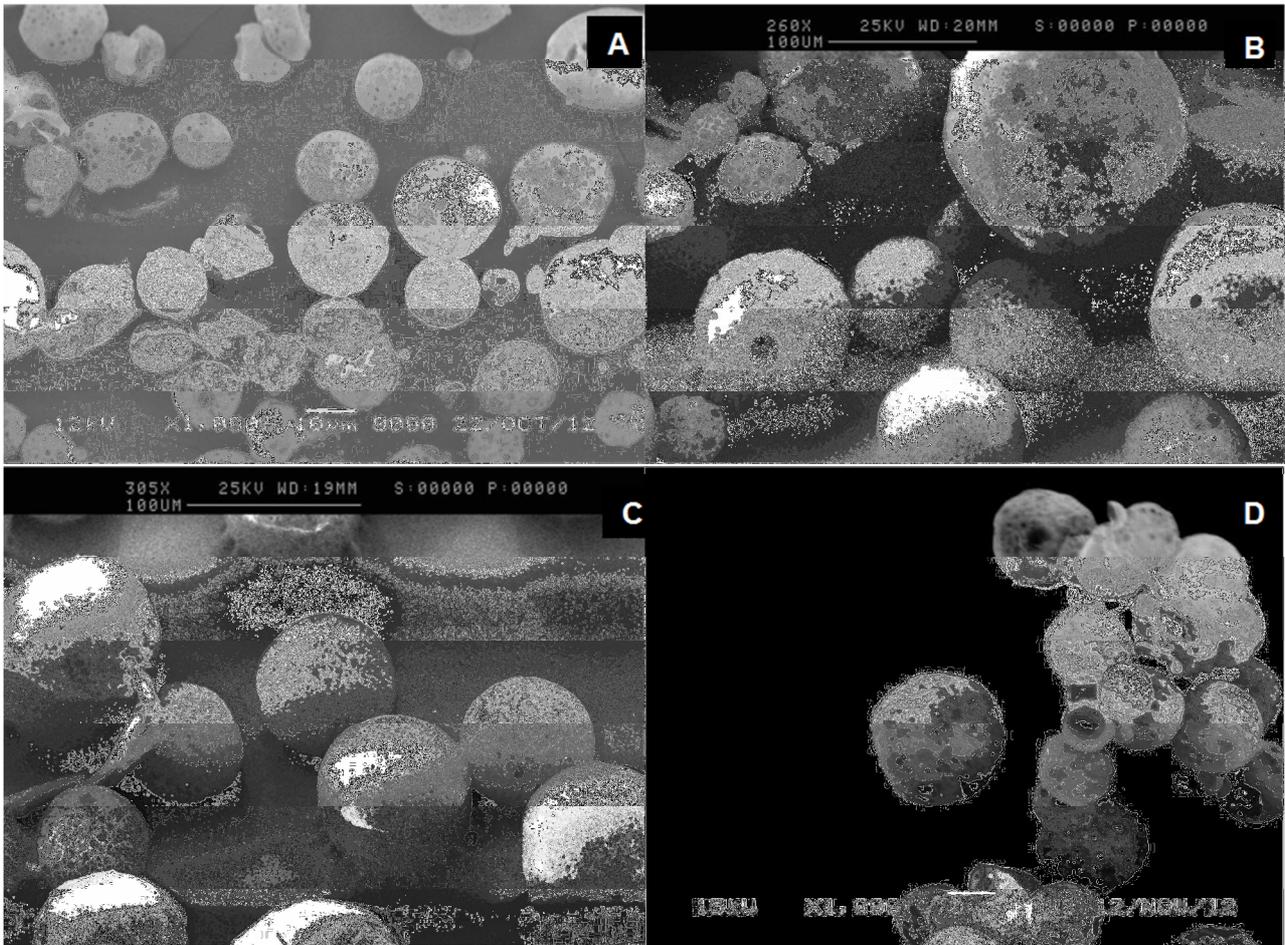


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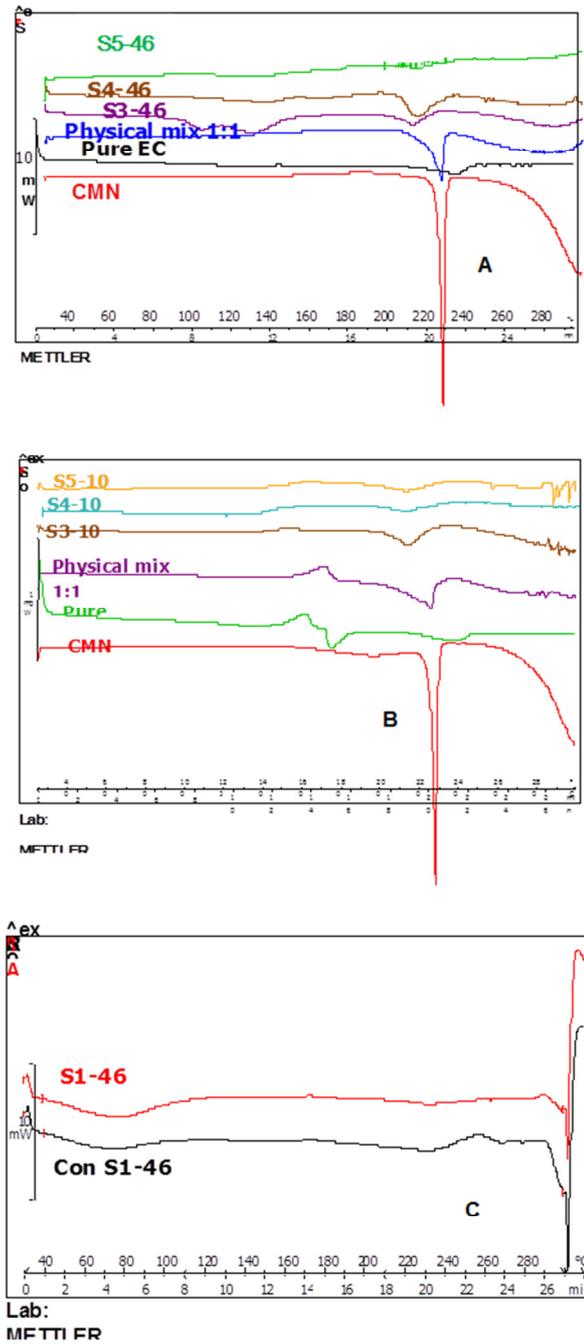


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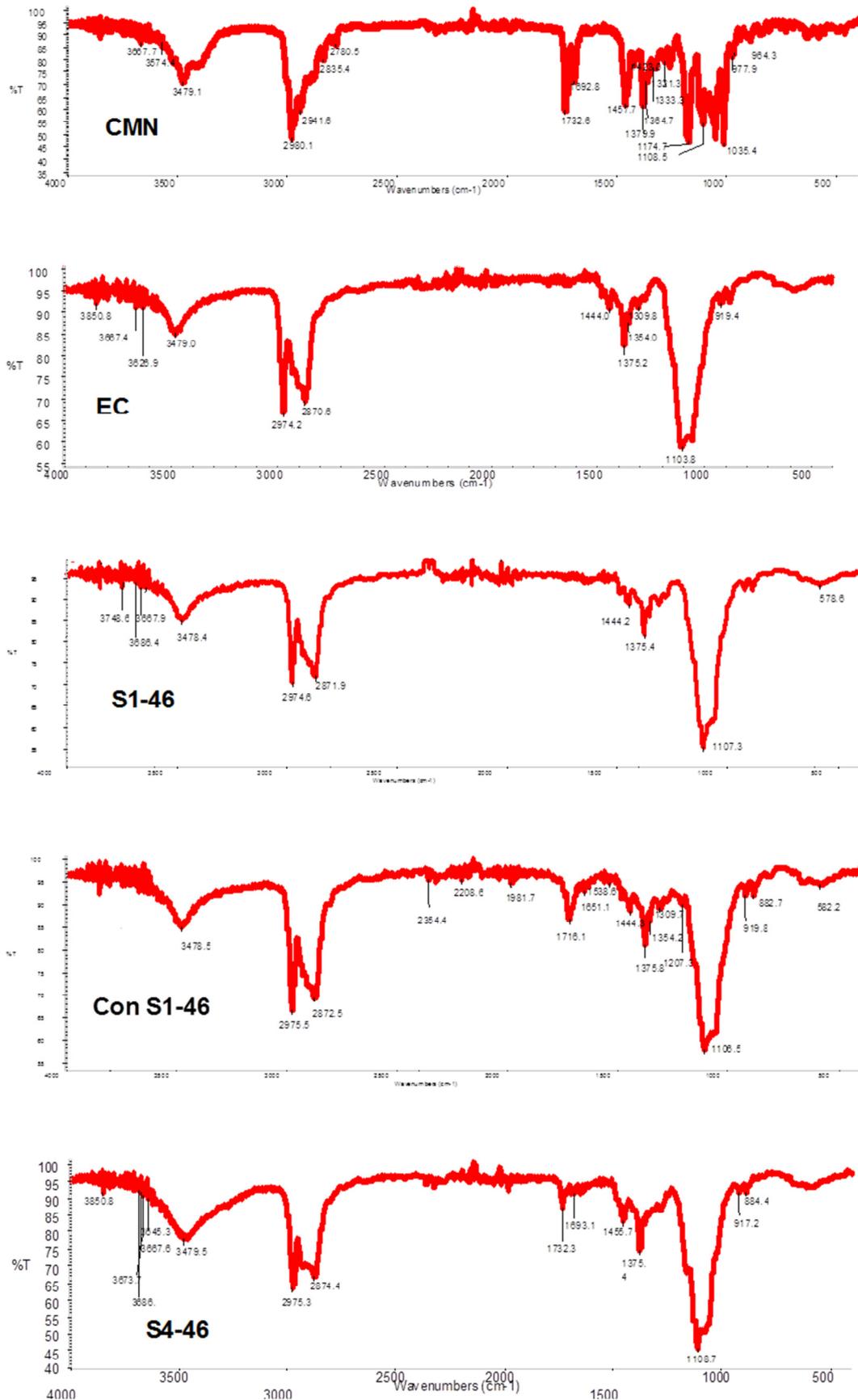


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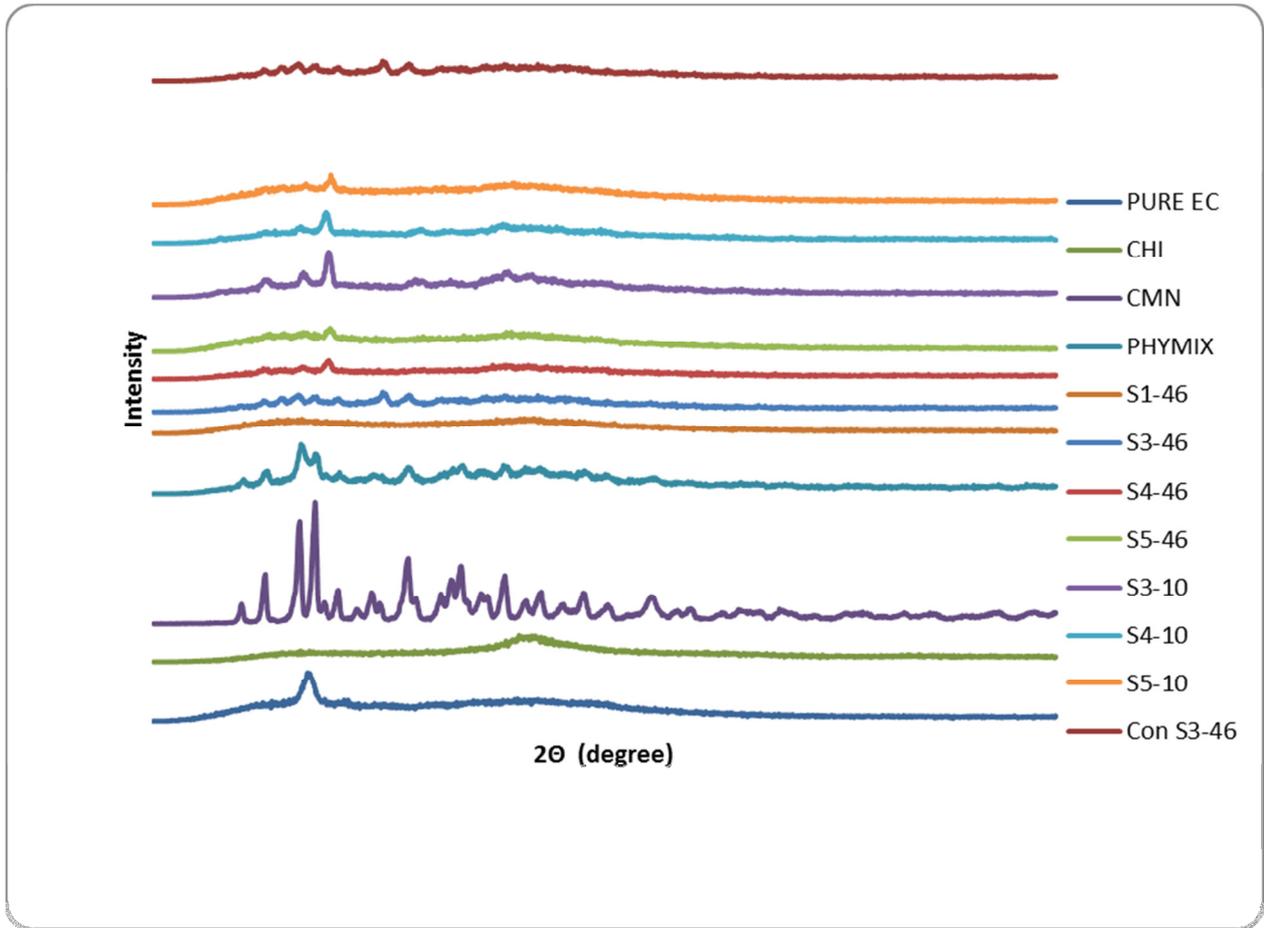


Figure 6: P-XRD of microspheres

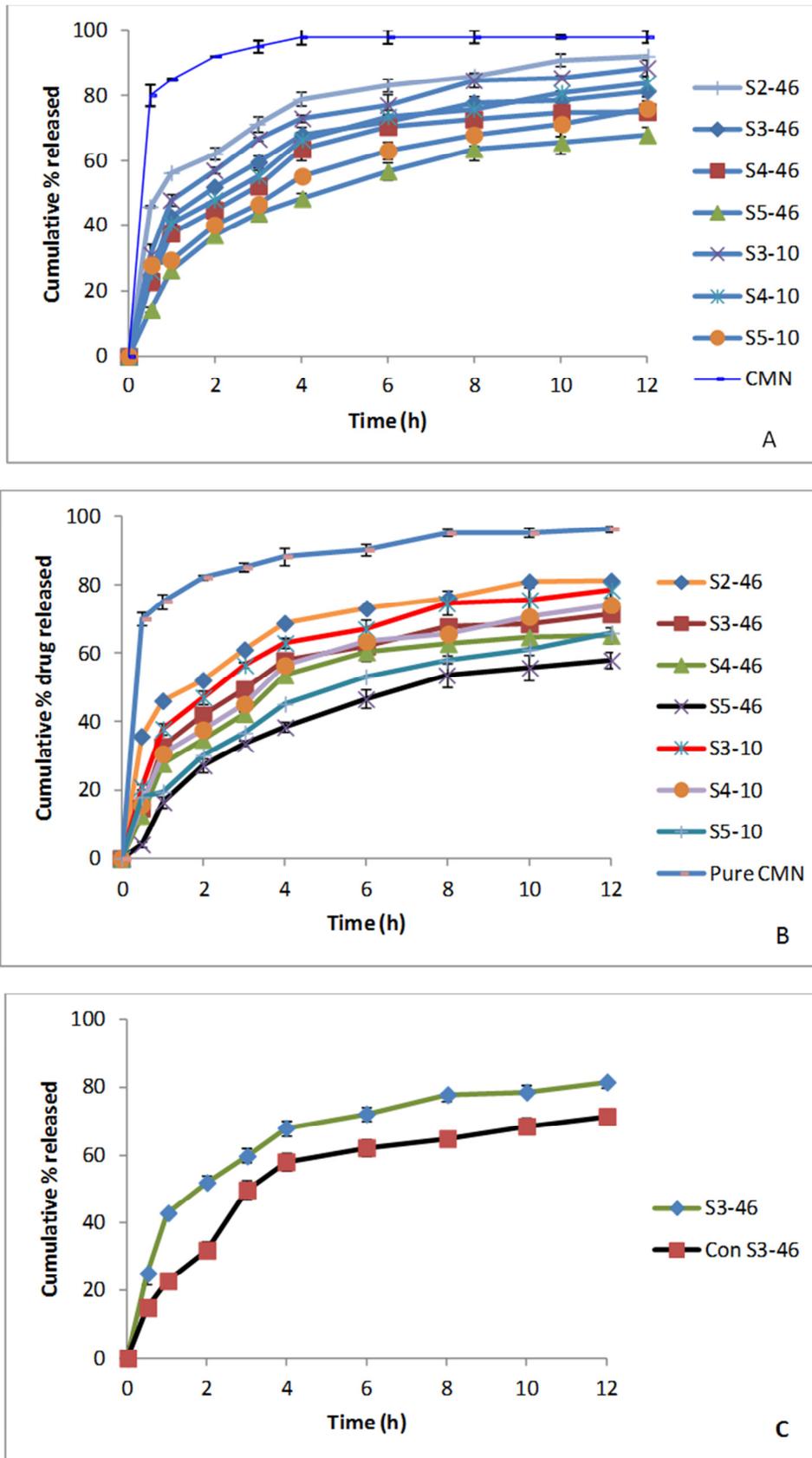


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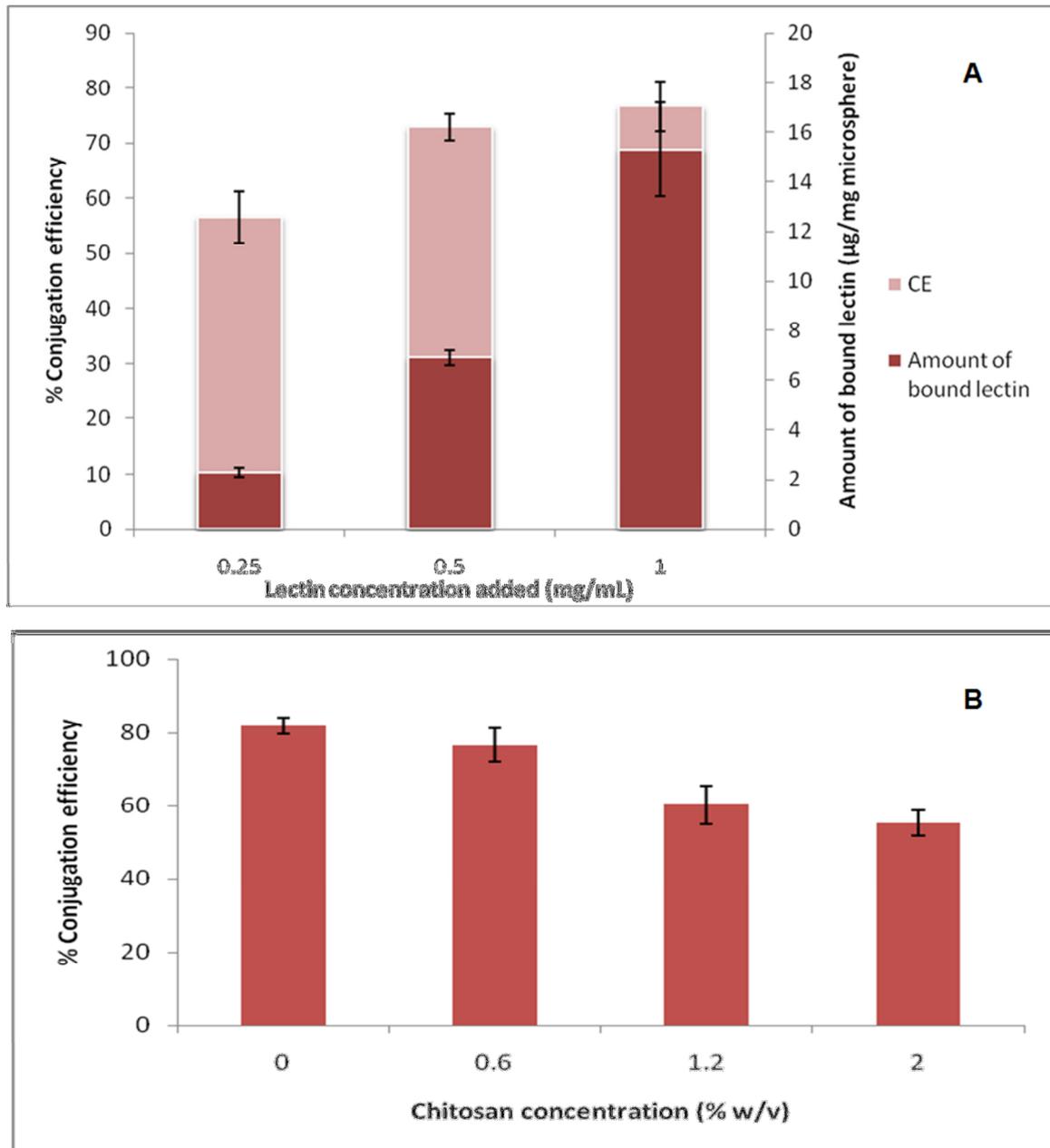


Figure 8: Effect of A) initial lectin concentration and B) Chitosan concentration on CE.

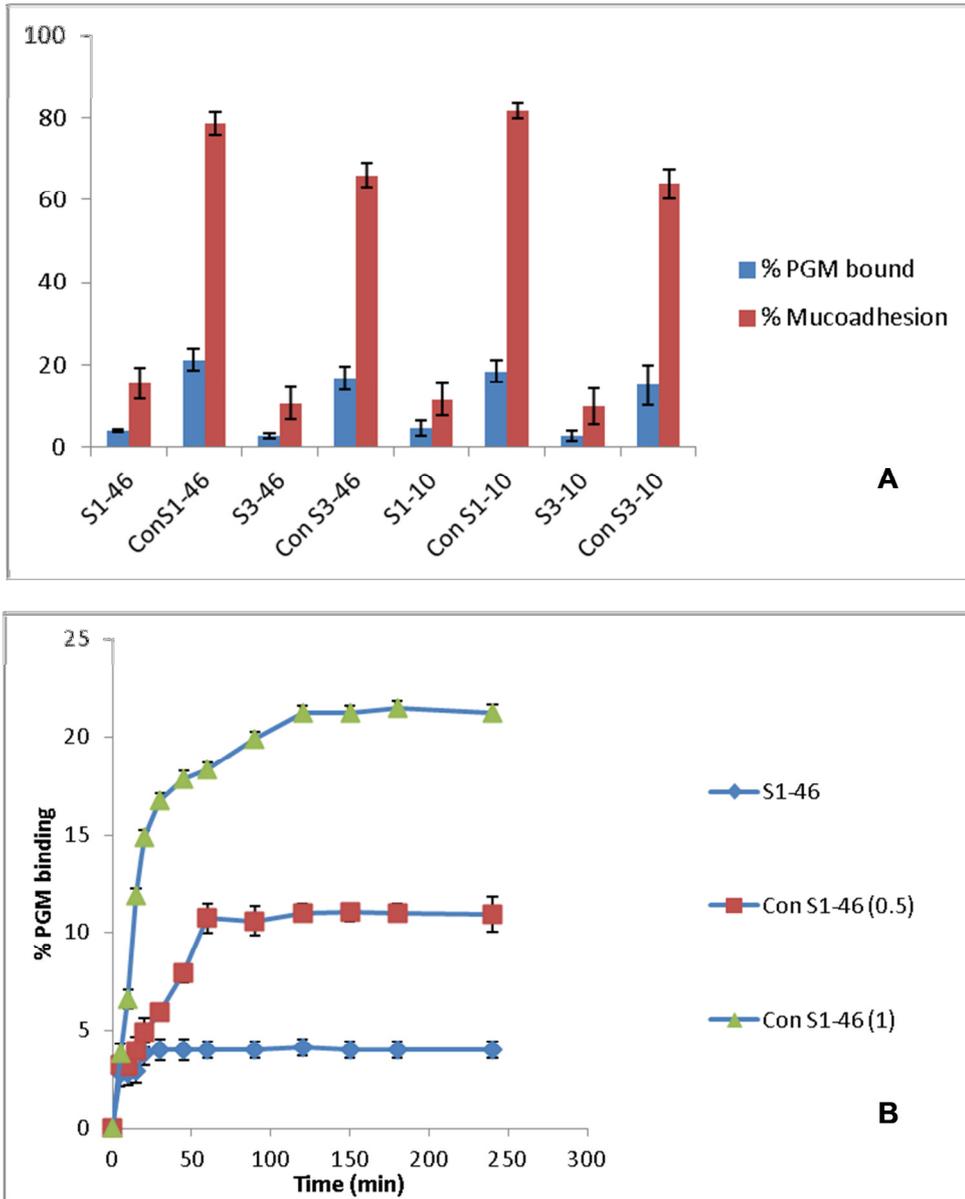


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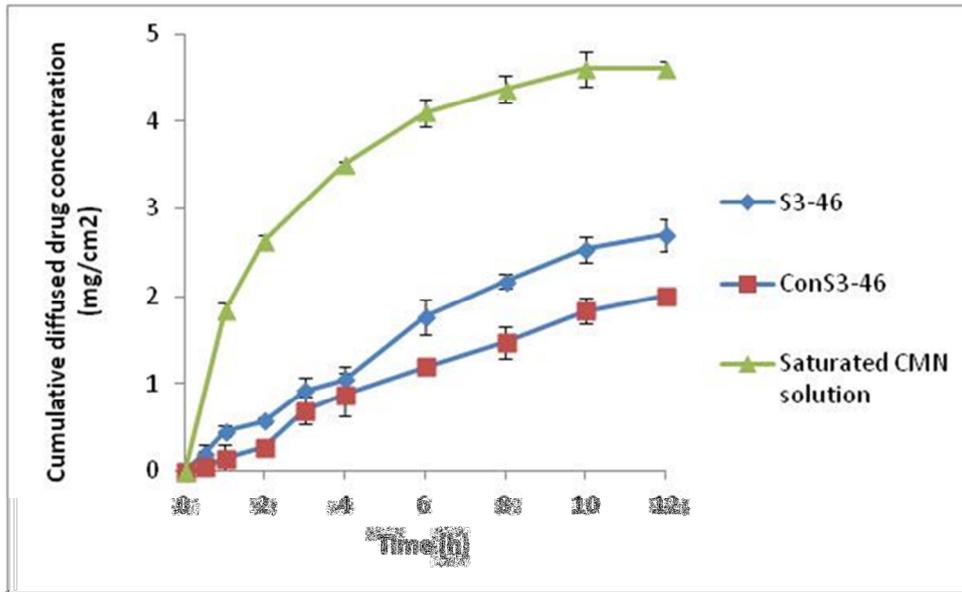


Figure 10 : Franz cell diffusion studies of the microspheres in mucin suspension (pH 2). Results presented as mean \pm SD (n=3).

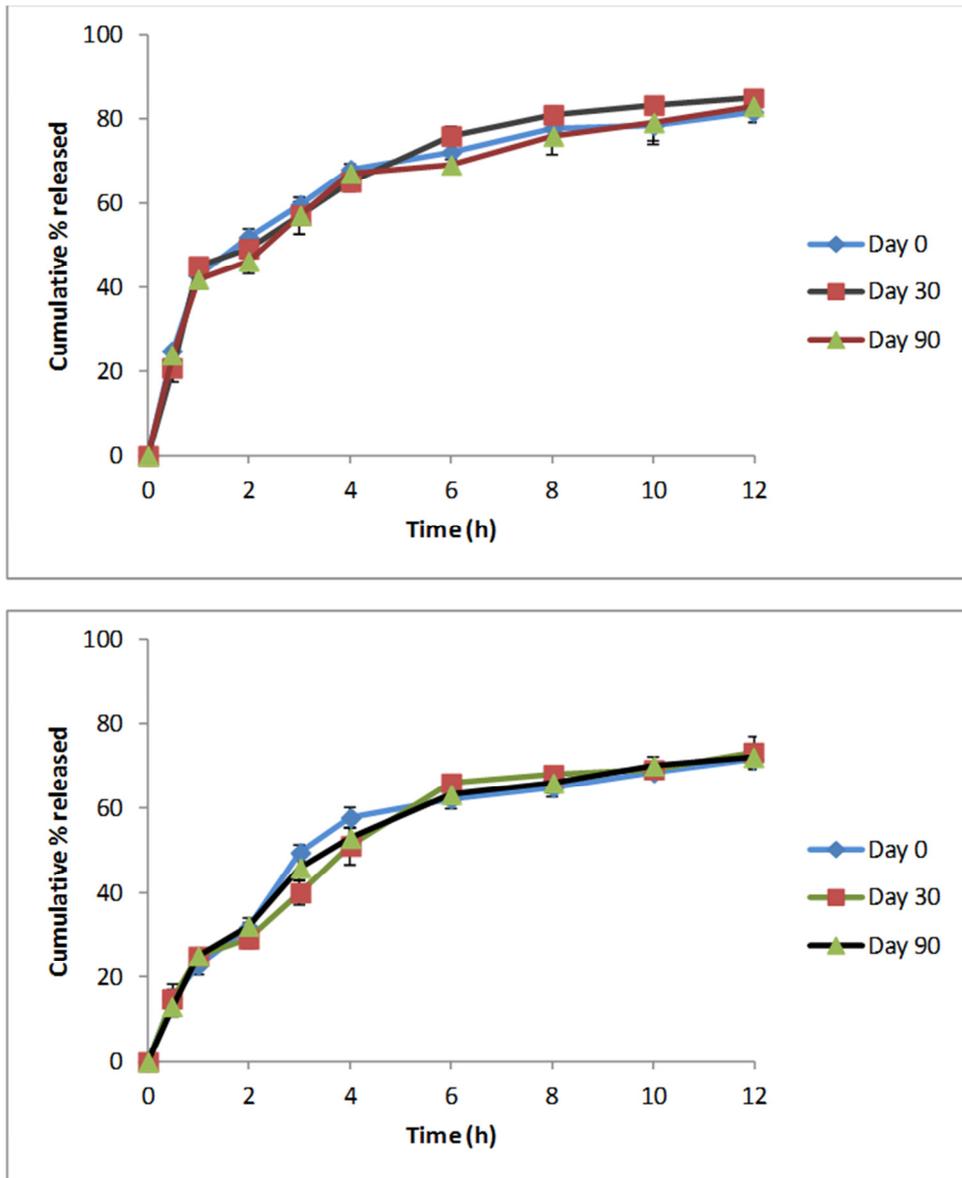


Figure 11 : Release studies of drug from microspheres stored at 4 °C: a) S3-46; b) Con S3-46

| Code | EC (%w/w) | Chitosan (%w/w) | CMN (%w/w) | Con A (mg/ml) |
|------------------|---------------------|---------------------------|----------------------|-------------------------|
| S1-10 | 4 | 0.6 | - | - |
| S2-10 | 2 | 0.6 | 4 | - |
| S3-10 | 4 | 0.6 | 4 | - |
| S4-10 | 8 | 0.6 | 4 | - |
| S5-10 | 12 | 0.6 | 4 | - |
| S1-46 | 4 | 0.6 | - | - |
| S2-46 | 2 | 0.6 | 4 | - |
| S3-46 | 4 | 0.6 | 4 | - |
| S4-46 | 8 | 0.6 | 4 | - |
| S5-46 | 12 | 0.6 | 4 | - |
| Con S1-10 | 4 | 0.6 | - | 1 |
| Con S3-10 | 4 | 0.6 | 4 | 1 |
| Con S1-46 | 4 | 0.6 | - | 1 |
| Con S3-46 | 4 | 0.6 | 4 | 1 |

Table 1: Drug, polymer and lectin ratios (S-10 and S-46 series are made from EC-10 and EC-46 polymers)

| Code | % Yield | Particle size (μm) | % Drug loading | % DEE | % Buoyancy | Conjugation efficiency (%) | Bound lectin (μg lectin/mg microspheres) |
|-----------------|----------------|---|---------------------------|----------------|-----------------------|---|---|
| S1-10 | 56.7 \pm 4.8 | 51.67 \pm 2.56 | - | - | 95.9 \pm 5.9 | - | |
| S2-10 | 62.9 \pm 2.8 | 60.65 \pm 3.65 | 35.5 \pm 2.3 | 63.9 \pm 4.6 | 80.8 \pm 2.9 | - | |
| S3-10 | 68.8 \pm 6.9 | 95.47 \pm 5.62 | 30.7 \pm 2.6 | 70.6 \pm 2.1 | 81.7 \pm 5.4 | - | |
| S4-10 | 76.9 \pm 2.7 | 131.73 \pm 4.74 | 22.9 \pm 1.7 | 75.5 \pm 5.5 | 87.9 \pm 3.9 | - | |
| S5-10 | 79.7 \pm 3.9 | 183.17 \pm 8.12 | 20.1 \pm 1.6 | 86.2 \pm 4.1 | 96.8 \pm 8.8 | - | |
| S1-46 | 69.9 \pm 3.7 | 66.75 \pm 0.32 | - | - | 92.8 \pm 3.6 | - | |
| S2-46 | 78.2 \pm 2.6 | 75.54 \pm 2.76 | 39.3 \pm 3.2 | 70.7 \pm 6.4 | 78.9 \pm 5.8 | - | |
| S3-46 | 75.8 \pm 4.6 | 104.18 \pm 0.96 | 35.9 \pm 2.2 | 82.8 \pm 5.1 | 75.8 \pm 3.8 | - | |
| S4-46 | 81.3 \pm 5.6 | 133.45 \pm 1.51 | 25.6 \pm 1.2 | 84.5 \pm 3.9 | 84.8 \pm 4.9 | - | |
| S5-46 | 82.6 \pm 6.5 | 190.44 \pm 8.28 | 22.1 \pm 2.3 | 94.8 \pm 3.8 | 93.2 \pm 4.9 | - | |
| ConS1-10 | ND | | - | - | 92.7 \pm 2.7 | 71.2 \pm 1.1 | 14.4 \pm 0.2 |
| ConS3-10 | ND | | 21.7 \pm 3.9 | 49.8 \pm 9.2 | 76.8 \pm 4.9 | 55.9 \pm 1.6 | 11.2 \pm 0.4 |
| ConS1-46 | ND | | - | - | 90.5 \pm 4.5 | 76.7 \pm 4.5 | 15.3 \pm 1.9 |
| ConS3-46 | ND | | 30.9 \pm 1.3 | 70.9 \pm 3.8 | 79.9 \pm 5.8 | 64.9 \pm 3.7 | 12.9 \pm 2.1 |

Table 2: Parameters for microparticles, results presented as mean \pm SD (n=3)

| Release kinetics models | | | | | | | | |
|-------------------------|----------------|------------------------------------|----------------|-----------------------------------|----------------|--------------------------------------|------------------|------|
| Code | Zero order | | First order | | Higuchi model | | Korsmeyer-Peppas | |
| | R ² | K _o (%/h ¹) | R ² | K ₁ (h ⁻¹) | R ² | K _h (%/h ^{1/2}) | R ² | n |
| S2-10 | 0.9025 | 13.09 | 0.9082 | 0.112 | 0.9824 | 43.76 | 0.9132 | 0.24 |
| S3-10 | 0.9204 | 11.06 | 0.9759 | 0.109 | 0.9787 | 38.66 | 0.9877 | 0.27 |
| S4-10 | 0.9456 | 10.39 | 0.9672 | 0.089 | 0.9815 | 31.99 | 0.9764 | 0.31 |
| S5-10 | 0.9683 | 7.05 | 0.9913 | 0.057 | 0.9903 | 22.67 | 0.9907 | 0.37 |
| S2-46 | 0.9513 | 9.35 | 0.9849 | 0.110 | 0.9746 | 28.62 | 0.9848 | 0.23 |
| S3-46 | 0.9987 | 8.30 | 0.9662 | 0.097 | 0.9942 | 24.85 | 0.9873 | 0.29 |
| S4-46 | 0.9635 | 6.83 | 0.9715 | 0.074 | 0.968 | 23.25 | 0.9707 | 0.44 |
| S5-46 | 0.9544 | 4.99 | 0.9883 | 0.042 | 0.9942 | 19.84 | 0.9921 | 0.39 |
| Con S3-46 | 0.9667 | 6.03 | 0.9873 | 0.091 | 0.9936 | 21.71 | 0.9844 | 0.65 |

Table 3: Dissolution parameters of the microspheres (pH 2.0)

| | S3-46 | Con S3-46 | S3-10 | Con S3-10 |
|-----------------------------------|--------------|------------------|--------------|------------------|
| Particle size (µm) | | | | |
| Day 0 | 104.18±0.96 | 121.75±3.44 | 95.47±5.62 | 111.81±3.79 |
| Day 30 | 97.46±4.27 | 123.67±1.25 | 92.58±2.68 | 115.22±4.75 |
| Day 60 | 98.64±2.84 | 119.01±0.87 | 98.52±4.82 | 108.15±3.52 |
| Day 90 | 102.87±4.22 | 122.34±2.76 | 99.47±3.11 | 109.28±6.84 |
| Zeta potential (mV) (pH 2) | | | | |
| Day 0 | -13.4±1.51 | +10.3±1.63 | -7.32±2.01 | +9.21±2.89 |
| Day 30 | -15.7±3.35 | +12.2±3.87 | -5.72±1.96 | +11.43±2.77 |
| Day 60 | -12.9±3.38 | +13.8±4.53 | -7.84±3.99 | +8.95±2.63 |
| Day 90 | -9.89±3.86 | +14.23±4.95 | -6.45±2.98 | +10.74±2.78 |
| % DEE | | | | |
| Day 0 | 75.79±3.76 | 70.98±3.85 | 70.64±2.07 | 49.80±9.17 |
| Day 30 | 70.58±3.96 | 73.78±1.75 | 68.93±4.23 | 52.86±2.64 |
| Day 60 | 68.95±4.32 | 71.43±2.57 | 71.35±1.24 | 50.64±3.77 |
| Day 90 | 73.84±2.64 | 69.31±4.23 | 71.64±0.86 | 51.33±3.63 |
| % Mucoadhesion | | | | |
| Day 0 | 10.65±3.83 | 65.98±5.85 | 9.87±4.37 | 63.98±3.58 |
| Day 30 | 12.11±5.78 | 69.77±3.95 | 6.21±3.99 | 67.91±4.42 |
| Day 60 | 10.28±4.23 | 70..21±5.83 | 10.53±2.71 | 68.31±6.32 |
| Day 90 | 9.34±5.21 | 68.53±4.22 | 8.21±3.94 | 71.45±6.13 |

Table 4: Stability of microspheres stored at 4 °C over a period of 3 months

| | S3-46 | Con S3-46 | S3-10 | Con S3-10 |
|-----------------------------------|--------------|------------------|--------------|------------------|
| Particle size (µm) | | | | |
| Day 0 | 104.18±0.96 | 121.75±3.44 | 95.47±5.62 | 111.81±3.79 |
| Day 30 | 102.31±5.31 | 116.55±4.67 | 97.34±6.31 | 109.53±5.32 |
| Day 60 | 103.33±6.89 | 123.85±3.95 | 98.11±8.77 | 110.57±6.21 |
| Day 90 | 105.45±8.31 | 126.52±6.81 | 98.32±3.37 | 116.53±4.34 |
| Zeta potential (mV) pH 2.0 | | | | |
| Day 0 | -13.41±1.51 | +10.3±1.63 | -7.32±2.01 | +9.21±2.89 |
| Day 30 | -13.62±4.33 | +11.12±2.69 | -8.20±1.96 | +10.95±1.84 |
| Day 60 | -14.12±2.41 | +12.62±1.64 | -8.29±2.94 | +10.32±0.54 |
| Day 90 | -13.94±2.66 | +12.29±3.24 | -9.73±1.55 | +11.32±1.73 |
| % DEE | | | | |
| Day 0 | 75.79±3.76 | 70.98±3.85 | 70.64±2.07 | 49.80±9.17 |
| Day 30 | 76.21±4.14 | 74.38±2.11 | 69.31±2.24 | 48.42±3.92 |
| Day 60 | 74.33±3.85 | 69.21±3.94 | 70.93±0.26 | 51.93±1.34 |
| Day 90 | 75.88±4.32 | 70.22±2.84 | 70.89±2.94 | 50.65±2.74 |
| % Mucoadhesion | | | | |
| Day 0 | 10.65±3.83 | 65.98±5.85 | 9.87±4.37 | 63.98±3.58 |
| Day 30 | 11.34±3.84 | 59.77±5.23 | 9.28±4.28 | 54.87±5.48 |
| Day 60 | 11.28±4.69 | 60.21±3.24 | 11.92±3.81 | 55.29±5.11 |
| Day 90 | 10.49±2.57 | 58.53±5.22 | 10.99±4.53 | 51.23±4.34 |

Table 5: Stability of microspheres stored at room temperature (20 °C) over a period of 3 months