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Controlled release study of cell envelope proteins of *Vibrio cholerae* from alginate/HPMC microcapsule

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ABSTRACT

Cholera is an acute diarrheal disease mainly endemic in low income countries. The present study was made to develop the cell envelope protein (CEPs) loaded sodium alginate/HPMC microcapsules for oral controlled vaccine delivery. The alginate/HPMC microcapsules were prepared by ionic gelation technique with different polymer/protein ratio and evaluated. The morphology was evaluated in different physical states (fully swollen, dried & reswollen) and the particle size remains same for all formulations. The Swelling studies indicated the increase of swelling behavior after addition of HPMC. The protein release profile has indicated that the addition of HPMC increases the release rate. According to release kinetics applied for various formulations, kinetics was varied between zero order release and higuchi kinetics.

Keywords: Cell envelope proteins, *Vibrio cholerae*, alginate/HPMC microcapsules.

INTRODUCTION

Cholera is an acute diarrheal disease caused by vibrio cholera which spreads mainly through contamination of water and food by infected persons. It has been estimated that more than 150000 deaths occurs every year from cholera [1]. During clinical infection patients developed antibodies to a number of outer membrane proteins, which provides prolonged immunity against cholera [2] The outer membrane protein of *Vibrio cholerae* is immunogenic in nature but Oral delivery of protein are less immunogenic, thus they require adjuvant to enhance the immune response [3-4]. Biodegradable polymeric microspheres are one of the approaches for oral protein delivery [5]. These types of microcapsules enhance the both systemic and mucosal immune responses [6] .It can overcome problems associated with the conventional therapy, it delivers the protein antigen to the target site in a controlled manner. These microspheres can be effectively taken up by the macrophages and produced long lasting immune response [7].

Alginate is a naturally occurring linear anionic polysaccharide obtained from brown seaweeds. It's biocompatible and biodegradable, composed of β-D-mannuronic acid and α-L-guluronic acid residues linked by a 1, 4-glycosidic bond. The microcapsules are prepared through ionic gelation technique resulting from ionotropic effect between sodium alginate and divalent cation to form the gel. These alginate gels are pH sensitive which protects the protein

from degradation in the acidic environment of stomach. Alginate microcapsules are most widely used system for the controlled protein delivery [8]. Alginates formed viscous gel in the dissolution fluid that releases the protein in a controlled manner [9].

Hydroxy Propyl Methyl Cellulose (HPMC), propylene glycol ether of methyl cellulose, is used as a hydrophilic carrier in the oral controlled drug delivery system. It has high swellability which has an important effect on the release kinetics of incorporated drug [10]. After exposure to the dissolution fluid, it hydrates to form a gel which has high rigidity and viscosity. Further, the hydrated gel will act as a barrier and release the protein slowly [11-12].

The aim of the present study was to prepare the CEPs loaded alginate/HPMC microcapsules for oral vaccine delivery and evaluation of the effect of different alginate/HPMC ratio on the morphology, particle size, loading efficiency, swelling ratio and the release behavior.

MATERIALS AND METHODS

2.1. Materials

Low viscosity (250 cps of 2% solution) alginic acid sodium salt (NaAlg), and calcium chloride dehydrate ($CaCl_2.H_2O$), were purchased from Sigma – Aldrich, Hydroxy Propyl Methyl Cellulose (HPMC, Viscosity 50 cps in a 2% w/v aqueous solution at 20ºC) was obtained from Central Drug House (CDH,Mumbai).*Vibrio cholerae* MTCC 3906 (Serotype) were purchased from Institute of Microbial Technology(IMTECH),Chandigarh. The colony morphology, biochemical identification of strain was done using Thiosulfate citrate bile sucrose (TCBS) agar medium (selective media for identification and characterization of *Vibrio* species [13].

2.2. Isolation of cell envelope proteins (CEPs)

The Cell envelope proteins (CEPs) were isolated according to method developed by Manning et al. with minor modifications [14]. The selected strain of *Vibrio cholerae* were cultured in Luria Broth (LB) medium at 37º C for 24h under shaking condition and then harvested. The harvested cells were suspended in TM buffer (10 mM Tris, 5 mM $MgCl_2$, pH 7.5) and cell density were determined using Mcfarland standard equivalent to 10^{11} cells/ml. The suspended cells were sonicated in sonicator (Vibracell, Sonics & Materials, Inc., USA) at 20 kilocycles/s until all cells were broken (six cycles of 30 s each) and cells were centrifuged at 10, 000 X g for 1 min in a cold Centrifuge to remove cell debris. After centrifugation, cell envelope proteins from supernatant were pelleted by centrifugation at 10,000 X g for 10 min and then, after washing, CEPs were freeze dried (OPERON MPS -55, Yangchon-Myon, Korea) and stored at 4º C.

2.3. Preparation of sodium alginate/HPMC microcapsules

The CEPs loaded alginate/HPMC microcapsules were prepared by ionic gelation technique. Sodium alginate and HPMC were dissolved in 20ml of water under magnetic stirring to form a homogenous solution.100 mg of CEPs were added and mixed thoroughly. This dispersion was added drop wise into calcium chloride solution and left for 30 min in order to complete the gelation process. The microcapsules were rinsed gently with ultrapure water and dried at room temperature at 37ºC [15]. Nine different formulations were prepared with various ratios of sodium alginate and HPMC. The prepared microcapsules are summarized in Table.1

Table 1: Composition of the prepared alginate/HPMC microcapsules and their loading efficiency

S. No	Formulation code	Ratio of Polymer (Sod-Alg)/ HPMC	% Ratio of Polymer (Sod- Alg)/ HPMC
1	CEPs 1	5:2	2.5:1
2	CEPs 2	5:1	2.5:0.5
3	CEPs 3	5:0	2.5:0
4	CEPs 4	4:2	2:1
5	CEPs 5	4:1	2:0.5
6	CEPs 6	4:0	2:0
7	CEPs 7	3:2	1.5:1
8	CEPs 8	3:1	1.5:0.5
9	CEPs 9	3:0	1.5:0

2.4. Morphological characterization

The morphology of the prepared microcapsules was studied using digital photography at different physical states: swollen state (after preparation), dry state & reswollen state (in PBS for 5 hrs). The Cell envelope proteins loaded sodium alginate and HPMC microcapsules were placed on a conductive carbon tape. After air-drying at room temperature, the samples were gold coated using a JEOL JFC-1600 Autofine coater (JEOL, Japan). The coated samples were observed using JSM 6390LV Scanning Electron Microscope (JEOL, Japan).

2.4.1. Particle size analysis

The particle size of the prepared microcapsules was detected by optical microscopy. The eye piece micrometer and stage micrometer were calibrated and the microcapsules of different formulation were evaluated. The determination was done for at least 300 microcapsules.

2.4.2. Loading efficiency

The cell envelope proteins loaded microcapsules (100 mg) were washed with phosphate buffer and then microcapsules were kept into the phosphate buffer (pH-7.4, 100 ml) for 24 hours and sonicate for 1hr at room temperature to break the microcapsule completely. The sonicated solution was centrifuged at 1000 g for 10 minutes to remove the polymeric debris. The clear Supernatant was analyzed for the protein content at the λ max value of 750nm by Lowry protein assay method. The % loading efficiency was calculated as follows.

Loading efficiency (%) = [(total amount of CEPs – free CEPs) / Total CEPs)] X 100

2.4.3. Swelling studies

Swelling studies were performed in dry beads. An accurately weighed amount of dry particles were suspended in Phosphate Buffer Saline (PBS) for 5hrs. After 5hrs, swollen particles were removed & weighed. The swelling ratio was calculated according to the following formula.

Swelling ratio (Qs) = [(Ws – Wd/Wd)] X 100

Qs is the swelling ratio,Wd is the weight of the dry particles,Ws is the weight of the particles in swollen state.

2.5. Zeta potential

The zeta potential of cell envelope proteins were measured by photon correlation spectroscopy using Zetasizer (NanoZS; Malvern Instruments, Worcestershire, UK) equipped with 4.0 mW He-Ne Red laser (633 nm). The preparations were diluted with double distilled water for measurement of zeta potential. All measurements were done at 25º C in triplicate.

2.6. In vitro release study

In vitro release studies were performed using in phosphate buffer saline (PBS, pH 7.4) at 37ºC. Accurately weighed amount of microcapsules were suspended in 20ml of PBS in a conical flask. At definite time intervals, 1ml of samples were withdrawn from release medium up to 30hrs and replaced with equal amount of fresh PBS. The amount of protein released from each sample was estimated by Lowry protein assay method.

Table 2: Applied release models

Model	Equation
Zero order	$Mo - Mt = Kt$
First order	$\ln Mo - \ln Mt = K_1 t$
Higuchi	$Mt = K_H \sqrt{t}$
Hixson – Crowell	$(Wo)^{1/3} - (Wt)^{1/3} = K_{HC} t$
Peppas	$Mt/M\alpha = k_p t^n$

M, amount of protein released in time t, K, K1, K_H, K_{HC}, K_p release rate constants, n release exponent.

2.7. Dissolution data analysis

Cell envelope proteins (CEPs) release kinetics was analyzed by various mathematical models, which were applied considering the release of proteins from 0 to 30 hours. The equation of applied release models has been summarized in Table 2.

RESULTS AND DISCUSSION

3.1. Morphological characterization

The morphology of CEPs loaded microcapsules in different physical states: swollen state (after preparation), dry state & reswollen state (in PBS for 5 hrs) have been depicted in Figure 1. The fully swollen capsules are semitransparent and spherical in shape. After drying the size of microcapsules were reduced. The size of the reswollen microcapsules were opaque and presented smaller particle size with less spherical shape in contrast to the fully swollen state.

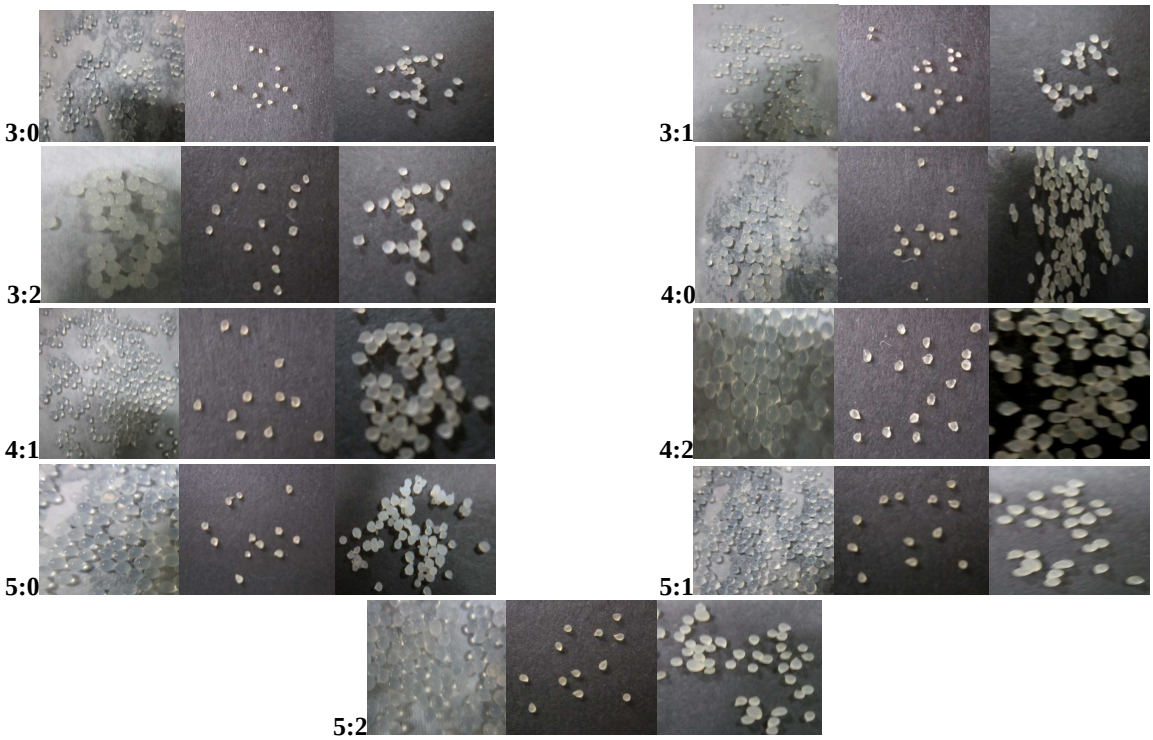

Figure 1 Digital photographs of the prepared microcapsules at different physical state: swollen, dry and reswollen

3.2. Particle size and Swelling studies

The particle size distribution of the prepared microcapsules has been depicted in Figure 2. The particle size of cell envelope proteins loaded sodium alginate HPMC microcapsules were determined by optical microscopy and data for different formulations has been summarized in Table 3. The result indicates that the addition of HPMC in the formulations has not significantly affected the particle size. The only slight variation was observed in formulation CEPs 1 and CEPs 3 with sodium alginate:HPMC ratio 5:2 and 5:0 respectively. The swelling ratio of the prepared microcapsule has been shown in Fig.3.The swelling has been increased with increasing the amount of HPMC due to hydrophilic nature of HPMC. The swelling ratio in pure alginate microcapsules for CEPs 3, CEPs 6, CEPs 9 were varied from 105.4, 91.7 and 90.6 respectively. The difference for pure alginate microcapsules were 14% among CEPs 3, CEPs 6, CEPs 9 formulations while the difference of swelling ratio was almost 75% between CEPs 1 to CEPs 3 formulation. This result clearly indicates the hydrophilic behavior of HPMC which leads to high swell ability of alginate HPMC microcapsules.

Table 3: Particle size, Swelling ratio and Loading efficiency of prepared formulations

S. No.	Formulation code	Polymer ratio	Particle size	Swelling ratio	Loading Efficiency (%)
1	CEPs 1	5:2	878.67 ± 4.2	179.4	65.56 ± 4.2
2	CEPs 2	5:1	865.86 ± 3.5	118.6	57.35 ± 1.6
3	CEPs 3	5:0	767.28 ± 2.2	105.4	57.24 ± 4.5
4	CEPs 4	4:2	782.25 ± 4.3	168.8	50.15 ± 2.6
5	CEPs 5	4:1	766.20 ± 2.5	107.5	46.89 ± 1.6
6	CEPs 6	4:0	753.42 ± 1.8	91.7	45.64 ± 1.7
7	CEPs 7	3:2	754.25 ± 2.9	144.6	47.93 ± 3.7
8	CEPs 8	3:1	736.83 ± 1.6	101.8	44.16 ± 2.2
9	CEPs 9	3:0	722.46 ± 4.1	90.6	44.67 ± 1.9

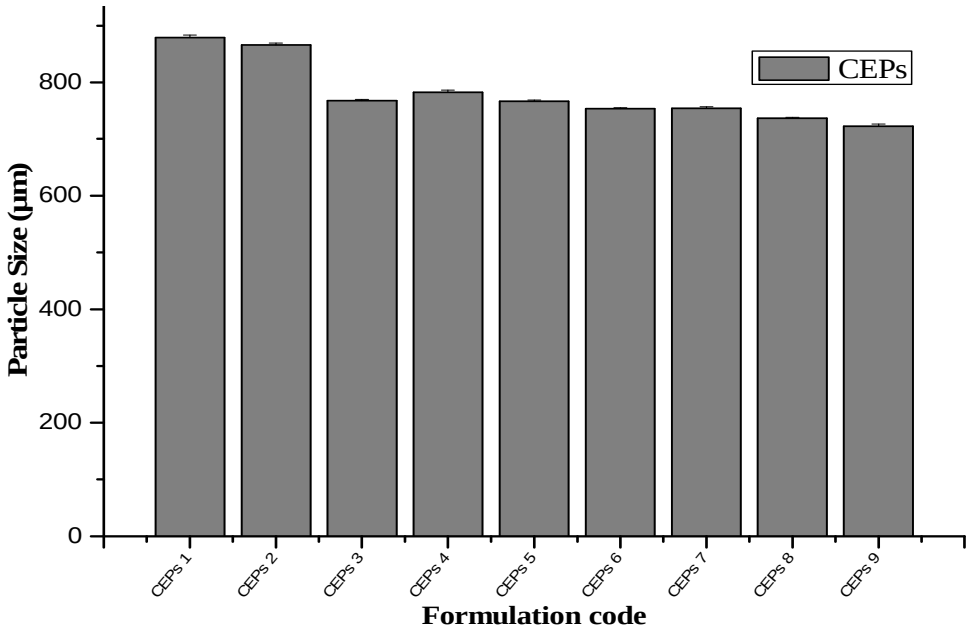

Figure 2 Particle size distribution of CEPs loaded alginate/HPMC microcapsules

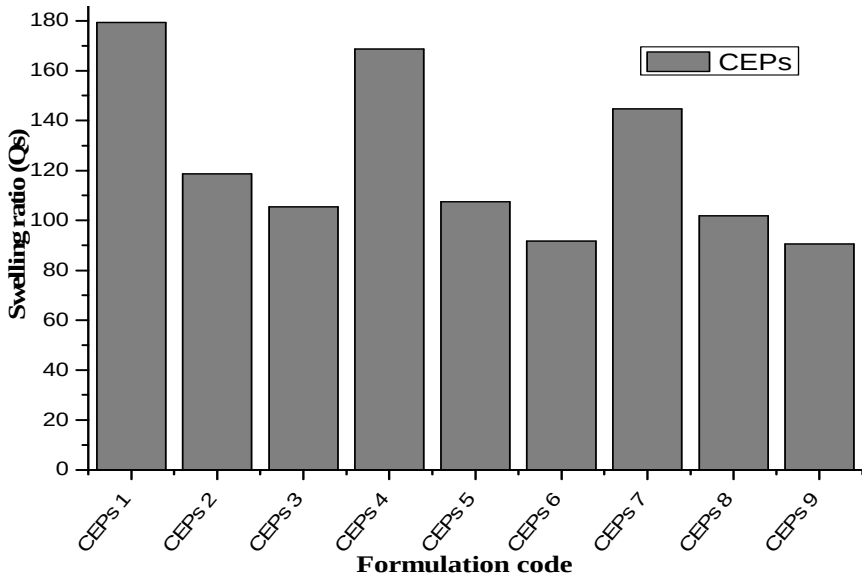

Figure 3 Swelling ratio of different formulation after immersion for 5 hours

3.3. Loading efficiency

In this study, the loading efficiency of cell envelope proteins in alginate/HPMC microcapsules was varied from 65.56% to 44.67%. The data for loading efficiency has been summarized in Table 3. In another study, it was demonstrated that loading of bovine serum albumin at a pH value lower than the protein isoelectric point (pI) was higher than that at a pH similar to the pI due to an electrostatic interaction between the positively charged protein and the polyanionic alginate. Again, the loading efficiency at pH values higher than the isoelectric point of the protein is related to the capacity of polymeric chains to entrap the protein [16].As revealed by zeta potential (NanoZS, Malvern Instruments, Worcestershire, UK) measurements, CEPs were negatively charged (-25.3 mV). It may be concluded from zeta potential measurement that loading efficiency of CEPs was mainly due entrapment in the polymeric chains of alginate. Further, the higher loading efficiency of CEPs in higher ratio of alginate and HPMC has confirmed the above hypothesis. In another study, it was observed the high loading of bovine serum albumin in calcium alginate microspheres prepared by emulsification technique. The degree of gel formation is also important for the retention of protein inside the gel polymer. The complete gel formation further increases the loading efficiency [17-18]. In this study it was also observed that increase in more than 12% loading efficiency in the case pure alginate microcapsule.

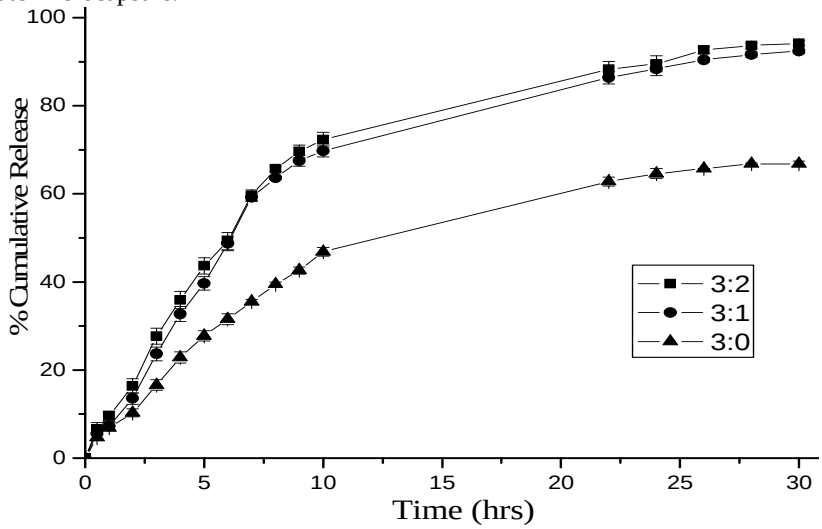

Figure 4 Cumulative releases of CEPs in PBS from pure calcium alginate microcapsule and in different ratio of alginate/HPMC microcapsule

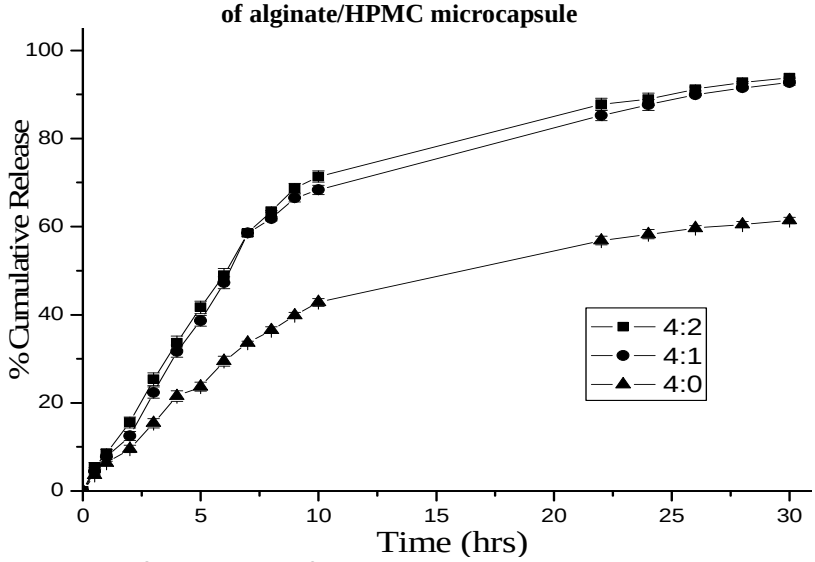

Figure 5 Cumulative releases of CEPs in PBS from pure calcium alginate microcapsule and in different ratio of alginate/HPMC microcapsule

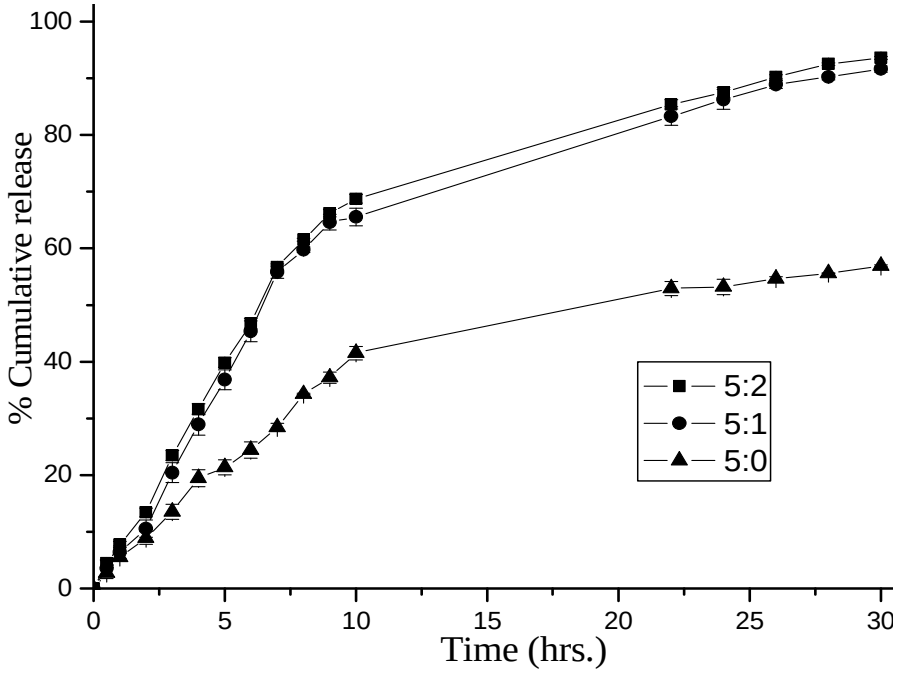

Figure 6 Cumulative releases of CEPs in PBS from pure calcium alginate microcapsule and in different ratio of alginate/HPMC microcapsule

3.4. In vitro release study

The cumulative release of nine formulations has been shown in figure 4, figure 5 and figure 6 respectively. It was observed from the release experiments that release of CEPs from Alginate/HPMC microcapsule increases with the increase in HPMC in the formulations. Further, it was observed that only 28.48% CEPs were released from pure alginate microcapsules in 7hrs while 56.73% CEPs release was occurred in the case of alginate/HPMC microcapsules.The overall release studies showed that the increase in release of CEPs due to incorporation of small amount of HPMC in the alginate solution. Therefore, it may be concluded that presence of HPMC increases the release rate due to high swellability [19].

Table 4: Release rate constants and determination coefficients of produced formulation

	05:02	**05:01**	**05:00**	**04:02**	**04:01**	**04:00**	**03:02**	**03:01**	**03:00**
zero order									
K	6.541	6.4829	1.688	2.8514	2.8591	1.9113	2.8345	2.8427	2.11
R^2	0.9828	0.98	0.8793	0.82	0.827	0.8809	0.8138	0.817	0.8883
first order									
K_1	0.2216	0.2505	0.0761	0.082	0.0854	0.0807	0.0795	0.0836	0.0808
R^2	0.7668	0.805	0.5576	0.4772	0.4945	0.5412	0.4647	0.486	0.5413
Higuchi									
K_H	19	18.908	11.341	18.964	18.931	12.458	18.911	18.895	13.716
R^2	0.9503	0.9475	0.9691	0.9444	0.944	0.9743	0.9432	0.9399	0.977
Peppas									
K_p	6.416	5.52	4.303	7.143	6.325	4.937	7.841	6.771	5.462
R^2	0.8065	0.8338	0.8585	0.782	0.8078	0.8349	0.7581	0.791	0.8195
n	0.9049	0.9488	0.8532	0.8737	0.9084	0.8335	0.8444	0.8869	0.8277
Hixson- crowell									
K_{HC}	0.0882	0.0907	0.0703	0.0861	0.0883	0.0763	0.0843	0.087	0.0787
R^2	0.5669	0.5814	0.6058	0.5501	0.5652	0.591	0.5384	0.5556	0.5972

3.5. Release kinetics

The *in vitro* CEPs release data from alginate/HPMC microcapsules were estimated by using different kinetic models to explain the release kinetics and mechanism [20]. The parameters calculated by this models and the determination

coefficient (R^2) has been summarized in Table 4. Based on these estimations the fit of each model was predicted. Considering R^2 value,the calculated Higuchi model fitted correctly for most of formulations however, and some formulations has shown zero order release as well. All other models like Hixson crowell, first order were not able to fit the CEPs release profile.

Among all models Higuchi model was considered as the best fitted model with the highest value of R^2 0.9503 to 0.977.The release data next fitted with zero order all the formulations showed good linearity with R^2 from 0.9828 to 0.8883.From the peppas equation the 'n'value range from 0.9049 to 0.8277 indicating the nonfickian or anomalous release indicating the CEPs release from the formulation due to diffusion [21-22].

CONCLUSION

In this study the ionic gelation method was used for the preparation of sodium alginate microcapsule for the incorporation of cell envelope proteins isolated from *Vibrio cholerae*. Being the negative charge of cell envelope proteins, the microcapsule has exhibited medium loading efficiency which may possible due to entanglement of CEPs within polymer chains. The release experiments suggested the high release in alginate/HPMC microcapsule as compare to pure calcium alginate microcapsule due to swell ability of HPMC which release the CEPs by diffusion. All the formulations presented higuchi release kinetics while some formulation obeyed the zero order release. So this formulation may be useful for the delivery of CEPs as active immunizing agent through oral route.

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