CHITOSAN MICRO AND NANOPARTICLES FOR DELIVERY OF DRUGS AND THERAPEUTIC PROTEINS

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ABSTRACT

Absorption of proteins into cationic chitosan microparticles through electrostatic interaction is a common process suitable for oral delivery of proteinaceous drugs. In this research work in order to achieve a good stable delivery system different combinations chitosan, acetic acid Sodium tripolyphosphate and model protein bovine serum albumin was taken. It was found that Chitosan of 4mg/ml and 5mg/ml concentration had loading efficiency more than 60% and with a particle size near about 300 nm. Morphological characterization of the particles was done using zeta sizer and SEM (scanning electron microscope). In vitro release of BSA from chitosan microparticles was checked taking different time intervals at pH7.4 using PBS (phosphate saline buffer). Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) assay chitosan microparticles could effectively protect in a small extent the BSA from degradation or hydrolysis in acidic condition for at least 2 hours using HCl pH 2.0.

Key words: Chitosan, SEM, SDS-PAGE, Microparticle, Bovine serum albumin.

INTRODUCTION

Peptides and proteins have become the drugs of choice for the treatment of numerous diseases as a result of their incredible selectivity and their ability to provide effective and potent action [1] due to their fewer side effects and higher potency to cure diseases. Pharmaceutical scientists have produced variety of protein and peptide drugs in commercial scale using biotechnological techniques [1, 2, and 3]. In recent years, there has been a significant increase in the number of targeting mechanisms available to the pharmaceutical scientist to provide site-specific delivery in the gastrointestinal (GI) tract [4]. Generally, it is

highly beneficial to target a drug to a particular site within the GI tract either to maximize a therapeutic response or to reduce side effects caused by drug delivery to an inopportune region of the gut [4, 5]. Drug absorption differences in various gastrointestinal segments. In general, drug absorption is moderately slow in the stomach, rapid in the small intestine, and sharply declining in the large intestine. Administering drugs orally is so far the most widely used route of administration, although it is generally not feasible for peptide and protein drugs. The main reasons for the low oral bioavailability of biological are presystemic enzymatic degradation and poor penetration of the intestinal membrane. Much has been learned in the past few decades about macromolecular drug absorption from the gastrointestinal (GI) tract, including the barriers that restrict GI absorption. Various strategies have been followed to overcome such barriers and to develop safe and effective oral delivery systems for proteins. Most oral delivery strategies for biological are based on system equipped to protect against enzymatic degradation and provide high transfer of drugs across the epithelial mucosa. Certain particles can be taken up by the Payer's patches without employing additional penetration enhancers. In developing oral protein delivery systems with high bioavailability, three practical approaches might be most helpful: (1) modification of the physicochemical properties of macromolecules; (2) addition of novel function to macromolecules; or (3) use of improved delivery carriers [6].

In design of oral delivery of peptide or protein drugs, one important aspect is that natural polymer like chitosan (CS) has attracted increasing attention since most of the synthetic polymers are immunogenic and the incorporation of proteins into these polymers require a harsh environment which may denature and inactive the desired protein [7].

Chitosan, a natural cationic polysaccharide derived from chitin is a copolymer of glucosamine and N-acetyl glucosamine units [8], has gained an increased attention in biomedical as well as pharmaceutical purposes due to its biocompatible properties such as non-toxicity, biodegradability [7, 9 and 10], muco-adhesive properties etc. This biodegradable polymer is synthesised by alkaline deacetylation of chitin by the enzyme chitin deacetylase as given in figure 1.

Chitosan micro/nanoparticles can be easily prepared by ionic gelation method using tripolyphosphate (TPP) as precipitating agent [9]. In spite of all superior qualities like non-toxicity, biodegradability etc chitosan has an apparent pKa of 5.6 and is only soluble in acidic

solutions. When incubated in physiological fluid environment, chitosan will lose its capacity of mucoadhesive properties and permeation enhancing effect due to the deprotonation of chitosan, which would make chitosan carriers lose its advantage compared with other carriers for mucosal vaccine [11].

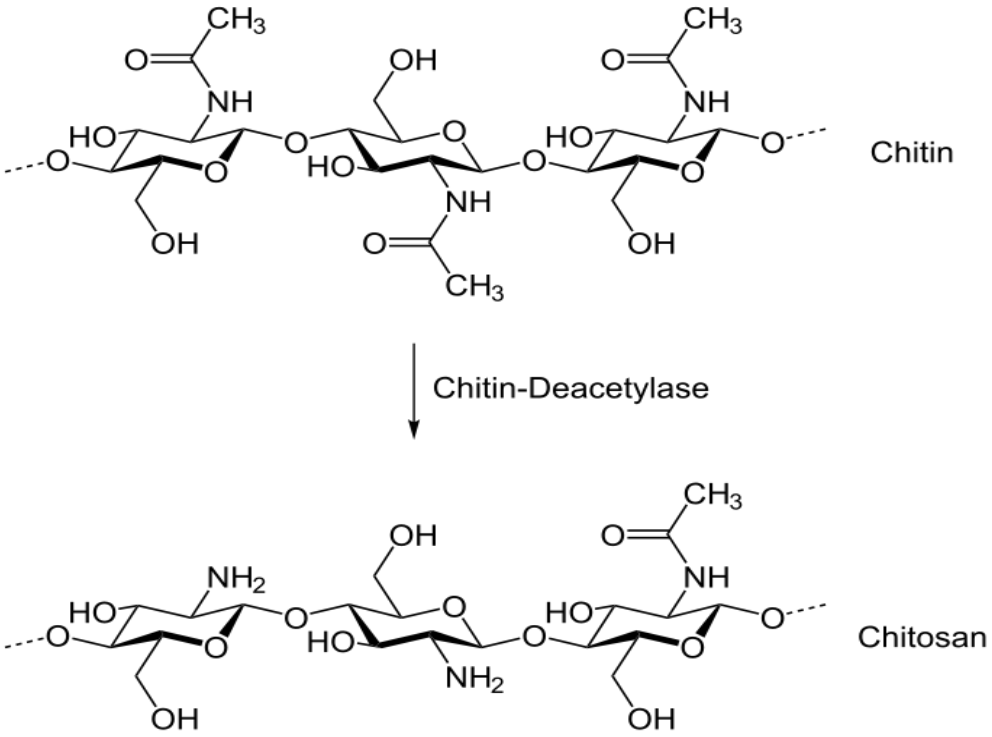


Fig.1 Production of chitosan by the deacetylation of chitin by the enzyme chitin deacetylase.

Bovine serum albumin has been reported as a model protein by so many authors for protein drug delivery [11, 12, 13 and 14]. It is a protein of molecular weight 66 kDa. It has been well characterized and the physical properties of this protein are well known thus it can be used as a model protein.

In this work, we hope to develop a novel oral drug delivery system to act as a carrier for therapeutic proteins with highest efficiency by using chitosan polymer. The model protein (BSA) was adopted to evaluate the chitosan microparticles. Morphological behaviours and release behaviours (physiological pH and also in acidic conditions) and stability of chitosan microparticles have been studied.

MATERIALS AND METHODS:

Chitosan was purchased from Sigma Aldrich; (New Delhi, India).Sodium Tripolyphosphate was purchased from Central Drug House (Delhi, India).Bovine serum albumin fraction V was purchased from Hi Media. BCA™ Protein Assay Kit was provided by thermo scientific. Acetic acid was from Hi media. Water purified by reverse osmosis was used, (Milli Q, Millipore USA) to prepare aqueous solutions.

Preparation of chitosan microparticles

Chitosan microparticles were prepared by the ionic gelation of chitosan solution with anionic sodium tripolyphosphate. First, chitosan was dissolved in 1% and 2%(v/v) acetic acid aqueous solution at different concentrations i.e.(2.5,3.0,4.0,5.0,10.0) mg/ml. Then, TPP was dissolved in distilled water at the concentration of 6mg/ml. Subsequently, 5 ml of TPP solution was added drop wisely into 20 ml of chitosan solution of all concentration. Now chitosan colloid microparticles were formed spontaneously under mild agitation at room temperature on a magnetic stirrer. After 15-20 minutes, chitosan colloid microparticles were centrifuged at 10,000 rpm for 15 min. Then, the supernatant was discarded and the deposit was re-dispersed in distilled water for further use. Colloid chitosan microparticles were re-dispersed in 25ml of distilled water under continuous ultrasonication to disaggregate the chitosan microparticles. For each formulation ultrasonication was done for 10 minutes.

Loading bovine serum albumin fraction-V (BSA-V) to chitosan microparticles and calculation of loading efficiency by BCA™ protein estimation kit.

The loading procedure was performed by incubating different concentrations of BSA-V (1.0, 2.0,4.0,8.0)mg/ml with chitosan microparticles under mild agitation at room temperature for 15 min. Loading efficiency (LE) of BSA-V on chitosan microparticles were detected in an indirect way by determining the free BSA-V remained in the supernatant after the performance of centrifuge, and the method was shown as following. One millilitre of BSA loaded chitosan microparticles suspension was centrifuged at 13,000 rpm for 20 min and the amount of BSA in the supernatant was measured by BCA™ kit using a standard curve formulated through spectrophotometer. The supernatant of blank chitosan

microparticles was adopted as the blank to correct the absorbance reading value of the BSA-
loaded chitosan microparticles. The corrected optical density (OD) value was then used to calculate the concentration of BSA in the supernatant.

The loading efficiency (LE) was calculated by following formula

$$LE(\%) = \frac{\text{total amount of BSA} - \text{free BSA}}{\text{total amount of BSA}} \times 100$$

Morphological characterization

Colloidal microparticles were first lyophilized for 24 hours. Then morphological characteristics of microparticles were examined by scanning electron microscope (SEM). For SEM, a specimen is normally required to be completely dry, since the specimen chamber is at high vacuum. Microparticles were sputtered with gold and maintained at room temperature for complete dryness before the observation.

***In vitro* release and checking of protein degradation by SDS-PAGE**

After loading the protein (BSA) to the microparticles in vitro release was studied by using PBS (phosphate saline buffer) pH 7.4. One milliliter of microparticles suspension was first centrifuged and the deposit was incubated in 1 ml of phosphate buffer saline (PBS, pH7.4) in eppendorf tube (EP tube). Then, the EP tube was placed in an air shaker bath at 100 rpm/min (at 37°C) for *in vitro* release. At scheduled time, samples were centrifuged at 14,000 rpm for 20 min and the supernatant was replaced with fresh PBS (previously warmed to 37°C). The amount of BSA presented in the supernatant was determined by BCA™ kit using spectrophotometer. Then protein degradation was checked by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) method in which release of protein was done in pH 2.0 by using HCl (hydrochloric acid) for 2 hours (37^{o}C)then sample was centrifuged at 14,000 rpm for 20 minutes and the supernatant was replaced with fresh PBS (previously warmed to 37°C).

RESULTS AND DISCUSSIONS

In this research paper we have prepared chitosan microparticles by ionic gelation method using sodium tripolyphosphate as a precipitating agent. BSA was used as a model protein for evaluation of the properties of the alginate coated chitosan microparticles. The BSA with isoelectric point (PI) of 4.8 was encapsulated into cationic chitosan microparticles at aqueous solution (pH 7.0) via electrostatic interactions. BSA was used as a model protein for evaluation of the properties of the chitosan microparticles. Prepared particles and their codes are given in table 1 and 2.

Table1.Description of sample name with respective compositions

Sl.No	Sample code	Sample description		
		Acetic acid concentration (%)	Chitosan (mg/ml)	Bovine serum albumin(mg/ml)
1.	1250	1	2.5	0
2.	1251	1	2.5	1
3.	1252	1	2.5	2
4.	1254	1	2.5	4
5.	1258	1	2.5	8
6.	130	1	3.0	0
7.	131	1	3.0	1
8.	132	1	3.0	2
9.	134	1	3.0	4
10.	138	1	3.0	8
11.	140	1	4.0	0
12.	141	1	4.0	1
13.	142	1	4.0	2
14.	144	1	4.0	4
15.	148	1	4.0	8
16.	150	1	5.0	0
17.	151	1	5.0	1
18.	152	1	5.0	2
19.	154	1	5.0	4
20.	158	1	5.0	8
21.	1100	1	10.0	0
22.	1101	1	10.0	1
23.	1102	1	10.0	2
24.	1104	1	10.0	4
25.	1108	1	10.0	8

To each sample 1mg /ml TPP was added with suitable volume.

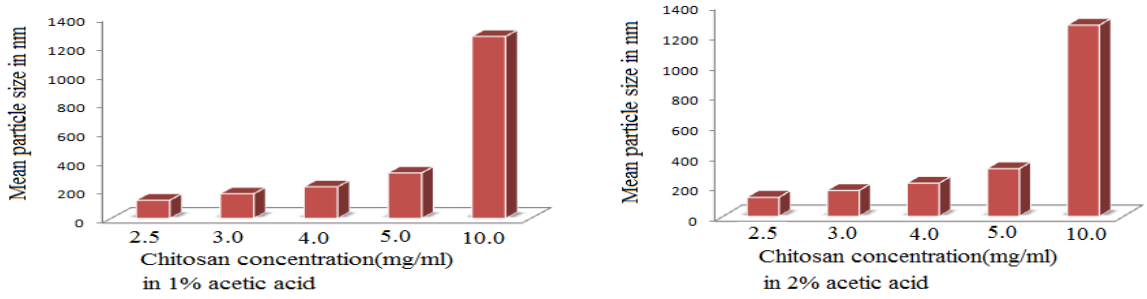
Table2.Description of sample name with respective compositions

Sl.No	Sample code	Sample description		
		Acetic acid concentration (%)	Chitosan (mg/ml)	Bovine serum albumin(mg/ml)
26.	2250	2	2.5	0
27.	2251	2	2.5	1
28.	2252	2	2.5	2
29.	2254	2	2.5	4
30.	2258	2	2.5	8
31.	230	2	3.0	0
32.	231	2	3.0	1
33.	232	2	3.0	2
34.	234	2	3.0	4
35.	238	2	3.0	8
36.	240	2	4.0	0
37.	241	2	4.0	1
38.	242	2	4.0	2
39.	244	2	4.0	4
40.	248	2	4.0	8
41.	250	2	5.0	0
42.	251	2	5.0	1
43.	252	2	5.0	2
44.	254	2	5.0	4
45.	258	2	5.0	8
46.	2100	2	10.0	0
47.	2101	2	10.0	1
48.	2102	2	10.0	2
49.	2104	2	10.0	4
50.	2108	2	10.0	8

To each sample 1mg /ml TPP was added with suitable volume.

Analysis of chitosan microparticles

Sample 1250,130,140,150,1100,2250,230,240,250,2100 are the chitosan microparticles without loading of BSA were analysed in the zeta sizer to evaluate particle size, PDI and zeta potential by taking refractive index of chitosan 1.523. It was found that with increase in chitosan concentration the particle size increases. The particle size increased from 126.4nm to1321nm when chitosan concentration was increased from 2.5mg/ml to 10 mg/ml in case where chitosan was dissolved in 1% acetic acid (table 3 and fig2).



(a) (b)

Fig 2. Variation of chitosan microparticles size with increase in chitosan concentrations. (a)Different concentrations (mg/ml) of chitosan dissolved in 1% acetic acid and (b) Different concentrations (mg/ml) of chitosan dissolved in 1% acetic acid.

Table 3. Formulations of chitosan microparticles with 1% acetic acid and their mean particle size, PDI and zeta potential.

Sl.No	Sample name	pH	Mean particle size	PDI	Zeta potential	Loading efficiency (%)
1.	1250	3.48	126.4	0.507	+43.9	---
2.	1251	--	215.0	0.337	+33.3	44.0
3.	1252	--	243.8	0.410	+24.5	32.0
4.	1254	--	256.1	0.370	+14.2	24.0
5.	1258	--	289.6	0.432	+9.4	17.0
6.	130	3.62	174.1	0.383	+46.2	---
7.	131		259.8	0.447	+35.5	47.0
8.	132	--	294.5	0.387	+26.6	35.0
9.	134	--	327.0	0.471	+16.5	26.0
10.	138	--	353.9	0.337	+10.9	18.0
11.	140	3.82	236.5	0.468	+53.5	---
12.	141	--	309.4	0.337	+36.1	64.0
13.	142	--	326.7	0.391	+28.5	52.0
14.	144	--	347.3	0.379	+17.3	40.0
15.	148	--	379.9	0.399	+11.1	29.0
16.	150	3.97	316.4	0.476	+45.4	---
17.	151	--	402.3	0.447	+36.8	69.0
18.	152	--	419.0	0.509	+29.4	56.0
19.	154	--	443.4	0.534	+20.7	42.0
20.	158	--	478.7	0.584	+11.9	31.0
21.	1100	4.19	1321.0	0.802	+54.9	---
22.	1101	--	1357.0	0.900	+43.5	66.0
23.	1102	--	1378.1	0.943	+34.9	61.0
24.	1104	--	1398.5	0.954	+31.2	50.0
25.	1108	--	1402.3	0.977	+23.9	44.0

--Not checked

---No loading of protein

Table 4. Formulations of chitosan microparticles with 2% acetic acid and their mean particle size, PDI and zeta potential.

Sl.No	Sample name	pH	Mean particle size	PDI	Zeta potential	Loading efficiency (%)
26.	2250	3.34	126.0	0.455	+40.2	---
27.	2251	--	213.6	0.339	+34.3	48.0
28.	2252	--	243.8	0.440	+23.6	30.0
29.	2254	--	256.9	0.370	+14.4	25.0
30.	2258	--	283.4	0.439	+9.9	19.0
31.	230	3.40	170.9	0.445	+46.2	---
32.	231	--	269.3	0.497	+35.8	50.0
33.	232	--	299.8	0.387	+26.0	38.0
34.	234	--	329.1	0.481	+16.5	30.0
35.	238	--	358.9	0.387	+10.9	22.0
36.	240	3.49	219.9	0.456	+48.0	---
37.	241	--	309.9	0.357	+34.9	59.0
38.	242	--	336.7	0.399	+28.8	52.0
39.	244	--	348.8	0.379	+19.8	41.0
40.	248	--	372.2	0.390	+11.9	29.0
41.	250	3.55	315.8	0.384	+45.4	---
42.	251	--	400.3	0.497	+34.8	67.0
43.	252	--	419.8	0.589	+29.4	60.0
44.	254	--	448.9	0.584	+20.2	42.0
45.	258	--	473.9	0.584	+13.9	31.0
46.	2100	3.81	1266.0	0.905	+54.2	---
47.	2101	--	1352.0	0.899	+48.5	66.0
48.	2102	--	1373.1	0.948	+34.9	61.0
49.	2104	--	1390.8	0.959	+34.2	49.0
50.	2108	--	1400.8	0.979	+23.9	41.0

--Not checked

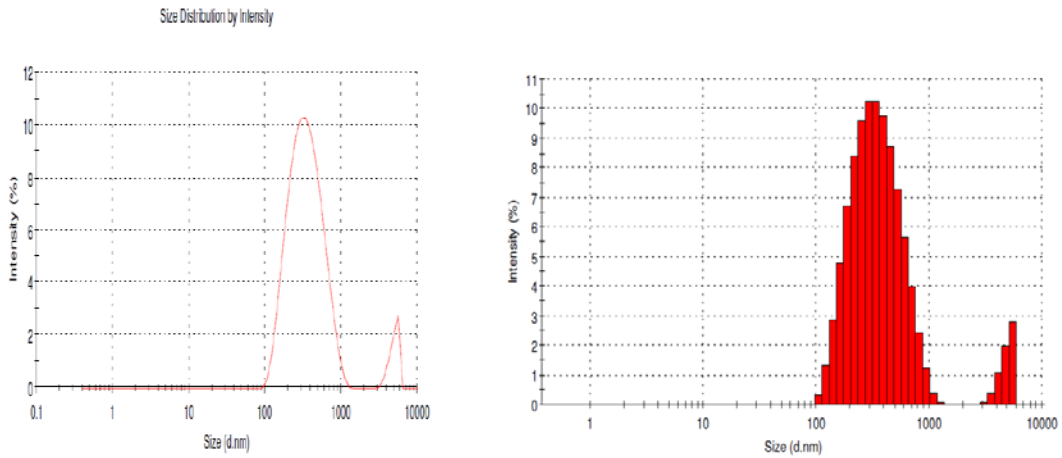
---No loading of protein

Similarly the particle size increased from 126.0 nm to 1266 nm when chitosan concentration was increased from 2.5mg/ml to 10 mg/ml in the case where chitosan was dissolved in 2% acetic acid as given in table 4.Thus it can be also concluded that more the concentration of acetic acid less will be the particle size (but to a smaller extent), probably

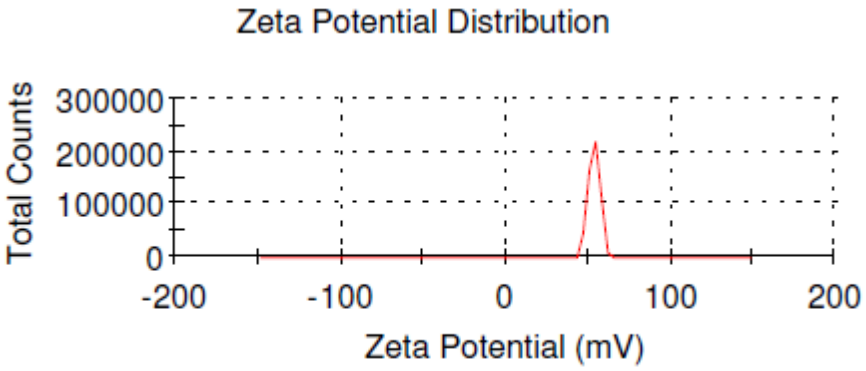
because chitosan were dissolved finely in more concentrated acetic acid. Respective PDI values of the formulations were evaluated.Polydispersity index (PDI), a term in polymer chemistry referring to the molecular weight distribution of polymers. Zeta potential is a scientific term for electro-kinetic potential [15]. The significance of zeta potential is that its value can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate as outlined in the table5.

Zeta potential [mV]	Stability behaviour of the colloid
From 0 to ±5	Rapid coagulation or flocculation
From ±10 to ±30	Incipient instability
From ±30 to ±40	Moderate stability
From ±40 to ±60	Good stability
More than ±61	Excellent stability

Table 5 derived from Zeta Potential of Colloids in Water and Waste Water", ASTM Standard D 4187-82, American Society for Testing and Materials, 1985 [16]



(a) (b)



(c)

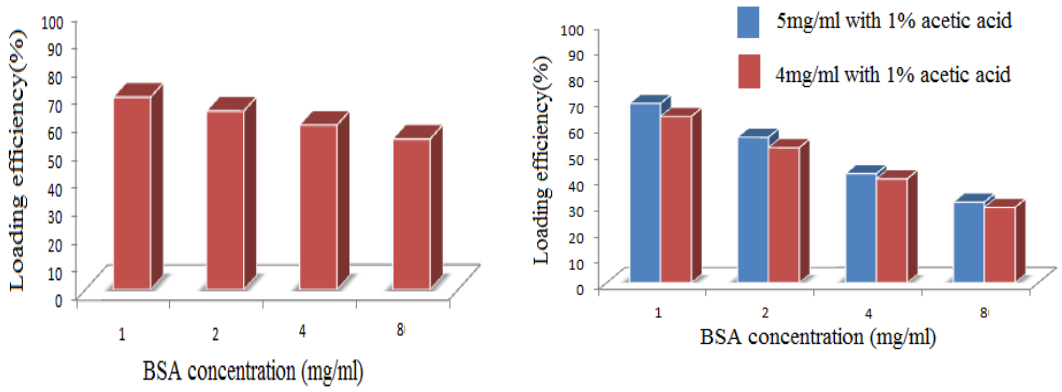
Fig 3.Characteristics of chitosan microparticles (5mg/ml) (a) Size distribution by intensity (b) statistics of size distribution(c)Zeta potential of distribution.

Zeta potential of maximum samples were found to be from +30 to +60(samples loaded with 1mg/ml and 2mg/ml BSA) i.e. chitosan colloid particles loaded with 1mg/ml and 2mg/ml BSA were having moderate to good stability. On the other hand chitosan colloid particles loaded with 4mg/ml and 8mg/ml BSA, zeta potential were varies from +10 to +30 (maximum case) i.e. chitosan colloid particles loaded with 4mg/ml and 8mg/ml BSA were having incipient stability. In some case of chitosan microparticles loaded with 8mg/ml BSA, zeta potential was less than +10 means sample were having rapid coagulation or flocculation properties.

Procedure of BSA loading and Effect of BSA concentration on chitosan microparticles

The chitosan microparticles were incubated in aqueous solution with different BSA concentrations at a relatively mild condition to obtain suitable loading efficiency LE). Cationic microparticles can easily absorb anionic protein or DNA via electrostatic interaction [17, 18]. Cationic chitosan microparticles prepared in this work had a potent capacity to absorb the model anionic protein (BSA) in aqueous solution (pH 7.0) via electrostatic interaction. The obtained chitosan microparticles at different concentrations were adopted to evaluate particle size, zeta potential, loading efficiency. The loading efficiency was calculated using BCA standard curve for BSA protein by using spectrophotometer and ELISA instruments. As presented in Table 2 and 3 effect of BSA concentration on the properties of chitosan microparticles were investigated. The mean diameter of chitosan

microparticles increased accompanied with decrease in zeta potential (table 2 and 3) when BSA concentration increased from 1 mg/ml to 8 mg/ml. This might be attributed to the fact that negatively charged BSA absorbed onto chitosan microparticles and neutralized part of zeta potential of chitosan microparticles resulted in increase of particle size and decrease of zeta potential [19]. It was seen that when BSA concentration was less than 2mg/ml or 2mg/ml then the loading efficiency was more as compared to other formulations with BSA concentration 4mg/ml or 8 mg/ml. This interesting phenomenon might be attributed to the saturated absorption was achieved as BSA concentration at about 2 mg/ml, the more addition of BSA was seldom adsorbed onto chitosan microparticles which leaded to the great decrease in loading efficiency [11]. The loading efficiency of 141, 151, 1101, 1102, 251, 252, 2101 and 2102 were found to be more than 60% while sample 241 was found to be 59%(table 5 and 6).The mean particle size of the formulation 1101, 1102.2101 and 2102 were more than 1350 nm.Thus these were not taken into account because of their large particle size. Here in this article formulation with chitosan 4mg/ml, 5mg/ml were the best formulations to study further.



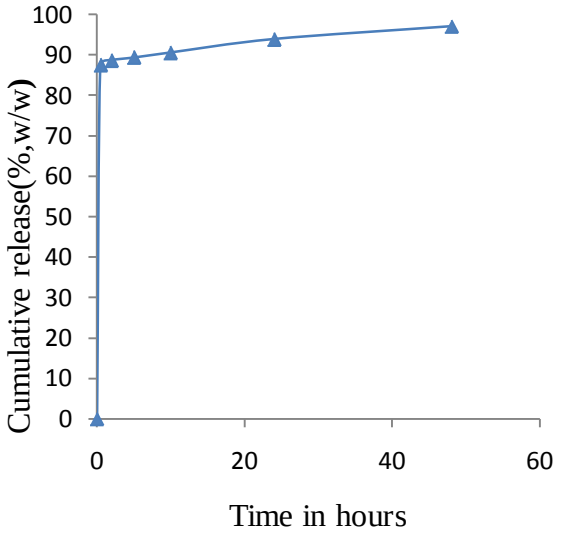
(a) (b)

Fig.4.Effect of different BSA concentration on loading efficiency (a) .Effect of different BSA concentration on loading efficiency (LE) of Chitosan microparticles 5mg/ml dissolved in 1% acetic acid, (b) Effect of different BSA concentration and chitosan concentration on loading efficiency (LE).

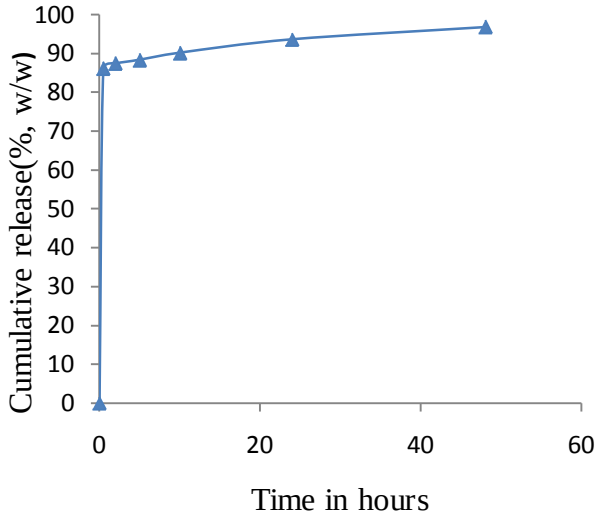
In vitro Protein release study

The release profiles of BSA from chitosan microparticles in phosphate buffer (PBS, pH7.4) were shown in Fig 4 and 5. It was observed that the initial burst release of BSA from chitosan

microparticles 141,142,151 and 152 was about 87.5%,87.9%,86.2% and 85.3% respectively occurred in the first 0.5 hour, followed by release of 97.1% ,97%,96.9% and 96.5% respectively in 48 hours. The burst release might be attributed to the fact that BSA macromolecules were loosely bound onto chitosan microparticles by ionic interaction which could be easily desorbed at ionic environment [20].



(a)



(b)

Fig.5 Release study (a) Release study of sample 141(b) Release study of sample 151.

Detection of Acidic degradation of proteins by SDS-PAGE

In this paper, we have tried to design an oral drug delivery system for protein. As we know, pH value of gastric fluid is approximately 2 which will destroy the integrity and structure of antigens (protein, DNA, etc) after the oral administration without any protection [11]. Here, BSA released from chitosan microparticles under acidic medium (pH2.0) and PBS medium (pH7.4) was analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). According to Fig.6 BSA 2mg/ml and 4mg/ml(Lane 8 and Lane 9 respectively) were taken as control and the different formulations of BSA loaded chitosan microparticle incubated with PBS (pH7.4) for 24 h exhibited a clear band (Lane 5,Lane 6 and Lane 7) at about at same distance as control(Lane 8 and Lane 9). However, BSA pre-treated with 0.01 M HCl for 2 hours then incubated with PBS for 24 hours had a faint band (Lane 1 ,Lane 2, Lane 3 and Lane 4) at same and above the distance of control (Lane 8 and Lane 9) which indicated the serious degradation or hydrolysis of BSA in acidic medium.

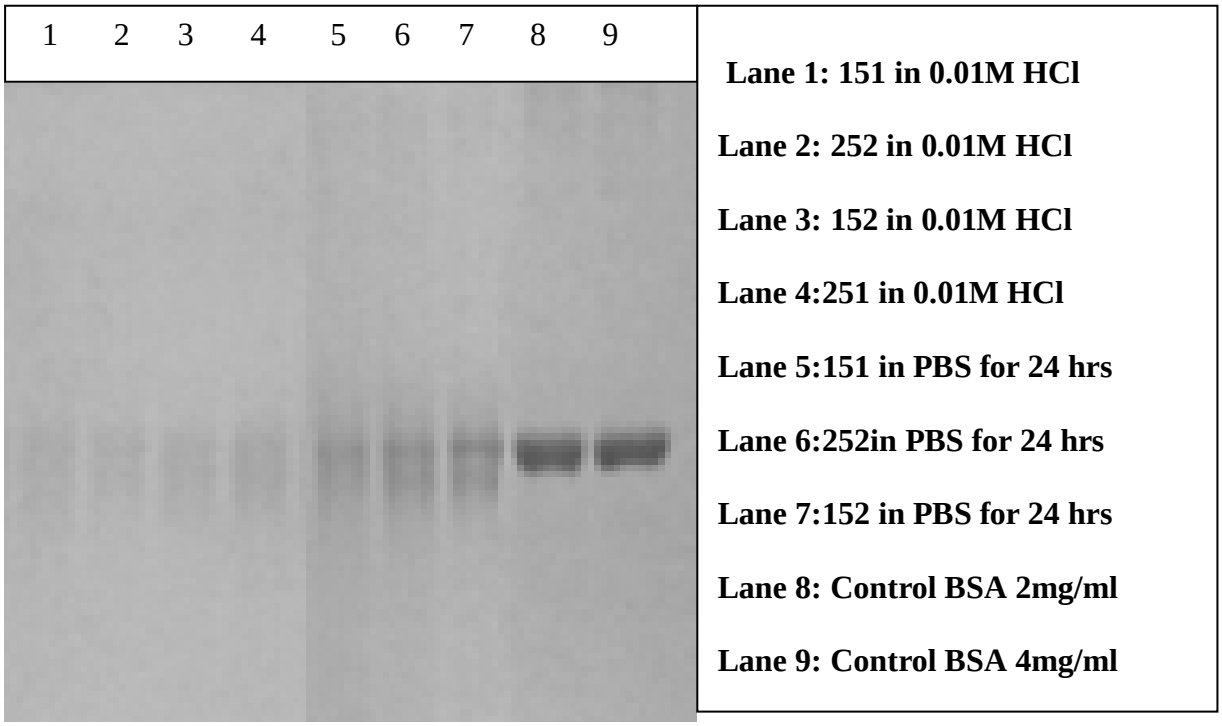


Fig 6. SDS-PAGE for BSA loaded chitosan microparticles for analysis of protein degradation.

CONCLUSION

The prepared BSA loaded chitosan microparticles, with mean diameter of about 0.4μm, were suitable for oral administration due to their small particle size. It has high encapsulation efficiency but have lesser stability as it was shown by SDS-PAGE protein degradation analysis, degradation of BSA occurred at pH 2. So for stabilization of chitosan microparticle some coating of other material required. As chitosan microparticles have high loading efficiency, thus they can be used for carriers for proteinaceous drugs.

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