RESEARCH ARTICLE

Understanding the mechanism of ionic gelation for synthesis of chitosan nanoparticles using qualitative techniques

Sijumon Kunjachan[1,2], Sajan Jose[1], Twan Lammers[2]

[1]Department of Pharmaceutical Sciences, Mahatma Gandhi University, Kerala, India, [2]Department of Experimental Molecular Imaging (EXMI), University Clinic, RWTH Aachen, Germany

We focused on qualitatively exploring the basic mechanisms involved in the *Ionic gelation* (IG) process, a method quite frequently used for synthesis of chitosan (CS) microparticles (MPs) and nanoparticles (NPs). We synthesized CS MPs and NPs using the Ionic gelation and microemulsion methods, and characterized the CS NPs and MPs at different stages of formulation using scanning electron microscopy (SEM) and fluorescence microscopy. Fourier Transform Infrared (FTIR) analysis was carried out to confirm effective cross-linking. Moreover, for the first time, we reported the mechanisms of IG technique for CS NP and MP synthesis with qualitative proof: (1) Complex formation of long chain oligomers with polyanions (long beaded structures) (2) cleavages at weak sites on addition of acid (HCl) (3) formation of CS NPs on chain scission. The versatility of IG for the synthesis of CS MPs and NPs was proved and compared with the microemulsion technique, thereby enhancing the wide spectrum of its use in therapeutics and biomedical applications.

Key words: *Chitosan, cross linking, ionic gelation, microparticles, nanoparticles*

INTRODUCTION

Ionic gelation (IG), earlier known as '*ion-induced gelation*', results in nanoparticles (NPs) and microparticles (MPs) with defects such as improper surface morphology, fragile particulate system, high dispersibility index, and lack of proper surface modification sites to attach functional moieties.[1-3] Here, we attempt to overcome the defects associated with IG synthesis and formulate chitosan (CS) NPs, and in principle, compare them with the already established microemulsion formulation protocols and then to discern their usefulness in drug delivery applications such as pulmonary inhalations, mucoadhesive systems, oral delivery systems for treating peptic ulcers, and finally for intra-tumoral drug targeting. All above-mentioned 'defects' can be resolved by the novel insights gained from the understanding of the synthesis mechanisms[4,5] of the *IG method*.

CS, a natural linear biopolyaminosaccharide obtained by alkaline deacetylation of chitin, and second abundant polysaccharide next to cellulose.[6] Rendering amino ($-NH_2$) and hydroxyl (-OH) groups, CS enables a high degree of chemical modification. The N-deacetylated product of chitin is an interesting biopolymer to prepare MPs and NPs, owing to its unique polymeric cationic character, good biocompatibility, non-toxicity, biodegradability, its mucoadhesivity, and absorption-enhancing effects. Chitin is a straight chain homopolymer composed of (1, 4)-linked N-acetyl glucosamine units, while CS comprises of copolymers of glucosamine and N-acetyl glucosamine.[7,8] CS has one primary amino group and two free hydroxyl groups for each C6 building unit [Figure 1]. Due to the availability of free amino groups, it carries a positive charge and reacts with many negatively charged surfaces such as the cell membrane, mucus lining (due to negatively charged sialic acid residues), and also with other anionic polymers.[9] CS is a weak base, insoluble in water and organic solvents, however, it is soluble in dilute aqueous acidic solution (pH $<$ 6.5), which can convert the glucosamine units into a soluble form of protonated amine ($R–NH_3^+$).[10] CS gets precipitated in alkaline solutions or with polyanions and forms a gel at a lower pH.[11] Particle size, density, viscosity, degree of deacetylation, and molecular weight are important parameters of CS, influencing its pharmaceutical formulations. The mucoadhesive properties of CS[12,13], due to molecular attractive forces formed by electrostatic interaction between positively charged CS

Address for correspondence:
Mr. Sijumon Kunjachan, Experimental Molecular Imaging (EXMI), University Clinic, RWTH Aachen, Germany.
E-mail: sijumon@gmail.com, skunjachan@ukaachen.de

DOI: 10.4103/0973-8398.68467

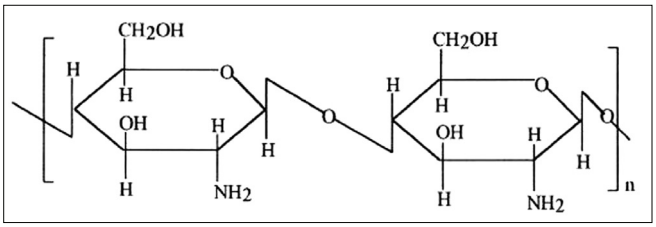


Figure 1: Schematic chemical structure of CS

and negatively charged mucosal surfaces is well documented. These properties may be attributed to (a) strong hydrogen bonding groups like –OH, –COOH,[14] (b) strong charges,[15] (c) high molecular weight,[16] (d) sufficient chain flexibility,[17] and (e) surface energy properties favoring spreading into the mucus. CS MPs or NPs are the most widely studied drug delivery systems for controlled release of drugs, namely, antibiotics, antihypertensive agents, anticancer agents, proteins, peptide drugs, and vaccines.

MATERIALS AND METHODS

Light liquid Paraffin, heavy liquid Paraffin, hexane, methanol, sodium nitrate, and glacial acetic acid (GAA) were procured from CDH Chemicals Laboratory, India. CS (degree of deacetylation: 85% and viscosity: 800 – 1000 cps), span 85, tween 20, sodium chloride, potassium dihydrogen phosphate, and dextran sulfate were obtained from Sigma Chemicals, USA. Glutaraldehyde, toluene, orthophosphoric acid and triethylamine (TEA) were purchased from Qualigens Fine Chemicals, India. The dialysis bag membrane (MWCO: 3500) was purchased from Sigma, USA.

Optimized procedure for the formulation of CS MPs using the microemulsion cross-linking method

CS was procured at an average molecular weight ranging between 3800 and 2,000,000 Da and deacetylated to 66 to 95%, and was used for these studies. The method used for preparation was a modified protocol reported by Wang Y *et al.*[18] Light and heavy liquid paraffin oil (1:1) was taken and a mixture of Span 85, Tween 20 (7 – 8% v/v) in a ratio of 10:1 was added to this, which was then homogenized at 16,000 rpm for two minutes, to ideally disperse the surfactants in the oil base. During homogenization, the CS solution (1% w/v) was slowly injected into the oil phase to form an o/w emulsion. Homogenization was carried out at 16,000 rpm for about 30 minutes that resulted in the formation of primary emulsion. The cross-linking agent, toluene-saturated-glutaraldehyde (1:8), was added to it stage by stage (every hour for a period of three hours). The preparation was transferred to a magnetic stirrer and left for four to five hours, for stabilization and cross-linking. The whole process was performed under light protective conditions and the temperature was maintained at 37±1°C throughout the experiment. The formulation was centrifuged at 8000 g and the sediment recovered and washed thrice with n-hexane and acetone. The residue was washed with triple distilled water. It was then freeze-dried using liquid N_2 to generate rocky ice, followed by sublimation at -85°C and 05 m torr. The freeze-dried product was a fine and free flowing powder, which was collected and stored in an air-tight container at 4°C. Optimization of the procedure was carried out by varying the process parameters such as surfactant concentration, stabilizing time (using cross-linking agent), concentration of CS (aqueous phase), and homogenizing time and speed.

Optimization of process parameters

Several parameters such as change in surfactant concentration, change in stabilizing time, change in concentration of CS, change in homogenization time and homogenization speed were tested and optimized. Surfactant concentration was varied from 2 to 7% v/v and it was noticed that the optimized surfactant concentration (7 – 8% v/v) produced an ideal emulsion with improved dispensability. However, it was observed that a surfactant concentration of more than 8% (v/v) resulted in an agglomeration of particles and ultimately bigger particles (unpublished data). The stabilizing / cross-linking time was varied and toluene-saturated-glutaraldehyde (8:1) was added step by step. In spite of starting with two hours of cross linking time, the optimized procedure ended in four hours, during which, bound water molecules from CS MPs evaporated and cross-linking took place effectively (reaction between amino group of CS and aldehyde group of glutaraldehyde occurred eventually). Stabilizing for a period of four to five hours was found to be effective for formulating good cross-linked particles (unpublished data). Change in the concentration of the CS solution had a direct effect on the particle size of the formulation. Different formulations using CS solution of varying concentrations were prepared and particle size was analyzed. An ideal concentration of 1% (v/v) was used for the formulation of CS MPs. Homogenization was carried out for different time intervals, and the optimized time for homogenization was 30 minutes, above which it could generate heat, produced due to shear force, and result in agglomeration of the emulsion (as observed under the light microscope). A significant change in particle size was observed with a change in homogenizing speed. Varying homogenizing speeds from 13,000 to 16,000 rpm resulted in the formulation of finer particles (unpublished data).

Cross-linked and non-cross-linked MPs

Cross-linking of MPs during formulation was carried out using toluene-saturated glutaraldehyde. Cross-linking of particles provided compact fine particles, which dispersed quite easily once formulated. During cross-linking of CS MPs, the amino group of CS reacted with the aldehyde group of glutaraldehyde and resulted in the formation of the amino-aldehyde complex (Schiffs base). Cross-linked and non-cross linked MPs were analyzed using FTIR (Qualitative approach).

Optimized procedure for the formulation of CS MPs using the Ionic gelation method

Degradation of high molecular weight CS into low molecular weight CS (LMWCS)

Low molecular weight oligomers were obtained by unspecific digestion. One percent (w/v) sodium nitrite solution was used to digest the CS polymer into oligomers and monomers, and incubated for one week in a shaking water bath (37°C). Digested CS was suspended in a dialysis bag membrane containing de-ionized water, which restricted the flow of oligomers, but allowed the perfusion of water soluble monomers in a water bath, thus retaining uniform-sized oligomers for the IG method, for synthesis of CS NPs.

Formulation of CS NPs using the IG method

LMWCS (1% v/v) dissolved in acetic acid solution (1% w/v) was used throughout the studies in the IG formulation method. LMWCS (5 ml) was gently dripped into the 10% v/v tripolyphosphate (TPP) and the solution was allowed to stand still for five minutes to allow gelation to occur. pH was monitored and observed to shift toward alkaline conditions (≥7.5) on addition of TPP; and was adjusted to acidic pH using 1M HCl. The precipitated NPs were washed thrice with water to remove excess unreacted TPP and freeze-dried. Mannitol 1% v/v (cryoprotectant) was used for lyophilization at -80°C using a freeze dryer.

Characterization of CS MPs and NPs

Fourier Transform Infrared spectrometry was used to analyze the structural purity of the formed CS MPs. Both cross-linked and non-cross-linked MPs were analyzed using FTIR.

RESULTS AND DISCUSSION

Chitosan microparticles were synthesized using the emulsion cross-linking technique and the particles exhibited ideal shape morphology [Figure 2].

Characterization of cross-linked and non-cross-linked MPs using FTIR spectrum

FTIR analysis was carried out to find out the cross-linking of MPs. In emulsion cross-linking, the amino group of CS reacted with the aldehyde group of glutaraldehyde to form an amino-aldehyde complex, characterized by FTIR spectroscopy [Figures 3 and 4]. The FTIR spectrum highlighted the –NH = CH- prominence in the data. Hiding of the amino and the aldehyde peaks when compared with that of the non-cross-linked MPs provided an adequate proof for cross-linking of MPs. Prominent bands in the FTIR spectra of non-cross-linked CS MPs are reported in [Tables 1 and 2]. With the data procured, it was found that the (N-H) bending band and the (O-H) stretching bands were prominent. This further confirmed the presence of active –OH and $–NH_2$ groups in the CS MPs formulated. Active hiding of the amino peak in the cross-linked CS MPs gave the evidence of cross-linking in the MPs. It was observed that (N-H) bending in this FTIR

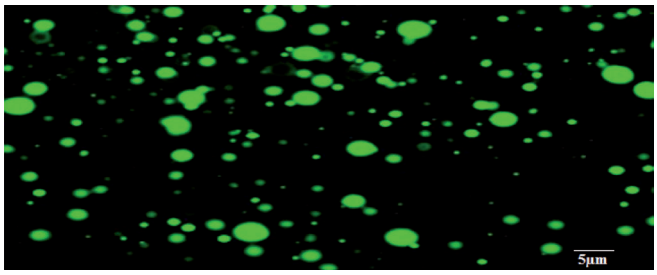


Figure 2: CS MPs prepared using the emulsion cross-linking method (intrinsic auto fluorescence of Doxorubicin)

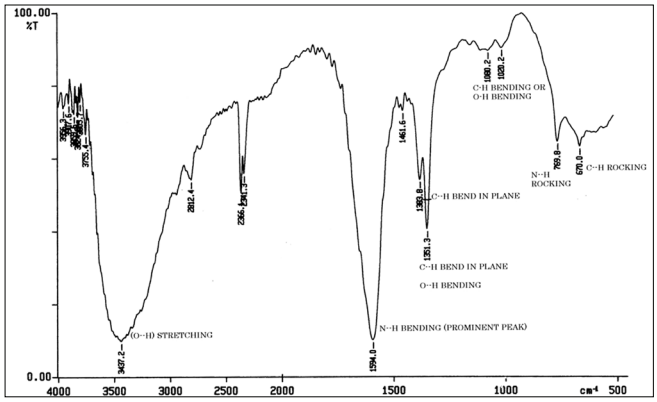


Figure 3: FTIR spectrum of CS MPs

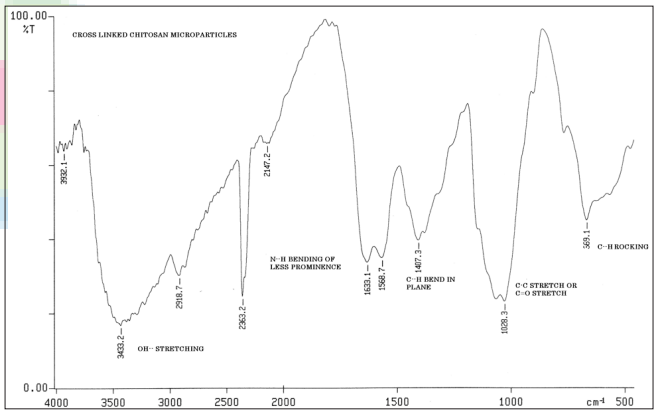


Figure 4: FTIR spectrum of cross-linked CS MPs

Table 1: Change in particle size with concentration of chitosan

Concentration of chitosan (%w/v)	D(0.1)	D(0.5)	D(0.9)*
0.1	0.066	0.128	0.674
0.2	0.065	0.126	0.765
0.3	0.083	0.134	1.014
1.0	0.286	0.533	1.049
2.0	0.076	0.146	1.307

*D(0.9)-Indicates the size of 90% of the particles

Table 2: Swelling Index calculations in different solvents

Solvent used	Dry weight (mg)	Wet weight (mg)	Swelling index
PBS (pH7.4)	2.040	2.230	1.01
Normal saline	2.109	2.113	1.001
Triple distilled water	2.154	2.203	1.022

spectrum was less prominent (there was active hiding of the $–NH_2$ group) proving the involvement of a reaction between the amino group of CS and the -CHO group of glutaraldehyde, resulting in the formation of an aldehyde-amino complex (or Schiffs base). Hence, these data clearly interpret that our optimized formulation protocol rightly generated CS MPs.

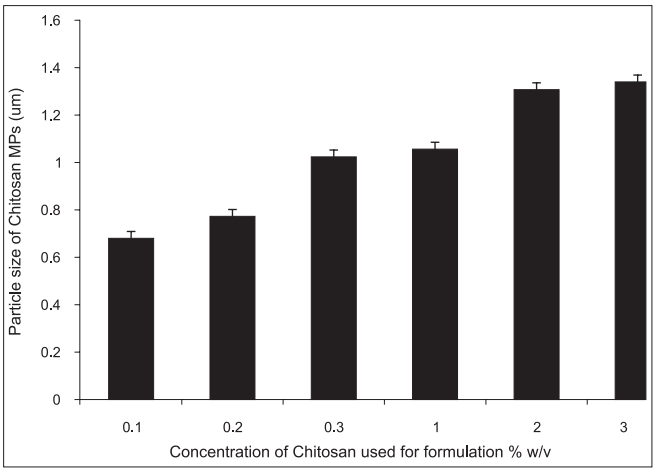
In brief, CS MPs were synthesized using the IG method and the inner mechanisms were traced using our imaging approach supplemented with polymer chemistry studies. The molecular weight of CS played a crucial role in the IG method. Particle size could be controlled by varying the molecular weight of CS [Graph 1]. CS was digested to low molecular weight oligomers or monomers using the sodium nitrite-based degradation technique, which ligated specifically at specific chemical links (glycosidase). These digested or degraded oligomers were further used for IG formulation studies. CS oligomers assumed a straight chain confirmation when suspended in GAA [Figure 5a], due to charge-to-charge repulsion at protonated amino groups ($-NH_3^+$).

Formation of long chain beaded structures

On reaction with TPP, CS oligomers lose their straight chain confirmation and appear looped with or without entanglements [Figure 5b]. The degree of entanglement depends on the molecular weight of the CS used (LMWCS plays an important role). As TPP is an alkaline solution, it contains hydroxyl ions and tripolyphosphoric ions (OH^-, $P_3O_{10}^{5-}$). There is competition between both these ions to bind to the $–NH_3^+$ sites of CS. Random attachment of both OH^- and $P_3O_{10}^{5-}$ occurs resulting in structures as shown in Figures 6a and 6b. On immediate addition of TPP, charge neutralization occurs, randomly substituting the positive charges of $–NH_3^+$ with negative charges of polyphosphate ion, resulting in a complex formation of chain-like beads. They appear like a beaded chain, linked to one another, with possible sites for scission [Figure 6a and 6b]. They represent the formulation reacting with TPP at two minutes of addition of TPP. This process of chain formation is totally random, but maintains uniformity in terms of its surface morphology. The concentration of LMWCS and TPP plays a major role in this formulation step.

Formation of cleave sites

On further observation, at four minutes time interval, several points of chain scission develop on acidification [Figure 7a and 7b]. These weak sites are capable of detachment or scission (mechanical stirring or gentle shaking is also beneficial). To retain the ionic cross-linked structure and to exclude the remaining hydroxyl group, HCl is added to the solution resulting in H^+ ions, which first react with $P_3O_{10}^{5-}$ ions causing "*Chain Scission*" to occur, resulting in the formation of CS MPs or NPs [Figure 8]. On further addition of HCl, certain $–NH_2$ groups exist due to the removal of water and the chain assumes a randomly coiled arrangement in the form of particles [Figure 9]. Amino ($-NH_2^+$) sites act



Graph 1: Change in particle size with concentration of chitosan

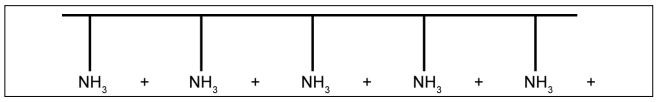


Figure 5a: CS oligomers straight chain in GAA (1%v/v)

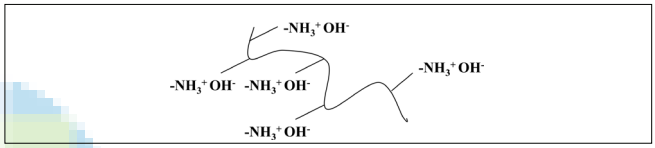


Figure 5b: Schematic diagram of CS oligomers with TPP

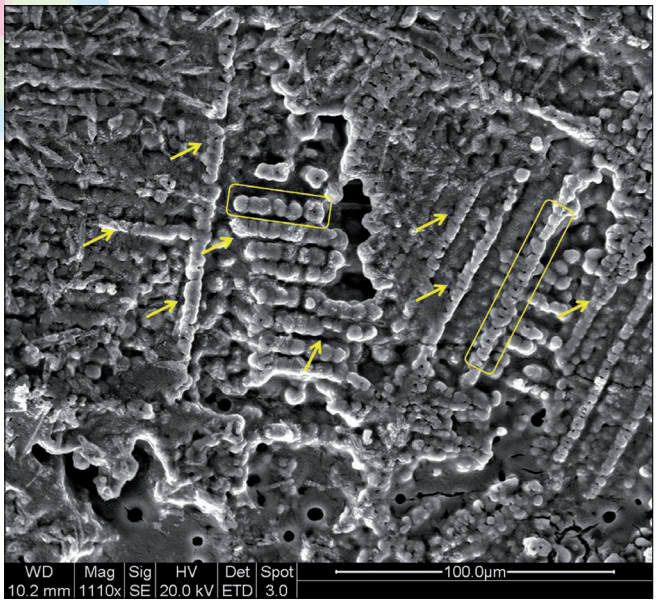


Figure 6a: SEM images of long chain CS beads

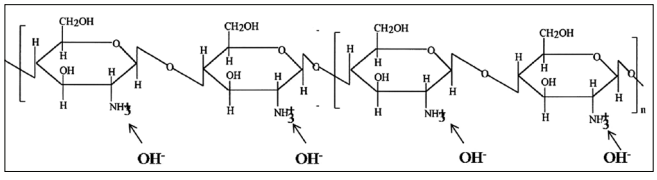


Figure 6b: Schematic chemical structure of CS with respect to long chain bead formation

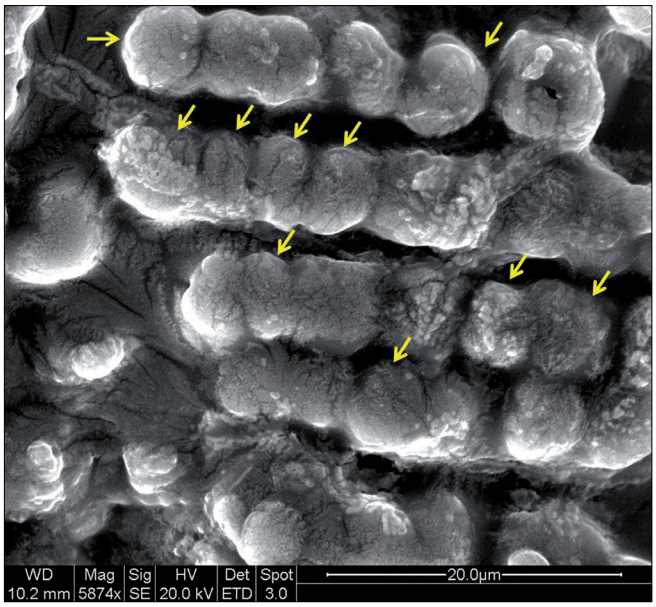


Figure 7a: SEM image showing cleaves at the weak sites of CS beaded chains (represented by arrow marks)

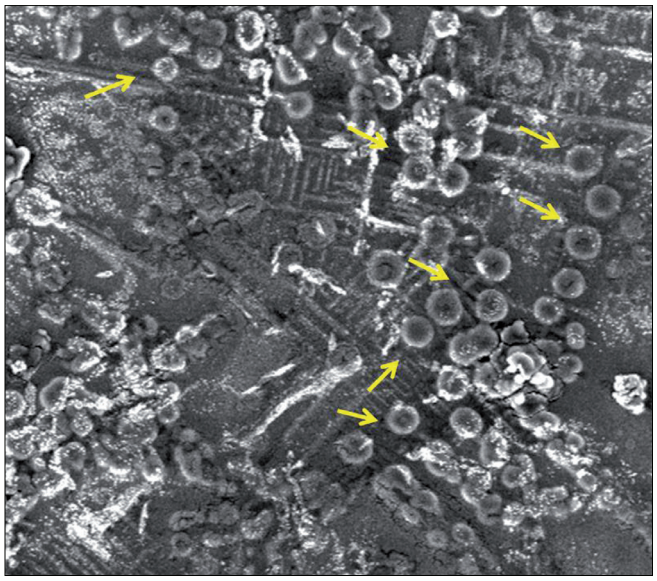


Figure 8: Formation of CS NPs (shown by marked arrows)

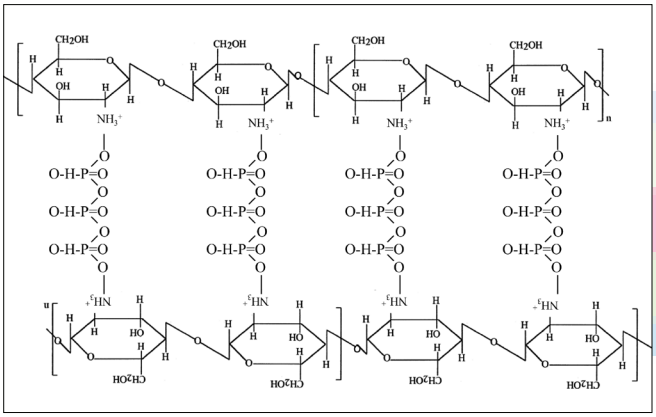


Figure 7b: Schematic representation of ionic cross linking

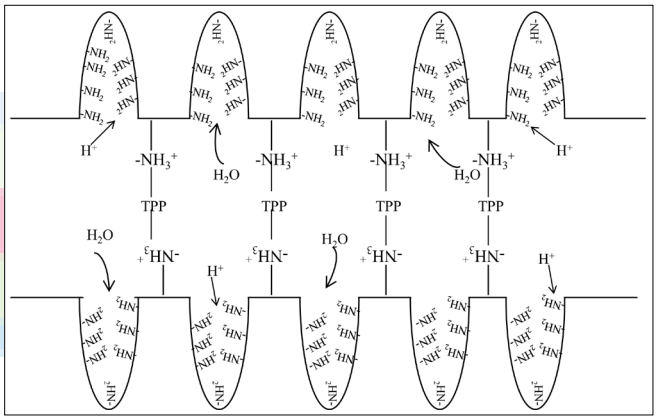


Figure 9: Schematic depiction of lower cross-linked CS bead-acid resistive property

as buffer sites against protons and aid in acid resistance by formation of protonated amines ($-NH_3^+$). Such systems benefit in drug targeting in gastrointestinal diseases, where drug intervention is needed at acidic pH.

Comparison of microemulsion and IG methods

Both methods exhibited *controlled cross-linking* (cross-linking with TPP and cross-linking with glutaraldehyde) depending upon the concentration of cross linkers used. The swelling index is an important parameter for iv formulations, and it also relies largely on the degree of cross-linking of the CS. Cross-linking also decides the remnant unreacted groups left for the 'acid resistive' function of CS at the acidic pH. Particle formation is largely influenced by the pH of the TPP solution and the ionic cross-linking density. Using both the IG and microemulsion techniques, the degree of cross-linking can be modulated using simple formulation techniques such as varying the ratio of complex formation by changing the amount of TPP and CS used in case of IG, and changing the amount of toluene-saturated GAA in case of the cross-linking technique. Ideally, these formulation techniques resist the pH change (by acting as a buffer) and can be potentially used for fabricating a tumor-specific drug delivery system. On account of its swelling property, tissue fluid can diffuse inside CS MPs and NPs via surface pores and sense the core (which might contain the drug molecule), thereby acting as a smart polymeric drug delivery system.

Although the surface morphology of CS MPs prepared by emulsion cross-linking appears fine, the size distribution is very broad and does not comply with the pharmaceutical standards. Also, polydispersity of CS MPs is far from optimum according to pharmaceutical standards per se, when compared to the IG method [Figures 2 and 8]. Apart from the simplicity of the IG method, the microemulsion formulation technique involves the use of harsh chemicals and oil bases

required for the formation of primary emulsion and cross-linking, followed by its purification. However, IG uses only chitosan and TPP majorly and the excess TPP is washed off using solvents (TDW). Unlike the microemulsion method, another advantage of the IG method is the ease with which the degree of cross-linking and therefore the 'buffering' activity can be manipulated. Systematic stability studies for particles generated by both techniques are essential before it can be directly extrapolated for industrial scale up.

ACKNOWLEDGEMENT

SK acknowledges BSBE department, Indian Institute of Technology Kanpur for the SEM images.

REFERENCES

1. Patel VM, Prajapati BG, Patel MM. Design and characterization of chitosan-containing mucoadhesive buccal patches of propranolol hydrochloride. Acta Pharm 2007;57:61-72.
2. Badawi AA, El-Laithy HM, El Qidra RK, El Mofty H, El dally M. Chitosan based nanocarriers for indomethacin ocular delivery. Arch Pharm Res 2008;31:1040-9.
3. Wu P, He X, Wang K, Tan W, He C, Zheng M. A novel methotrexate delivery system based on chitosan-methotrexate covalently conjugated nanoparticles. J Biomed Nanotechnol 2009;5:557-64.
4. Ghosn B, Kasturi SP, Roy K. Enhancing polysaccharide-mediated delivery of nucleic acids through functionalization with secondary and tertiary amines. Curr Top Med Chem 2008;8:331-40.
5. Muzzarelli RA. Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. Mar Drugs 2010;8:292-312.
6. Baxter A, Dillon M, Taylor KD, Roberts GA. Improved method for i.r. determination of the degree of N-acetylation of chitosan. Int J Biol Macromol 1992;14:166-9.
7. Singla AK, Chawla M. Chitosan: Some pharmaceutical and biological aspects–an update. J Pharm Pharmacol 2001;53:1047-67.
8. Kato Y, Onishi H, Machida Y. Application of chitin and chitosan derivatives in the pharmaceutical field. Curr Pharm Biotechnol 2003;4:303-9.
9. Onsoyen E, Skaugrud O. Metal recovery using chitosan. J Chem Technol Biotechnol 1990;49:395-404.
10. Chandy T, Sharma CP. Chitosan–as a biomaterial. Biomater Artif Cells Artif Organs 1990;18:1-24.
11. Kas HS. Chitosan: Properties, preparations and application to microparticulate systems. J Microencapsul 1997;14:689-711.
12. Jain AK, Chalasani KB, Khar RK, Ahmed FJ, Diwan PV. Muco-adhesive multivesicular liposomes as an effective carrier for transmucosal insulin delivery. J Drug Target 2007;15:417-27.
13. Needleman IG, Smales FC. *In vitro* assessment of bioadhesion for periodontal and buccal drug delivery. Biomaterials 1995;16:617-24.
14. Schipper NG, Olsson S, Hoogstraate JA, deBoer AG, Vårum KM, Artursson P. Chitosans as absorption enhancers for poorly absorbable drugs 2: Mechanism of absorption enhancement. Pharm Res 1997;14:923-9.
15. Lee YM, Kim SS, Kim SH. Synthesis and properties of poly(ethylene glycol) macromer/beta-chitosan hydrogels. J Mater Sci Mater Med 1997;8:537-41.
16. Kotzé AF, Luessen HL, de Leeuw BJ, de Boer AG, Verhoef JC, Junginger HE. Comparison of the effect of different chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). J Control Release 1998;51:35-46.
17. Chen RH, Tsaih ML. Effect of temperature on the intrinsic viscosity and conformation of chitosans in dilute HCl solution. Int J Biol Macromol 1998;23:135-41.
18. Wang YM, Sato H, Adachi I, Horikoshi I. Optimization of the formulation design of chitosan microspheres containing cisplatin. J Pharm Sci 1996;85:1204-10.

Source of Support: Nil, **Conflict of Interest:** None declared.