INTRODUCTION
Chitosan, a natural linear biopolyaminosaccharide is obtained by alkaline deacetylation of chitin, which is the second abundant polysaccharide next to cellulose\(^1\). It is a weak base and is 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 R–NH\(_2\). A great number of chitosan derivatives have been obtained by grafting new functional groups on the chitosan backbone. The chemical modifications (quaternization, acylation, cyclodextrin, encapsulation, thiolation etc) afford a wide range of derivatives with modified properties for specific applications in biomedical and biotechnological fields\(^2\). Modification does not change the fundamental skeleton of chitosan but brings new or improved properties for e.g., mucoadhesion and permeation enhancement. The advantage of chitosan over other polysaccharides is that its chemical structure allows specific modifications without too many difficulties at C-2 position. Specific groups can be introduced to design polymers for selected applications. The main reaction easily performed involving the C-2 position is the quaternization of the amino group or a reaction in which an aldehydic function reacts with amino group by reductive amination. This latter reaction can be performed in aqueous solution under very mild conditions to obtain randomly distributed substituents in a controlled amount along the chitosan chain. This method has been proposed for introduction of different functional groups on chitosan cross-linking through a reaction between the nitrile group and the amine group\(^3\). Figure 1 shows some examples of chemical derivatization of chitosan.

The cross-linking may be achieved in acidic, neutral, or basic environments depending on the method applied. Chemical cross-linking is the most straightforward method to produce permanent networks using covalent bonding between the polymer chains. The available amino and hydroxyl groups on chitosan are active sites capable of forming a number of linkage, including amide and ester bonding as well as Schiff base formation\(^4\). These networks formed by using small molecule cross-linkers, polymer–polymer reactions between activated functional groups. Cross-linked chitosan are highly useful in the pharmaceutical field for the formulation of various novel drug delivery systems like microspheres, nanospheres, hydrogels and films/membranes. The release of drug from chitosan microspheres is dependent upon the molecular weight of chitosan, drug content and density of cross-linking. Various therapeutic agents such as anticancer, anti-inflammatory, antibiotics, antithrombic, steroids, proteins, amino acids, antidiabetic and diuretics have been incorporated in cross-linked chitosan microspheres to achieve controlled release as well to enhance bioavailability or for drug targeting to specific areas of the body\(^5\).

CROSS-LINKING AGENTS
Cross-linking occurs when a reagent (namely a cross-linking agent) introduces intermolecular bridges and/or cross-links between polysaccharide macromolecules. The cross-linking step is a well-known and well-documented reaction, and a relatively easy method to prepare polysaccharide-based materials. The cross-linking agent can react with macromolecules linear chains (cross-linking step) and/or itself (polymerization step) in an alkaline medium. Cross-linking drastically reduces segment mobility in the polymer and a number of chains are interconnected by the formation of new inter chain linkages. A three dimensional network is then formed. If the degree of reticulation is sufficiently high, the matrix of the polymers becomes insoluble in water (but swellable in water) and in organic solvents\(^6\).

i) Classification of cross-linking agents: Cross-linking agents are broadly classified on the basis of interaction of cross-linkers with chitosan during cross-linking. These are:

a) Physical cross-linking
b) Chemical cross-linking

a) Physical cross-linking: In recent years there has been increase in interest in physical cross-linking. The main reason is that use of cross-linking agents is avoided. In it polysaccharides formed cross-linking network with counterions at the surface. High counterion concentration would require longer exposure times to achieve complete cross-linking of the polysaccharides. Physical cross-linking occurred by thermal cross-linking using citric acid, by using dextran sulfate or phosphoric acids.

b) Chemical cross-linking: Chemical cross-linking of polysaccharide is highly versatile method with good mechanical stability. In this cross-linking covalent bonds are present between different polymer chains or between polymer and cross-linker. During cross-linking counterions diffused into the polymeric and cross-linking agent reacts with polysaccharides forming either intermolecular or
intramolecular linkages. Factors which affect chemical cross-linking are concentration of cross-linking agent and cross-linking time. Chemical cross-linking is carried out by using glutaraldehyde, formaldehyde, vanillin, genipin, radial polymerization, 1, 6-hexamethylenediisocyanate or 1, 6-hexanediibromide.

ii) Characteristics, properties and advantages of cross-linking materials

a. Homogeneous cross-linked materials are easy to prepare with relatively inexpensive reagents and are available in a variety of structures with a variety of properties, and also in numerous configurations, e.g. gels, particles of irregular shape or regular beads, membranes, coatings, films, fibers, capsules and sponges.

b. They are insoluble in acidic and alkaline mediums as well as organic solvents. Cross-linked gels are very stable hydrophilic polymers. They become more resistant to shear, high temperature and low pH compared to their parent polysaccharide.

c. After cross-linking, they maintain their properties, original characteristics (except the crystallinity) and strength in acidic and basic solutions. These characteristics are important for an adsorbent so that it can be used in a lower pH environment. Furthermore, the swelling behavior of the beads in wastewater can be optimized.

d. Cross-linking reduces the amount of the crystalline domains in the polysaccharide and can then change the crystalline nature of the raw polymer. This parameter significantly influences the sorption properties because it may control the accessibility to sorption sites.

e. Cross-linked beads also have other advantages such as faster kinetics, increased ease of operation and interesting diffusion properties. Due to the hydrophilic nature of their cross-linking units, materials possess a remarkably high swelling capacity in water, and consequently their networks are sufficiently expanded as to allow a fast diffusion process for the pollutants.

f. Even though cross-linked polysaccharides have good sorption properties, their adsorption capacities can be improved by the grafting of various functional groups onto the polymer network or the polymer backbone. This increases the density of sorption sites. The presence of new functional groups on the surface of the beads results in an increase of surface polarity and hydrophilicity and this enhances the sorption of polar solbates and improves the sorption selectivity for the target pollutant.

iii) Importance and examples of various cross-linking agents:

Non cross-linked chitosan microspheres were unsuitable for the purpose of sustained/controlled released formulations as compared to cross-linked chitosan microspheres, which showed better results. Mi et al. prepared an injectable-chitosan based delivery system with low cytotoxicity. The chitosan microspheres with small particle size, low crystallinity and good sphericity were prepared by a spray-drying method followed by cross-linking with a naturally occurring cross-linking agent (genipin). The results of the study demonstrated that the genipin cross-linked chitosan microspheres had a superior biocompatibility and a slower degradation rate than the glutaraldehyde-cross-linked chitosan microspheres. It was concluded that the genipin-cross-linked chitosan microspheres may be a suitable polymeric carrier for long-acting injectable drug delivery. Therefore cross-linking agents have more importance in the pharmaceutical field. The various cross-linking agents used for the cross-linking of chitosan have been given in table 1.

iv) Relative comparison of cross-linking agents: Roy et al. prepared mefanamic acid chitosan microspheres both by thermal and glutaraldehyde cross-linking method and found that high level of entrapment of drug was obtained in glutaraldehyde cross-linking method. Kumbar et al. compared chitosan microspheres of diclofenac sodium containing different cross-linking agents, i.e. glutaraldehyde, sulfuric acid and physically by heat treatment, and demonstrated that among three different cross-linkers fastest drug release was found in heat treated microspheres and slowest in the glutaraldehyde cross-linked chitosan microspheres. Goncalves et al. prepared cross-linked chitosan microspheres for the controlled release of sodium diclofenac using epichlorohydrin as an alternative to cross-linking agents that react with the amine groups of chitosan. For the sake of comparison, glutaraldehyde was used as cross-linker. The effects of the cross-linking agents on chitosan microspheres were evaluated in relation to swelling, hydrolysis, porosity, cross-linking, diclofenac sodium impregnation, and consequently the release rate of sodium diclofenac. The chitosan microspheres cross-linked with epichlorohydrin or glutaraldehyde behaved in different ways with regard to various aspects, such as the degree of swelling, and the amount of drug impregnated, and consequently released. Similarly comparison of tripolyphosphate, formaldehyde and glutaraldehyde were made by preparing chitosan microspheres. It was found that % encapsulation efficiency; % erosion, % swelling, surface morphology and drug release rate of microspheres were controlled by adding different cross-linking agents.
MECHANISM OF CROSS-LINKING
The cross-linking reaction is mainly influenced by the size and type of cross-linker and the functional groups of chitosan. Smaller the molecular size of the cross-linker, the faster the cross-linking reaction, since its diffusion is easier. Depending on the nature of the cross-linker, the main interactions forming the network are covalent or ionic bonds. Covalently cross-linked hydrogels present the cross-linking degree as the main parameter influencing important properties such as mechanical strength, swelling and drug release. Such gels generally exhibit pH-sensitive swelling and drug release by diffusion through their porous structure. Therefore, hydrogels based on covalently and ionically cross-linked chitosan can be considered as good candidates for the oral delivery of drugs.

Concern over the disintegration of chitosan microparticles has led to their modification by cross-linking to make a rigid polymer for use as a core material in controlled drug release research. Ko et al. reported the use of complexation between polyanion tripolyphosphate and cationic chitosan by electrostatic forces resulting in a more homogeneous structure due to the more homogeneous cross-linking process.

The mechanism of cross-linking of chitosan with different cross-linkers has been described below:

i) Glutaraldehyde and epichlorohydrin cross-linking: The cross-linking bifunctional agents used to bind different reaction sites in the chitosan. The aldehyde groups of the glutaraldehyde form covalent imine bonds with the amino groups of chitosan, due to the resonance established with adjacent double ethylenic bonds via a Schiff reaction (Figure 2) while epichlorohydrin links through the carbon atoms, resulting in the rupturing of the epoxide ring and the removal of a chlorine atom (Figure 3).

ii) Genipin cross-linking: The cross-linking reaction mechanisms for chitosan with genipin are different at different pH values. Under acidic and neutral conditions, a nucleophilic attack by the amino groups of chitosan on the olefinic carbon atom at C-3 occurs, followed by opening the dihydropyran ring and attacked by the secondary amino group on the newly formed aldehyde group. In other words, genipin acts as a dialdehyde but its condensation products are much more stable compared to glutaraldehyde. In the product, short chains of condensed genipin act as cross-linking bridges.

Under basic conditions, the ring-opening reaction of genipin occurs via a nucleophilic attack by hydroxyl ions in aqueous solution to form intermediate aldehyde groups, which subsequently undergo aldol condensation. The terminal aldehyde groups on the polymerized genipin undergo a Schiff reaction with the amino groups on chitosan to form cross-linked networks. Therefore, the pH condition plays an important role in influencing the cross-linking reactions. Figure 4 represents the cross-linking mechanism of genipin with chitosan.

iii) TPP cross-linking: It acts by increasing the pH and ionic strength of the solution, forming a gel and promoting ionic interaction between the amino groups of chitosan and the anionic groups of TPP.

iv) Vanillin cross-linking: Cross-linking of vanillin occurred via Schiff base reaction.

v) Sulphuric acid and heating cross-linking: Cross-linking of chitosan with sulphuric acid and by heating has been shown in figure 5.

DETERMINATION DEGREE OF CROSS-LINKING
The cross-linking degree of chitosan beads can be determined by ninhydrin assay, which can determine the percentage of free amino groups in cross-linked chitosan beads. The ninhydrin solution prepared as follows: Solution A: 1.05 g citric acid, 10 ml (1 M) NaOH, and 0.04 g SnCl₂·2H₂O mixed, then de-ionized water added until 25 ml; Solution B: 1 g ninhydrin dissolved in 25 ml ethylene glycol monomethyl ether. Solutions A and B then mixed and stirred for 45 min and then stored in a dark bottle. For the assay, the microspheres lyophilized for 24 h and then weighed. A 1.5 mg lyophilized sample was heated to 100 °C in water bath with 1 mL ninhydrin solution for 20 min. The solution then cooled down to room temperature, diluted with 5 mL 50% isopropanol, and then the optical absorbance of the solution read with UV spectrophotometer. The amount of free amino groups in the test sample, after heating with ninhydrin, is proportional to the optical absorbance of the solution. The concentration measured is divided by sample weight, and multiplied by the sample molecular weight to obtain the mole NH₂/mole sample. Every sample was tested three times. The degree of cross-linking of sample is then calculated following the equation:

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\text{Cross-linking degree (\%)} = \frac{Y}{X} \times 100
\]

Where, x is the mole of free amino groups of non-cross-linked chitosan beads, and y is the mole of free amino groups of cross-linked chitosan beads.

IMPLICATION OR EFFECT OF CROSS-LINKING
i) Drug release: Drug release from chitosan-based particulate systems depends upon the extent of cross-linking, morphology, size and density of the particulate system, physicochemical properties of the drug as well as the presence of adjuvants. Illum et al. observed that cemetidine-loaded chitosan microspheres have shown burst effect in the early stages of dissolution. Increasing the cross-linking density can prevent the burst release. Genta et al. prepared chitosan microspheres by using glutaraldehyde solutions at concentration higher than 5% by the emulsion/separation method. The microspheres obtained were of good morphological characteristics, spherical shape and smooth surface. It was concluded that suitable drug release rate can be obtained with the lowest glutaraldehyde amount (7 or 15%) and the concentration of glutaraldehyde solution higher than 10% decreased the microsphere sizes. Jameela et al. prepared progesterone loaded chitosan microspheres by emulsion cross-linking method using glutaraldehyde as a cross-linking agent and demonstrated that the extent of drug release had a remarkable dependence on the cross-linking density of the microspheres. Highly cross-linked spheres released only 35% of the incorporated steroid in 40 days compared to 70% from spheres lightly cross linked. Desai et al. concluded that the % encapsulation efficiency, % erosion, % swelling, surface morphology and drug release rate of the spray dried chitosan microspheres could be properly controlled by adding different cross-linking agents into the chitosan solution.

ii) Stability: A few studies have reported the instability of chitosan microspheres (prepared by precipitation) in acidic medium. Kreuter et al. prepared chitosan microspheres by sodium sulfate precipitation but the acid stability of the microspheres was found to be poor. Further, Berthold et al. made an extensive investigation into the acid stability of the microspheres and observed that cross-linking with

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glutaraldehyde can increase the same. The acid instability of the microspheres can be explained by the manufacturing process of the microspheres. The addition of sodium sulfate to acetic acid solution of chitosan leads to poorly soluble chitosan derivatives by ionic neutralization of the positively charged amino groups. On addition of the acid (increased proton concentration) the equilibrium is shifted towards solubilization of chitosan and microspheres dissolve.

### iii) Novel application of cross-linked chitosan matrix

New applications of chitosan matrix have been appeared in the last few years. Although not much information can be found with regard to these applications we have tried to update the knowledge on the relationship between physicochemical properties and function.

**Imprinted Chitosan-Based Matrixes**

Molecularly imprinted polymer (MIP) represents a new class of materials that have artificially created receptor structures. This potential technology is a method for making selective binding sites in synthetic polymers by using a molecular template. MIPs have steric and chemical memory toward the template and hence could be used to rebind it.

As can be seen in table 2, chitosan imprinted matrixes have been prepared with different purposes by using different chitosan samples. In general, samples with DD ≥ 90% and wide range of Mw have been used to prepare chitosan imprinted materials. As far as we know, there is only one paper studying the effect of chitosan Mw (92, 47 and 29 kDa) on the rebinding properties of molecular imprinted chitosan matrixes. In this case, the sample with 47 kDa achieved the best rebinding capacities.

It is well known that the number of recognition sites in MIPs increases with network density. The cross-linking density is influenced by various parameters being favoured by high DD and Mw. Since crystallinity and surface area control the accessibility of the free amino groups to the chemical reagents, these parameters are expected to have an impact on the behaviour of the matrixes.

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**Figure 1 Examples of chemical derivatization of chitosan**

**Figure 2 Cross-linking process of chitosan treated with glutaraldehyde**
Figure 3 Cross-linking process of chitosan treated with epichlorohydrin

Figure 4 Genipin reacts with chitosan to yield two main cross-linking reactions. On the right, two chitosan chains (represented by their structural units) are cross-linked by one mole of genipin: the formula shows the two newly formed chemical groups, namely the monosubstituted amide and the tertiary amine. An additional reaction is the homopolymerization of genipin.

Figure 5 Cross-linking process of chitosan treated with sulphuric acid and by heating

Table 1: Various cross-linking agents used for the preparation of chitosan microparticulates

<table>
<thead>
<tr>
<th>Cross-linking agent</th>
<th>References</th>
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<tbody>
<tr>
<td>Glutaraldehyde saturated toluene solution (25% ml/ml)</td>
<td>13,14,15,16</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>17,18,19,20,21,22,23</td>
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<tr>
<td>Formaldehyde</td>
<td>24</td>
</tr>
<tr>
<td>2, 3-Dihydroxypropanal</td>
<td>25</td>
</tr>
<tr>
<td>Citric acid</td>
<td>26,27</td>
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<tr>
<td>Calcium Chloride</td>
<td>28,29</td>
</tr>
<tr>
<td>Genipin</td>
<td>12,30,31,32</td>
</tr>
<tr>
<td>Tripolyphosphate (TPP)</td>
<td>33,34</td>
</tr>
<tr>
<td>Vanillin</td>
<td>35</td>
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<tr>
<td>Sodium sulfate</td>
<td>36</td>
</tr>
<tr>
<td>Epichlorohydrin</td>
<td>37</td>
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<tr>
<td>Sulphuric acid</td>
<td>38</td>
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</table>
Chitosan has the desired properties for safe use as a pharmaceutical excipient. This has prompted accelerated research activities worldwide on chitosan microparticles as drug delivery vehicles. Cross-linked chitosan microspheres are highly suitable for the purpose of sustained/controlled drug delivery vehicles. Cross-linked chitosan microparticles as pharmaceutical excipient. This has prompted accelerated research activities worldwide on chitosan microparticles as a long acting biodegradable drug delivery vehicle: \textit{Chitosan Amphiphilic Derivatives. Hydrometallurgy 2003; 71:191-247.}

CORRECTION

CONCLUSION

Chitosan has the desired properties for safe use as a pharmaceutical excipient. This has prompted accelerated research activities worldwide on chitosan microparticles as drug delivery vehicles. Cross-linked chitosan microspheres are highly suitable for the purpose of sustained/controlled released formulations as compared to non cross-linked chitosan microspheres. Chitosan microspheres cross-linked with different cross-linking agents behaved in different ways with regard to various aspects, such as amount of drug impregnated and consequently released.

REFERENCES