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Tartrate/tripolyphosphate as co-crosslinker for water soluble chitosan used in protein antigens encapsulation

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ABSTRACT

In drug delivery research, several toxic chemical crosslinkers and non-toxic ionic crosslinkers have been exploited for the synthesis of microparticles from acetic acid soluble chitosan. This paper hypothesized the implementation of sodium potassium tartrate (SPT) as an alternative crosslinker for sodium tripolyphosphate (TPP) and SPT/TPP co-crosslinkers for synthesis of the microparticles using water soluble chitosan (WSC) for encapsulation of Bovine serum albumin (BSA) as a model protein, and Tetanus toxoid (TT) as a model vaccine. The crosslinking was confirmed by FT-IR, SEM with EDS. The XRD entailed molecular dispersion of proteins and thermal analysis confirmed the higher stability of STP/TPP co-crosslinked formulations. The resultant microparticles were exhibiting crosslinking degree (52–67%), entrapment efficiency (72–80%), particle size (0.3–1.7 μm), zeta potential (+24 to 46 mV) and mucoadhesion (41–68%). The superiority of SPT over TPP was confirmed by higher crosslinking degree and entrapment efficiency. However, co-crosslinking were advantageous in higher regression values for Langmuir adsorption isotherm, slower swelling tendency and extended 30 days controlled in-vitro release study. TT release obeyed the Quasi-Fickian diffusion mechanism for single and cocrosslinked formulations. Overall, in crosslinking of chitosan as biological macromolecules, STP/TPP may be alternative for single ionic crosslinked formulations for protein antigen delivery.

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1. Introduction

Chitosan [α-(1 → 4)-2-amino-2-deoxy-β-D-glucan], as biological macromolecules has received great attention in drug delivery research as a pharmaceutical excipient for its potential in the development of the controlled release delivery systems [1–3]. The certainly interesting intrinsic properties such as biodegradability, biocompatibility, special adhering feature to the mucosal surface, penetration enhancing by opening epithelial tight-junctions [4] and immune stimulating activity has increased attention to exploit chitosan as protein antigens delivery system [5]. Degradation of chitosan microparticles has also shown to be nontoxic, non-immunogenic and non-carcinogenic which makes it as a promising candidate for the development of single dose vaccines [6,7]. However, despite above desirable characteristic, its actual use is limited due to its poor solubility in water. The native chitosan dissolves only in aqueous acetic acid solution that induces undesirable cytotoxicity in the encapsulated formulations and increases sensitivity towards bioactive macromolecules such as peptide or protein drugs, genetic material and anticancer drugs [8,9]. Kurita [10] suggested that many chitosan derivatives are water soluble in a wide pH range and have unique biological activities and physicochemical properties. In addition, Tao et al. [11] reported that, use of water soluble chitosan (WSC) reduces mean particle size of the microspheres and has a preventive effect on diet-induced obesity.

The pKa value of the amino groups on chitosan is around pH 6.3; hence it behaves as a polycationic electrolyte in an aqueous medium [12]. For this purpose, a large number of studies on crosslinking agents involving bonds with chitosan amino groups have been carried out [13,14]. Also, to preserve the stability of chitosan under GI (gastrointestinal) tract delivery or enzymatic degradation and increase the time frame for drug delivery, amino groups on polymeric chains have to be fixed by crosslinking agents [15]. The crosslinking agents can react with macromolecules linear

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chains and reduces segment mobility in the polymer causing high degree of reticulation in dimensional network [16].

Chemical crosslinkers widely used such as glutaraldehyde, formaldehyde, glyoxal are either known to be relatively toxic, or their fate in the human body is unknown [17,18]. These agents would give rise to health concerns and causes undesirable side effects if unreacted crosslinkers remain in traces before the administration. Hence, it requires an additional purification and verification step, which further increases the overall cost of the formulations. Therefore, in the approach of using nontoxic crosslinkers, the mechanism of ionic crosslinking has attracted much attention because of the simplicity, the relative mild procedural conditions and avoidance of possible toxicity of the reagents commonly used for chemical crosslinking. Moreover, ionic crosslinking are advantageous in terms of homogeneous crosslinking, controlled release formulation in acidic and basic environments, compressible hydrophilic matrix systems, controlled hydration and gel forming tendency, smaller particle size, low crystallinity, good sphericity, good sorption properties, high loading efficiency, biocompatible property, *etc.* [19–22]. In this prospects, Sodium tripolyphosphate (TPP), a low molecular counter ion as ionic crosslinker was reported numerously in literatures for synthesis of encapsulated vaccine or drug delivery [23–26]. On the other hand, pharmacologically acceptable salts of tartrate were used in dextran crosslinking for the preparation of sustained release pharmaceutical compositions [27]. Further, potassium antimony tartrate was found to be an active component to crosslink hydrated natural gums at pH between 2.5–7.0 [28]. However, the polymeric microparticles crosslinked by single ionic crosslinkers are associated with some fundamentals limitations of drug delivery such as low crosslinking density, protein inactivation during the encapsulation process, poor mechanical strength, burst effect [29], inferior release tendency in basic condition, difficulties in controlling active protein releases [30], synthesis of mucosal delivery system below the pKa value of chitosan, *etc.* Also, to the best of our knowledge, there are no reports available for the implementation of sodium potassium tartrate (SPT) to crosslink commercial water soluble chitosan for delivery of vaccine antigens. Similarly, limited literatures could be obtained exhibiting the use of TPP to crosslink commercial water soluble chitosan for protein or vaccine delivery [31,32].

The present study involves the synthesis and characterization of chitosan microparticles using ionic crosslinkers TPP and SPT alone as well as SPT/TPP as a co-crosslinkers for encapsulation of BSA and TT to overcome the inherent constraints of single crosslinking formulations.

2. Experimental

2.1. Materials

Low molecular weight water soluble chitosan (Degree of deacetylation, 90.0% Min; Viscosity, 20–200 mPa.s) was purchased from Zhengzhou Sigma Chemical Co. Ltd China (CAS No. 9012-76-4). Tetanus toxoid (TT, 3000 Lf/ml) (Limes flocculation, International unit for vaccines), anti-tetanus serum (ATS), was obtained from the Serum Institute of India, Ltd., Pune. Mucin Type II (from porcine stomach), anti-horse Ig-G (whole molecule), sodium tripolyphosphate (TPP), were purchased from Sigma-Aldrich (USA). Bovine serum albumin cohn fraction V (BSA), sodium potassium tartrate (SPT), basic fuchsin as well as all other chemicals and reagents used for various analyses were of analytical grade and purchased from HiMedia Laboratories, Mumbai, India. Detergent compatible protein assay (DC) kit was obtained from Bio-Rad, USA.

2.2. Preparation of chitosan microparticles

Chitosan placebos, as well as protein encapsulated microparticles were prepared by coacervation method. Briefly, 4 ml chitosan was taken from 2% chitosan solution and dispersed in 50 ml of Milli-Q water. The mixture was mechanically stirred at 1500 rpm for 20 min to prepare chitosan solution prior to crosslinking reaction. For the preparation of BSA and TT encapsulated chitosan microparticles, 3.50 mg/ml BSA and 1500 Lf/ml TT in 10 mM PBS (pH 7.4) individually were dissolved in chitosan solution. The crosslinking reaction was carried out by using TPP and SPT aqueous solution of concentration 0.035 M each. The SPT/TPP ionic co-crosslinkers used was an aqueous solution comprising seven times diluted 1:1 vol of 0.035 M SPT and 0.035 M TPP crosslinkers. The crosslinker and co-crosslinkers solution was added drop wise into the chitosan solution in all formulations through a syringe needle at a rate of 0.2 ml/min. The colloidal suspensions were stirred at the speed of 1500 rpm till pH 6.0 of the system. After crosslinking reactions, the colloidal suspension of chitosan microparticles was centrifuged at 1500 rpm for 10 min. The obtained pellets were separated and successively washed two times with acetone and ethanol by centrifugation to remove residual crosslinking agents, then dried in air for 24 h to collect the final products. The prepared microparticles were designated as C1 for TPP crosslinked placebo microparticles, C2 for TPP crosslinked BSA encapsulated microparticles, C3 for TPP crosslinked TT encapsulated microparticles, C4 for SPT crosslinked placebo microparticles, C5 for SPT crosslinked BSA encapsulated microparticles, C6 for SPT crosslinked TT encapsulated microparticles, C7 for SPT/TPP crosslinked placebo microparticles, C8 for SPT/TPP crosslinked BSA encapsulated microparticles and C9 for SPT/TPP crosslinked TT encapsulated microparticles.

2.3. Crosslinking degree

The degree of crosslinking of the chitosan microparticles was determined by ninhydrin (NHN) assay [33]. The assay determines the percentage of free amino groups remaining in the microparticles after crosslinking. For the assay, 1.5 mg sample was heated to 100 °C in the water bath with 1 ml ninhydrin solution for 20 min. The solution was cooled down to room temperature, diluted with 50% isopropanol, and then the optical absorbance of the solution at 570 nm was read with a spectrophotometer (V-630, UV–vis Spectrophotometer, Japan). 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 of free NH_2 groups in the sample was determined from a standard curve of glycine concentration (5–25 μg/ml, $r^2 = 0.998$). The concentration measured was divided by sample weight, and multiplied by the sample molecular weight to obtain the mole NH_2/mole sample. The degree of crosslinking of the sample was then calculated using the following the equation:

$$\text{Degree of crosslinking} = \frac{(\text{NHN reactive amine})\,\text{fresh} - (\text{NHN reactive amine})\,\text{fixed}}{(\text{NHN reactive amine})\,\text{fresh}} \times 100 \quad (1)$$

where ‘fresh’ is the mole fraction of free NH_2 in non-crosslinked samples and ‘fixed’ is the mole fraction of free NH_2 remaining in crosslinked samples. Three samples of each type of microparticles were evaluated.

2.4. Encapsulation efficiency

Encapsulation efficiency of BSA was evaluated by reported procedure [34]. The amount of BSA entrapped in C2, C5 and C8

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