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Novel nanoparticles based on chitosan-dicarboxylate conjugates via tandem ionotropic/covalent crosslinking with tripolyphosphate and subsequent evaluation as drug delivery vehicles

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ABSTRACT

Chitosan-based nanoparticles prepared by ionotropic gelation are prone to stability issues. The aim of this work is to chemically modify chitosan by grafting to succinate, phthalate, glutarate and phenylsuccinate moieties and to investigate the suitability of the resulting polymers as covalently-crosslinked nanocarriers. Corresponding nanoparticles (NPs) were formulated by ionotropic gelation using tripolyphosphate (TPP) anion then they were covalently crosslinked using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). Infrared and thermal analysis confirmed the formation of phosphoramide bonds within the NPs indicating the involvement of TPP in covalent crosslinking. This is the first time to report phosphormide covalent crosslinking within nanoparticles matrices. The resulting NPs were found to resist drastic pH and calcium ion conditions. Size analysis indicated the NPs to be spherical and less than 500 nm in diameter. Loading studies using Safranine O showed enhanced NPs drug loading upon covalent crosslinking compared to ionotropic gelling. Doxorubicin-loaded NPs were of superior cytotoxic properties compared to free doxorubicin.

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1. Introduction

Chitosan is a linear polyaminosaccharide obtained by partial N-deacetylation of chitin (Fig. 1A). The polymeric hydroxyl and amino groups of chitosan confer hydrophilic character on this natural polymer and provide many options for chemical modifications (Zhang et al., 2008). When compared to other polymers, chitosan offers many advantages for the pharmaceutical industry, including its biocompatibility, biodegradability, safety, bacteriostatic, and muco-adhesive properties (Badwan et al., 2015; Bansal et al., 2011; Kumirska et al., 2011; Park and Kim, 2010). Chitosan is used as pharmaceutical excipient in tablets, granules, beads and microparticles (Bellich et al., 2016). One drawback of using chitosan in pharmaceutical preparations is its poor long-term stability as a result of gradual chain degradation (Szymańska and Winnicka, 2015).

To improve chitosan's stability and fine-tune its physicochemical properties, researchers took advantage of the primary amino and hydroxyl groups in the glucosamine units of chitosan to generate diverse chitosan derivatives. Examples on such modifications include: chitosan succinate (Aiedeh and Taha, 1999), carboxymethyl-chitosan (Zhao et al., 2011; Anitha et al., 2012), thiolated chitosan (Zhu et al., 2012), quaternary ammonium chitosan (Li et al., 2015; Mahjub et al., 2014), hydroxamated chitosan succinate (Aiedeh and Taha, 2006), chitosan-lactate-phthalate (Al Bakain et al., 2015), chitosan triethylene glycol phthalate (Taha et al., 2000), and PEG-grafted chitosan (Zhang et al., 2010).

Chitosan-based nanoparticles (NPs) attracted attention in the pharmaceutical industry owing to chitosan's safety and adaptability. Ionotropic gelation is one of the most investigated methods in chitosan NPs formulation. It is based on electrostatic interaction between cationic amine groups of chitosan and negatively charged

Abbreviations: $CaCl_2$, calcium chloride; DSC, Differential Scanning Calorimetry; DLS, dynamic light scattering; DOX, doxorubicin; EDC, *N*-Ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochlorides; FT-IR, Fourier Transform Infrared Spectroscopy; HCl, hydrochloric acid; LC, loading capacity; NPs, nanoparticles; PDI, polydispersity index; TPP, sodium tripolyphosphate; TEM, transmission electron microscopy; SO, safranin O; SD, standard deviation; SEM, standard error of the mean.

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polyanions such as sodium tripolyphosphate or dextran sulfate (Calvo et al., 1997; Katas et al., 2013). Advantages of ionotropic gelation include its relative simplicity, facile conditions, fast gelation and avoidance of organic solvents and high temperatures, thus allowing the successful encapsulation of fragile molecules like enzymes, vaccines and DNA (Zhao et al., 2016; Islam and Ferro, 2016). Still, despite its many advantages, ionotropic gelation suffers from some noticeable pitfalls, including: (i) Drug loss during NPs preparation, (ii) decreased control on matrix permeability, (iii) low mechanical strength, (iv) initial burst release, (v) tendency to aggregate and precipitate in response to subtle changes in pH and ionic strength, and (vi) the NPs can be rather large with wide polydispersity index (Katas et al., 2013).

Covalent crosslinking (e.g., using glutaraldehyde, genipin, glyoxal, etc) has been used to generate functionalized NPs by tethering functional groups to NP surfaces (Subbiah et al., 2010). However, only limited number of reports described the application of covalent crosslinking to stabilize NPs (Cadinoiu et al., 2012; Moraru et al., 2014; Soliman et al., 2014).

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is a water soluble carbodiimide used to couple carboxylic or phosphoric acids with amino groups to form amides or phosphoramides, respectively (Everaerts et al., 2008; Chen et al., 2015; Chu et al., 1983). EDC is widely used in fabricating different nanosystems implemented in drug/protein delivery and for surface crosslinking/immobilization of drugs onto NPs (Jafary et al., 2014; Jung et al., 2014; Shen et al., 2009).

We were prompted by the apparent pitfalls of ionotropic chitosan NPs to chemically graft chitosan with 4 dicarboxylic acids (succinic, phthalic, glutaric and phenylsuccinic) and prepare ionotropic NPs from the resulting derivatives using TPP. The resulting NPs were subsequently treated with EDC to affect covalent crosslinking within their chitosan matrices. Interestingly, infrared and DSC evidences indicated that TPP ions acted as covalent bridges across chitosan polymeric chains. This is the first time to report the dual use of TPP for ionotropic and covalent crosslinking in chitosan-based NPs. The resulting NPs were characterized *vis-à-vis* their stabilities (under different pH conditions and in the presence and absence of calcium ions), drug loading and release profiles using Safranin O (SO, Fig. 1B) and doxorubicin (DOX, Fig. 1C) as model drugs.

2. Materials and methods

2.1. Materials

Low molecular weight and medium molecular weight chitosans were purchased from Sigma-Aldrich (USA). High molecular weight chitosan was kindly provided by Jordan Pharmaceutical Manufacturing (JPM) Company (Na'or, Jordan). Pyridine, absolute ethanol and acetone were all of analytical grade and were purchased from Carlo Erba (France). Succininc, glutaric and phenylsuccinic anhydrides were purchased from Sigma Aldrich (USA) while phthalic anhydride was purchased from Fluka (Switzerland). Sodium Tripolyphosphate penta basic (TPP) was purchased from Sigma-Aldrich (Germany). Safranin O (SO) was purchased from Loba Chenie (India), Doxorubicin HCl (2 mg/ml) from Ebewe (Austria). *N*-Ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochlorides (EDC) from Sigma-Aldrich (Japan). TRIS buffer (Trizma® base) from Sigma-Aldrich (Germany). Hydrochloric acid (37%) from Carlo Erba (Spain) and sodium hydroxide from Scharlau (Spain). Aqueous solutions for size analysis were prepared using Milli Q water from Millipore Systems (USA) with conductivity less than 0.2 μS/cm. For other purposes deionized water was used. Dialysis tubing (for removing crosslinking byproducts and for loading and release studies) of a molecular weight cutoff range = 12–14 kDa was purchased from Medicell International Ltd. (UK). Human breast cancer cell line MCF-7 was provided from Faculty of Medicine (University of Jordan). CellTiter Non-Radioactive Cell Proliferation Assay Kit® from Promega (USA) was used to perform the MTT assay. RPMI 1640 medium and fetal bovine serum (FBS), L-glutamine, and penicillin–streptomycin were purchased from HyClone (USA). All chemicals used in this study were used as received without pretreatment or purification.

2.2. Preparation of NPs

2.2.1. Synthetic modifications of chitosan and preparation of corresponding NP

Different chitosan-dicarboxylic acid derivatives (i.e., chitosan-succinyl amide, chitosan-glutaryl amide, chitosan-phthalyl amide and chitosan-phenylsuccinyl amide) were prepared as previously reported with slight modifications (Aiedeh and Taha, 1999) as in

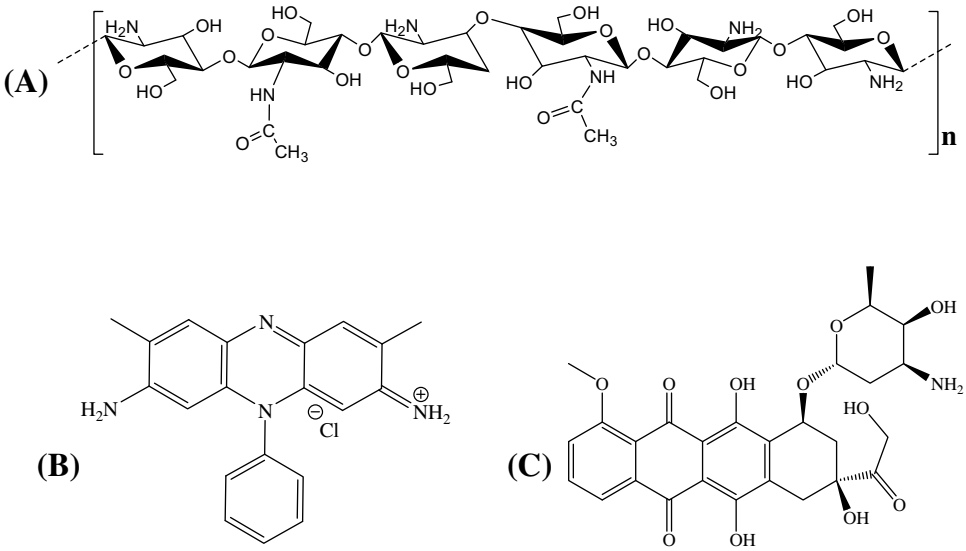

Fig. 1. Chemical structures of **(A)** chitosan, **(B)** Safranin O, **(C)** Doxorubicin.

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