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A novel serum-stable liver targeted cytotoxic system using valerate-conjugated chitosan nanoparticles surface decorated with glycyrrhizin

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ABSTRACT

The aim of this study was to target a naturally chemotherapeutic agent: ferulic acid to the liver using a biocompatible and an *in vivo* stable carrier. Accordingly, chitosan as a biopolymer was modified using a hydrophobic moiety and valeric acid in order to increase its *in vivo* stability. The structure of the newly synthesized product was confirmed using FT-IR and NMR techniques together with the ninhydrin assay. Ferulic acid was conjugated to the modified nanoparticles that were further characterized for particle size, PDI and zeta potential and subjected to *ex vivo* stability study in serum and cytotoxicity studies in HepG2 cell lines. Furthermore, the nanoparticles were surface-decorated with glycyrrhizin for active liver targeting. The *in vivo* biodistribution was experimented using radiolabeling assay where the liver scored the highest accumulation of the glycyrrhizin containing nanoparticles after 6 h reaching a value of 13.34% ID/g of the total injected dose of labeled drug compared to drug solution and glycyrrhizin free nanoparticles where the accumulation percent did not exceed 4.19% ID/g and 4.26% ID/g, respectively. As a conclusion, the conducted physico-chemical and biological investigations suggested that the proposed selected system can be efficiently utilized as a successful platform for targeting a natural chemotherapeutic agent *viz.* ferulic acid to the liver.

1. Introduction

Over the years, chitosan (Cs) has been extensively used as a promising drug carrier in the biomedical field. The great attention granted to this biopolymer is related to its interesting properties being cationic, biodegradable, biocompatible, non-toxic and non-immunogenic (Kumar et al., 2016). Besides, chitosan has some biological effects as antioxidant, anti-allergic, anti-inflammatory, anticoagulant, anti-bacterial and anti-obesity (Ngo et al., 2015). Notable studies have been carried out on chitosan modifications with the aim to functionalize the valuable biopolymer characteristics (Chiu et al., 2009; Huang et al., 2011; Millotti et al., 2014; Pereira et al., 2015). The reactive amino and hydroxyl groups in chitosan structure can be chemically derivatized under mild reaction conditions. Hydrophobic modification is a promising method used to prepare chitosan derivatives with superior blood compatibility. Hirano and Noishiki (1985) presented a study regarding the blood compatibility of chitosan and its acyl derivatives where the later showed improved *in vivo* hemocompatibility. After ten years, K.Y. Lee et al. (1995) also reported that N-acylated chitosan derivatives (N-valeric and N-hexanoic) showed delayed coagulation time and better antithrombogenicity than native chitosan. This strategy is of great importance especially with growing attention to use chitosan based nanomaterial for intravenous administration.

The enormous vital functions performed by liver make it one of the major vital organs in the body. Liver is responsible for maintaining continuous supply of glucose and lipids,

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detoxification and excretion of xenobiotics, secreting bile and storing vitamins and other essential materials. These tasks expose the liver to microbes, drugs and other toxic material, which could subject this organ to many diseases. Liver cancer is the sixth most common malignancy and is second to lung cancer in mortality associated with prevalence of hepatitis B, viral infection, alcohol consumption and other causes of liver cirrhosis (Reddy and Couvreur, 2011). Hepatocellular carcinoma (HCC) is the major pathological type of liver cancer and accounts for approximately 80% of cases (Cheng et al., 2016). Chemotherapy is the second line treatment after surgery but it is limited due to chemo-resistance, inadequate specificity and safety. Nanomaterial-based medicine can offer a great potential in delivering drugs to the target sites either passively or actively. Passive targeting can be mediated by the production of nanoparticles of sizes below 200 nm that can penetrate 100–200 nm fenestration in the endothelial wall of liver sinusoids (Li et al., 2010). Active targeting depends on attaching drugs to ligand that can interact with liver associated receptors and internalize into cells. The liver consists of hepatocytes (hepatic parenchymal cells), Kupffer cells and endothelial cells (hepatic nonparenchymal cells), where hepatocytes are the type of cells implicated in the development of liver cancer. Many studies have proved the presence of glycyrrhizin (GL) receptors on the surface of hepatocytes (Negishi et al., 1991). Tsuji et al. (1991) prepared unilamellar liposomes surface modified with glycyrrhizin that showed 4-fold accumulation in liver 4 h after intravenous administration than control unilamellar liposomes. The natural origin of GL, extracted from the root of *Glycyrrhiza glabra* (licorice), encouraged many researcher to use it and its aglycone derivative as liver targeting moiety (He et al., 2010; Tian et al., 2010, 2012; Huang et al., 2010; Shi et al., 2012; Mishra et al., 2014; Chopdey et al., 2015; Lin et al., 2008, 2009).

Ferulic acid belongs to a group of phenolic phytochemical, widely abundant in foods as grain, bran, citrus fruits, banana, coffee and broccoli. It is a promising nutraceutical with a well-documented antioxidant activity that can be used in treating various disorders as Alzheimer's disease, cardiovascular disease, diabetes and cancer (Zhao and Moghadasian, 2008; Ou and Kwok, 2004; Kumar and Pruthi, 2014). Its anticancer effect against breast cancer, colon cancer (Kawabata et al., 2000), skin cancer and liver cancer was previously reported in the literature (Vashisth et al., 2015). This antiproliferative effect may be explained by the ability of ferulic acid to modulate cell growth and proliferation, scavenge reactive oxygen species (ROS) and stimulate cytoprotective enzymes (Barone et al., 2009). Ferulic acid enhanced the cytotoxicity of paclitaxel *via* downregulation of p-gp (p-glycoprotein) expressing pump in multidrug resistant KB Ch^R 8-5 cell lines (Muthusamy et al., 2016). Also, it potentiated the UV irradiation effect on breast cancer by further reducing the S phase (Park, 2016).

To this end, the current work aims at developing modified chitosan nanoparticles (NPs) capable of targeting hepatocytes passively and actively. Chitosan was hydrophobically modified with a five-carbon chain length (valeric) moiety. Ferulic acid was selected for its potential cytotoxic effect. Different NPs fabrication variables were studied. The passive and active targeting methods were achieved by size adjustment or by using glycyrrhizin (GL) as a targeting ligand, respectively. Moreover, the *in vitro* cytotoxic effect of selected formulae as well as the *in vivo* drug biodistribution in mice was assessed.

2. Materials and methods

2.1. Materials

Chitoclear®, low molecular weight chitosan (viscosity <25 cps, deacetylation degree: 95% according to the data sheet of the manufacturer) was kindly obtained from Primex®, Siglufjordur, Iceland. Valeric anhydride, *trans*-Ferulic acid (Fer), glycyrrhizic acid ammonium salt (glycyrrhizin, <70%) (GL), ninhydrin reagent (2% solution), sodium tripolyphosphate (TPP), sodium periodate, poloxamer 407 (pluronic® F127), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), glacial acetic acid and ethanol were purchased from Sigma–Aldrich Chemical Co. Ltd., St. Louis, USA. Sodium hydroxide, hydrochloric acid and all other chemicals (analytical grade) were purchased from El Nasr Pharmaceuticals, Cairo, Egypt. All aqueous solutions were prepared using high-purity deionized water with conductance less than 1 $\mu S\,cm^{-1}$ (18.2 $M\Omega\,cm$). Spectra/Por® dialysis membrane, 12,000–14,000 molecular weight cut-off was purchased from Spectrum Laboratories Inc., Rancho Dominguez, Canada. Nalgene® Millipore filters of pore sizes 0.20, 0.45 and 0.80 μm and methanol (HPLC) were purchased from Thermo Fisher Scientific, Waltham, MA, USA.

2.2. Purification of chitosan

Chitosan powder was dissolved in 1% (v/v) aqueous acetic acid, in order to prepare 1% (w/v) solution, and stirred overnight (Heidolph, Schwabach, Germany) for complete dissolution. After, the solution was vacuum filtered using 0.8 m filter to remove insoluble matters. Chitosan precipitation was achieved by raising the pH to 8.5, using 4 N NaOH, which was then collected by centrifugation at 6000 rpm for 15 min. The precipitate was dispersed in deionized water and dialyzed against deionized water for three successive days with fresh water replacement every day. Finally, purified chitosan (PC) was collected and lyophilized (Christ, Osterode amHarz, Germany) (Abdel-Hafez et al., 2014).

2.3. Modification of chitosan

N-acylation of chitosan was done according to a previously reported method (Lin et al., 2009; K.Y. Lee et al., 1995). Briefly, 6 g of purified chitosan (PC) was dissolved using 300 ml 1% aqueous acetic acid solution under stirring overnight. Subsequently, 300 ml methanol was added portion wise. A solution of 150 ml of 2% valeric anhydride in methanol was added to the previous mixture. It is worth pointing out, that the feeding molar ratio of chitosan to valeric anhydride was 1:0.5. The reaction was performed twice for two different time periods (40 min and 4 h) to obtain two different modified polymers. The two polymers were coded MC and HC for 40 min and 4 h reaction respectively. Then the solution was filtered and washed thoroughly using methanol and diethyl ether. The slurry was subsequently dispersed in deionized water and dialyzed against deionized (1:1) water:methanol mixture for two successive days with replacement of the deionized water:methanol mixture every day. Finally, the modified chitosan was filtered once more and air dried for three days.

2.4. Determination of degree of N-substitution of valerate modified chitosan using ninhydrin assay

The ninhydrin assay was utilized to quantify the remaining unsubstituted amino groups after modification as described by K.Y. Lee et al. (1995), Curotto and Aros (1993) and Le Tien et al. (2003) with slight modification. An aliquot of 10 mg chitosan (native or modified) was dissolved in 50 ml 1% aqueous acetic acid solution. Acetate buffer (pH 5.5) was added to different volumes (250, 300, 350 and 400 μl) of the previous chitosan solution to make a final volume of 0.5 ml. Then, 2 ml of the ninhydrin reagent was added and the tubes were placed on a boiling water bath for 20 min. After cooling, 2.5 ml of (1:1) ethanol:water mixture was added while