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ORIGINAL RESEARCH PAPER

Preparation and evaluation of nanoparticles composed of thiolated methylated pyridinyl chitosan as a new strategy for bucal drug delivery of insulin

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ABSTRACT

Objective(s): The purpose of this study is about the preparation and evaluation of nanoparticles composed of thiolated methylated pyridinyl chitosan (TMPC) which are made P.E.C method, for bucal drug delivery of insulin.

Materials and Methods: First of all have to synthesize methylated pyridinyl chitosan and then for (TMPC) L-cysteine should be attached to methylated pyridinyl chitosan by the formation amid bounds. After of synthesis the (TMPC) nanoparticles were made and insulin loaded on them. The percentage of entrapment efficiency which was calculated for a nanoparticle which loaded by insulin is about to 91+2.6%.

Results: The release of insulin from nanoparticles was studied in vitro in Phosphate buffer solution (PBS) pH 6.8. After 240 minutes 71.3% of insulin released from (TMPC) and after 360 minutes 72.9% of insulin was released. By considering the specifications of nanoparticles which leads us to this result that the Zeta potential is 28.3 mv, poly dispersity index (pdi) is 0.33 and the size of the particles is about to 268 nm.

Conclusion: this study suggests that thiolated methylated pyridinyl chitosan can act as a potential enhancer for bucal delivery of insulin.

Keywords: *Bucal, Chitosan, Nanoparticles, Pyridinyl ,Thiolated*

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INTRODUCTION

There are many studies that consider about Insulin through bucal cavity. The main problem for the drug delivery through bucal cavity is the structure of mucosa in this area that cause poor absorption of Insulin. Different strategies used to prevalence to this problem, nanoparticulate delivery system can be a part of the solution and enhance drug absorption through mucus [1-3]. Utilization of mucoadhesive polymers such as Chitosan could increase the drug delivery through bucal cavity. Chitosan is a cationic polymer that formed from repeated unit of di glucose amine and N- acetyl glucose amine. It has been exploitation enormously in drug delivery systems due to its biodegradability, biocompatibility, permeation enhancing effects and non-toxicity [4-6]. Different derivates of chitosan have been synthesized, for example: triethyl chitosan (TEC) [7] and trimethyl chitosan (TMC) [8]. It has been proved that thiolated derivate of chitosan based on the immobilization of thiol bearing moieties on the backbone of chitosan resulted in promotion of mucoadhesion and permeation enhancing effect of chitosan due to

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formation of covalent disulfide bonds between the thiol group of chitosan and cysteine-rich subdominants of mucus glycoproteins because of disulfide exchange that leads to a gradient of drug concentration at the absorption site. Different derivatives of thiolated chitosan have been synthesized, such as chitosan cysteine [9], chitosan thiobutyl amidine [10] and chitosan thioglycolic acid [11].Thiolated chitosan make positively charge because of hydrophilic nature due to the presence of thiol groups with cationic properties on their side chains and this cationic charge lead to substantial electrostatic interaction with negatively charged molecules like DNA and also it can be used to deliver drugs or gene. Traditional methods of producing nanoparticles are very divergent and contain interfacial polymerization, emulsion polymerizations and solvent evaporation. These methods have disadvantages such as; heat, organic solvents residue, and strong agitation which can be harmful for sensitive biomaterials. In polyelectrolyte complexation (PEC) method can be a good approach to produce nanoparticles by self-assembly between oppositely charged polymers and drugs. The nanoparticles that synthesized by (PEC) method, make colloidal suspensions which have more homogenous dispersity and stability. Another advantage of this type of preparation is elimination of sonication and organic solvent. Also, (PEC) method can reduce the risk of drug degradation during the nanoparticle preparation [12]. In this study at the first step thiolated methylated of pyridinyl chitosan was synthesized then by using the (PEC) method, insulin nanoparticles were prepared and evaluated.

MATERIALS AND METHODS

Materials

Medium molecular weight of chitosan (95 % degree of deacetylation) was purchased from Primex (Iceland). 4-pyridinile carbaldehide, N-methyl pyrrolidone (NMP), sodium chloride, sodium borhydride,acetic acid, sodium hydroxide, methyl iodide were purchased from Merck (Darmstadt, Germany).

1-ethyl-3-(3- dimethylaminopropyl) carbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS) and L-Cysteine hydrochloride (Cys) were purchased from Sigma (St. Louis, Mo, USA). Insulin was obtained from Exir Pharmaceutical (Lorestan, Iran). Dialysing tube with a molecular cutoff of 12 kDa (D0530) was obtained from Sigma. Even though there are many other spectrometers including C-NMR and N-NMR, hydrogen (H-NMR) was the first and is the most common atom used in nuclear magnetic resonance spectroscopy.

synthesis of methylated pyridinyl chitosan

Methylated pyridinyl chitosan was synthesized as previously described [13]. In this study there is some modification. Briefly, Chitosan (1g) was dissolved in 1% acetic acid (100 ml). When it was dissolved completely 4-pyridinile carbaldehide added and the solution stirred for 24 hour at room temperature. The pH of the solution was adjusted to 4.5 by adding 1 N NaOH , the compounds were reduced by freshly prepared gaseous hydrogen prepared by gradual addition of sodium borohydride as the powder to the solution and keeping the solution to be stirred for a day at ambient temperature . Derivatives were precipitated from the solution by adding 1 N NaOH and adjusting the pH to10. The precipitates were then washed with methanol three times and dried. In next step NMP added to above sediment by the same weight and stirred for 6 hour. Above solution should reached to 55^0C under condenser and 5cc methyl iodide added each hour for 3 hours and stirrer for a day to have a pure solution with no sediment in it. In next step of process derivatives were precipitated from the solution by adding 500cc acetone and precipitate filtered by Bchner funnel and then dried by desiccators.

Synthesis of thiolated methylated pyridinyl chitosan

For thiolation of methylated pyridinyl chitosan we should attach, L-Cysteine (Cys) to methylated pyridinyl chitosan by the formation of amide bonds between the primary amino group of methylated pyridinyl chitosan and carboxylic acid group of Cys. At the first step we should dissolved, 2 g of Cys in 20 ml of demineralized water in order to activate of carboxylic acid group of Cys by adding (EDAC) and NHS achieving final concentration of 200 mM .Then 1g of above solution should be added after 2 hours stirring at room temperature and adjust PH to 5 by using 1M NaOH.

The environment of process should be incubated for 5h at room temperature without light. The produced thiolated methylated pyridinyl chitosan was purified by dialyzing at 4°C against aqueous HCl

for 5 days. Polymer solutions should lyophilize and stored at 4°C until use. [14]

Preparation of nanoparticles

In this study, insulin nanoparticles were prepared by PEC method[15]. For this derivative, ionic interaction was established between negatively charged insulin and positively charged polymer as a result insulin was trapped in polymer chains. Insulin was dissolved in pH 2.0 HCl and pH of insulin solution was adjusted to 8.0 by using 1 M NaOH to create the negatively charged. The polymer was dissolved in demineralized water and the pH of polymer solution was adjusted to 4 by using 1% acetic acid. In the next step both of solutions should be filtered by 0.45 m separately. Thereafter, the insulin solution was added drop wise to the equal volume of the polymer solution .These nanoparticles were centrifuged at 12,000 rpm for 20 min at 4^0 C. The supernatant was separated and analyzed by HPLC for computing the amount of entrapment efficacy and nanoparticles were resuspended in demineralized water.

Characterization of insulin nanoparticles

Determination of particle size and zeta potential

Laser Doppler anemometry and photon correlation spectroscopy respectively used to measure zeta potential and particle size. Utilizing a zetasizer 3000hs (Malvern Instruments, Malvern, UK) at 25 ÚC. The particle size distribution is reported as PdI. All the tests were completed in triplicate, and responses were reported as mean ± SD.

Determination of EE % of the nanoparticles

Indirect method was used for calculation of %EE (insulin encapsulation). In this method sample should be centrifuged and the supernatant of samples were injected to HPLC, the supernatant including non-encapsulated insulin, so by measure amount of free insulin were determined according to equation 1 we can calculate %EE.

$$\text{Loading Capacity} = \frac{(\text{The total amount of insulin}) - (\text{The amount of insulin in the ruby liquid})}{\text{Weight nanoparticles}}$$

As described previously by Dorkoosh et al (16), 20 micro liters of each sample were injected to Agilent 1260 infinity equipped with, 1260 Quat pump VL and 1260 DAD VL detector, required column is C18 (perfectSil 150*4.6 mm, 5 m. the mobile phase was composed of 30 % acetonitrile and 70 % buffer containing 0.1 M KH_2PO_4 and 1 % triethylamine adjusted to pH 2.8 with phosphoric acid. The flow rate is 1 mL/min, by 10 min run time and the wavelength were 214 nm. All samples were measured in triplicate.

In vitro release study

In vitro release study of insulin nanoparticles was carried out in PBS pH 6.8 which is representing the simulated environment of the oral mucosa. For this study, 20 mg of lyophilized nanoparticles were added to a tube containing 10 ml of PBS. The tube was situated in shaker incubator and temperature was set at 37± 0.5^0C. In a predetermined time intervals, 1 ml of release medium was aliquot and replaced with the same volume of fresh release medium preheated at 37^0 C. The amount of insulin determined by HPLC for each of samples. All samples were done in triplicate.

RESULTS

Characterization of thiolated methylated pyridine chitosan

Characterization of methylated pyridine chitosan

The ^{1}H-NMR spectrum of synthesized chitosan was recorded in Fig1. As shown in spectrum peaks were assigned uniquely. The sharp signal appearing at 4.8 ppm is attributed to D2O. The signal at 2.8 and 3.3 ppm represent methyl protons of

("N (CH_2)–) and ("N– $(CH_3)_2$), respectively. In 8.8ppm and 7.8ppm there is two duplicated peak that refers to proton of ("N=CH") and("C"CH"C") of pyridine respectively. Area under curve of aliphatic region at 4.2ppm to 3.7ppm used to calculate degree of quaternization (DQ) of the polymer (table 1). The SEM images related to the lone nanoparticles are shown in Fig 2.

Table 1. Characteristic of chitosan and synthesize area under curve of aliphatic region area under curve of aliphatic region area under curve of aliphatic region d derivatives

polymer	DQ (%)	–SH (µmol/g)	–S–S– (µmol/g)	Zeta potential (mV)
Methylated pyridinyl chitosan	14.48	-	-	28.3
Thiolated methylated pyridinyl chitosan	-	265	97	28.3
chitosan	-	-	-	18

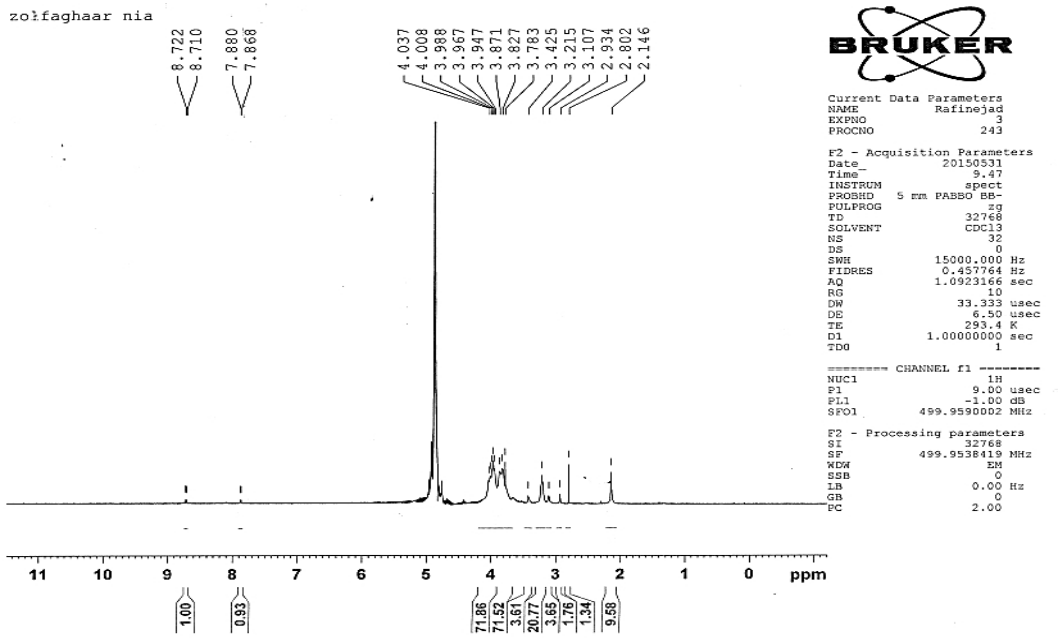

Fig.1. H-NMR spectrum of methylated pyridine chitosan

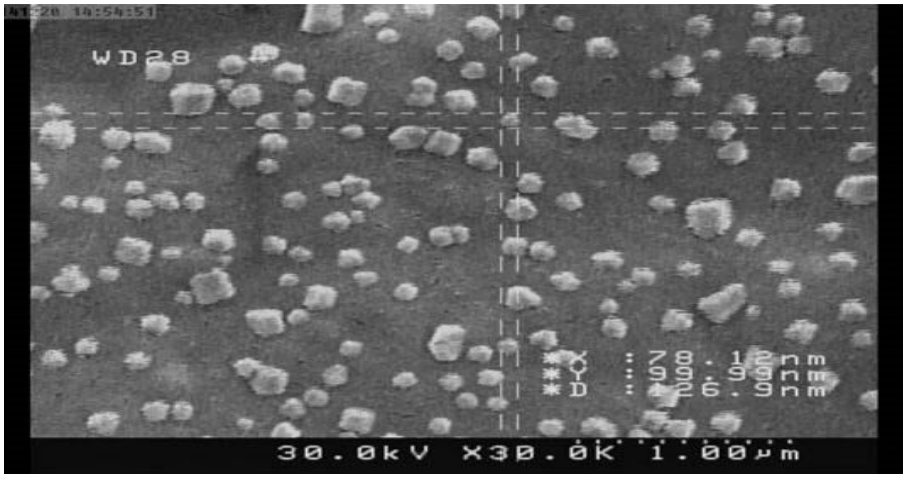

Fig .2. morphological view of nanoparticles in SEM

Characterization of insulin nanoparticles

Characterization of insulin nanoparticles was summarized in Table 2.

In vitro release study

The experiment was carried out in PBS pH = 6.8 and in 37±0.5^{0}C. Cumulative release of insulin from optimized TEC-Cys nanoparticles is described in Fig. 3. Burst release of insulin in first 30 minute was observed.

It could be depends on the method for preparation of nanoparticles [17].

DISCUSSION

Low solubility of plain chitosan and inability to open the tight junctions' shows requires modifying derivatives of chitosan.

Triethylated derivate of chitosan has been synthesized. Using traditional method could cause methylation in C3 and C6 hydroxyl group of chitosan

Table 2.Characteristics of nanoparticles

Size(nm)	Zeta potential (mV)	Pdi	EE%
268.0±23.9	28.3±0.95	0.33 ±0.05	91.0±0.8

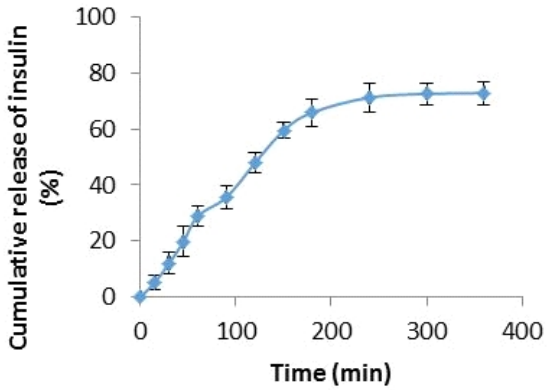

Fig .3. Cumulative release of insulin

so affecting the physicochemical properties of polymer. It is necessary to avoid from such reactions. As described previously by Mortazavian et al. [20], by omitting NaOH and NaI during alkylation process, ratio of o-alkylation/ n-alkylation was reduced significantly. Thiolation of methylated pyridinyl chitosan with cysteine could change the properties of permeation enhancing effect which will be explained in further. Amide bond formed by the reaction between primary amine of chitosan and carboxylic acid group of cysteine. At pH above 5, thiol group of chitosan oxidized and turned to S-S anions. This oxidized thiol group could form inter and intra disulphide bonds. So it could be presumed that pH value of thiolated polymer is a very important factor [18].In other hand formation of covalent bond between cysteine-rich sub domain of mucus layer and thiol group of polymer could increase mucoadhesion properties of polymer. As previously describe by Hornof et al [19-20], with thiolation of medium molecular weight chitosan, 100 times as many as plain chitosan in mucoadhesion. Furthermore, previous studies has been shown the effect of thiolated polymers in oral and bucal delivery of peptides, for example, in vivo experiment by Thanou [21] has been demonstrated that intestinal absorbance of buserelin (a peptide drug), could increase in rat by applying thiolated chitosan. Thiol group of polymer is unstable in presence of light and it is important to keep away from such conditions that could oxidize it rapidly. Nanoparticles seem to be appropriate carriers to improve biological effects and to protect sensitive drugs like insulin. For preparation of nanoparticles various methods have been investigated including poly electrolyte complexation (PEC) and iontoropic gelation method. PEC method appears to be more utilized in preparation of insulin nanoparticles than iontoropic gelation due to simple procedure for preparation of insulin nanoparticles. By using PEC method, nanoparticles with higher zeta potential and insulin loading could achieve [22]. Interaction between positively charged polymer and negatively charged insulin could explain high loading efficacy of obtained nanoparticles. Furthermore, PEC nanoparticles executed admissible impact on particle size, zeta potential, encapsulation efficacy and the release of insulin. Also lower PdI and smaller particle size have been achieved by PEC method.

Zeta potential could be raised by increasing chitosan concentration. This could be due to presence of positively charged amino groups. As it shown in table 2, quaternized result higher zeta potential. Because of formation of inter and intra disulphide anion bonds, thiolated chitosan displays lower zeta potential than plain chitosan.

Also lower pH of polymer, could lead protonation of free amino group and as a result zeta potential of nanoparticle will be increased. By increasing concentration of polymer, encapsulation of insulin could occur; therefore higher EE% could achieved. [23] Release study has been performed in PBS pH 6.8 in which both insulin and polymer have poor solubility. Behavior of insulin release can be affected by ionic interaction between polymer and insulin. As it is found in this study, the small burst release substantiated a suitable interaction between polymer and insulin by using PEC method.

CONCLUSION

In this study, thiolated methylated pyridinyl chitosan has been synthesized as a new derivative of thiolated chitosan. Insulin nanoparticles were prepared though PEC method that achieving higher zeta potential and entrapment efficiency and smaller particle size and PdI. The in vitro result has demonstrated that insulin nanoparticles composed of

thiolated methylated pyridinyl chitosan has sustained release characteristics. However, ex vivo and in vivo experiments would be needed to establish the efficacy of this derivate of chitosan for bucal delivery of insulin.

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CONFIICT OF INTEREST

The authors declare that there are no conflicts of interest.

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