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Enzyme coated beta-cyclodextrin for effective adsorption and glucose-responsive closed-loop insulin delivery

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ABSTRACT

Inconsistent dosage of insulin (INS) for type 2 diabetes patients lead to severe adverse effects like limb amputation, blindness and fatal hypo or hyper glycaemia. Hence, a drug delivery system (DDS) capable of consistent INS release by sensing changes in blood glucose level is essential. Herein, we report a glucose responsive DDS comprised of oleic acid-grafted-aminated beta cyclodextrin (OA-g-ACD) copolymer, coated with a dispersion of glucose oxidase (GOx) and catalase (CAT). The prepared DDS was characterised using FTIR, Optical Microscopy, H^1 NMR, DLS and SEM. Hydrophobicity and drug loading capacity was ascertained using contact angle measurements and confocal laser scanning microscopy (CLSM) respectively. Extent of swelling was observed to be a function of glucose concentration. INS release profile showed a cumulative release of 78.0 % after 240 min. Flow cytometry studies revealed greater population of INS on HeLa cells indicating application of DDS as potential candidate for the intravenous administration of INS.

1. Introduction

Diabetes mellitus is a serious life style disorder associated with the modern world, and is characterised by high glucose level in blood. There are mainly two types of diabetes. Destruction of pancreatic β-cells leading to insufficient production of INS is the characteristic feature of type 1 diabetes. In type 2 diabetes, impaired INS resistance in addition to deficient INS production is observed. By 2020, diabetes mellitus is believed to be the sixth and fourth fatal disease respectively in developed and developing countries that would take the lives of more than 3.7% of a nation's population [1]. Even though sub-cutaneous treatment for diabetes was established since the discovery of INS by Banting et al. [2] the amount of INS dosing, repeated administration, patient compliance, *etc.* remains a concern. In addition, repeated self-administration without assessing glucose level in blood possess a high risk factor, and sometimes, the administered drug may act as a potential toxic. In such a scenario, hybrid systems having the ability to sense deficiency of glucose level and simultaneously trigger INS release is highly beneficial. These pancreas mimicking hybrid materials are capable of substituting pancreatic β- cells and are widely used for closed-loop INS delivery where changes in glucose level are continuously monitored and INS is delivered accordingly. Even though there are previous reports on such closed-loop INS delivery systems, efficient INS loading and sustained release are rarely achieved [3–6].

INS, being a hydrophobic drug, polymers should be designed so as to have maximum hydrophobicity. Cyclodextrins, are very promising candidates in this regard as they are characterised by a unique torus like structure having a hydrophobic cavity helping to interact with guest molecules and a hydrophilic exterior enabling them solubility in aqueous medium [7,8].

Depending on the number of glucose units as 6, 7 or 8, they are named as α-, β- or γ- cyclodextrin. Among these, β-cyclodextrin (β-CD) serves to be the most potential candidate for drug delivery due to its biocompatibility, improved bioavailability of drugs, non-toxicity, *etc.* [9,10]. However, the hydrophobic cavity formed by the α-1,4-linkages of the D-glucose units in β-CD is of the order of 0.60–0.65 nm [11] and demands to be improved for efficiently accommodating large protein chains like INS. In the present work, we tried to prepare a hydrophobic DDS by grafting oleic acid (OA) onto β-CD *via* carbodiimide chemistry. OA, by virtue of its long alkyl chain can induce greater hydrophobicity to the β-CD cavity. In addition, the structural integrity offered by the hindered rotation of the double bond present in OA makes it an ideal choice unlike its saturated hydrophobic analogues. We also relied on previous reports [12] which stated that protein adsorption could be improved by forming amide bonds.

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Thus by introducing OA to β-CD skeleton *via* an amide bond, we could improve the hydrophobic cavity thereby increasing encapsulation of INS. In order to impart acid sensitiveness, β-CD was initially modified to aminated β-CD (ACD). For glucose responsiveness, use of GOx is a common procedure. GOx converts glucose to gluconic acid along with undesired H_2O_2. In order to adsorb H_2O_2, CAT is commonly used as a complementary enzyme to GOx. Hence, after injection, whenever the proposed DDS is exposed to hypoglycaemic concentrations, the enzyme converts glucose to gluconic acid thereby decreasing the environmental pH of the polymer. On decreasing the pH, the amine groups get protonated and the electrochemical forces of repulsion operate, leading to the swelling of the polymeric matrix. As a result, the entrapped INS comes out of the matrix. The present work is the first to report a glucose sensitive microgel with appreciable drug loading efficiency which facilitates deswelling of the copolymer after drug release with the same solution used for swelling.

2. Materials and methods

2.1. Materials

β-Cyclodextrin (β-CD, 1135 kDa, CAS No. 7585-39-9) was procured from HiMedia laboratories, Mumbai. *p*-toluene sulphonyl chloride (*p*-TsCl, CAS No. 98-59-9) was purchased from Sigma Aldrich, USA. Triethylene tetramine (TETA, CAS No. 112-24-3) and 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC, CAS No. 25952-53-8) were obtained from Loba chemie, India and Tokyo Chemical Industry (TCl), Tokyo respectively. Oleic acid (OA, CAS No. 112-80-1) was obtained from Otto chemie, Mumbai. Triethyl amine (TEA) (CAS NO. 121-44-8), enzymes for coating namely – glucose oxidase (GOx, 319.0 U/mg activity, CAS No. 9001-37-0) and catalase (CAT, 15130.0 U/mg activity, CAS No. 9001-05-2) were purchased from Merck, Mumbai. Monocomponent human insulin (INS) of biosynthetic r-DNA origin used for the study was purchased from torrent pharmaceuticals, India. Fluorescein-5-isothiocyanate (FITC, CAS No. 3326-32-7), used for conjugating INS was purchased from Alfa Aesar, Germany. All the solvents used in the experiment were purchased from Merck and was used as received. Distilled water was used for all studies.

2.2. Equipments and methods of characterization

All the experimental steps were monitored using Cary 630 ATR spectrophotometer (model-Spectrum 400, USA) between 4000 and 650 cm^{-1} by direct sampling technique. The absorbance measurements of drug loading and release steps were performed on a JASCO-530 UV–Vis spectrophotometer (model-V 530, Japan) at λ_{max} = 214 nm, characteristic of INS. Proton NMR of the prepared copolymer was determined using a 400 MHz Bruker Avance NMR spectrometer in $CDCl_3$. Tetra methyl silane (TMS) was used as the internal standard. The chemical shifts are reported in ppm relative to TMS. The surface morphology of all samples was observed using a JEOL JSM 6390 LA scanning electron microscopy (SEM) conducted at room temperature *via* low vacuum mode. For determining the surface changes of the polymer and to confirm glucose responsiveness, SEM images after treating the samples with respective glucose concentrations for about 4 h were taken. Optical photographs of all samples were taken after sonicating the solutions for about 2 min. One drop of the sample solution was then mounted onto a glass plate previously washed with PBS. Images were then recorded using a MOTIC SMZ161 stereo microscope attached with an image capturing camera. FITC conjugated INS was prepared according to a previously reported procedure [13] and was entrapped into the polymeric matrix for taking Confocal Laser Scanning Microscope (CLSM, Olympus Co. Ltd., Tokyo, Japan) with imaging software, Fluoview FV500. DLS studies were performed on Horiba SZ-100 equipped with a 532 nm Diode Pumped Solid State (DPSS) laser, operated at a temperature of 25 °C. Prior to DLS analysis, all samples were dispersed using an ultrasonicator for definite periods of time for avoiding aggregation (PCi Electronics, Mumbai, 230 V, 50 Hz). Flow cytometry studies were performed with a Coulter Elite – ESP flow cytometer (Beckman-Coulter, France) using a 15 mW air cooled argon–ion laser tuned at 490 nm with a beam width of 86.9 μm. pH measurements were made on a μ processor Systronic pH meter (model 361). A temperature controlled water bath shaker (Lab line shaking incubator) with a temperature variation of ±10 °C was used for temperature controlled shaking studies.

2.3. Preparation of OA-g-ACD

2.3.1. Preparation of aminated β-CD

Amination was done by a previously reported procedure [14] by initially tosylating the secondary alcohol of β-CD and then aminating it with TEA/TETA. Briefly, 20 g β-CD (16.3 mmol) suspended in 150 mL water was reacted with NaOH (54.7 mmol, 2.16 g) in 7 mL water, added drop wise for 6 min. To the homogenous yellow solution, *p*-TsCl (3.36 g, 17.6 mmol) in 10 mL acetonitrile was added dropwise for 8 min to cause an immediate white precipitate. After stirring for 24 h at room temperature, the precipitate was removed by filtration and was cooled overnight at 4 °C to obtain tosylated β-CD. 4.0 g of the obtained tosyl β-CD was then dissolved in 30 mL water containing 20 mL (w/v) TEA and was reacted with 0.8 g (w/v) TETA. The solution was stirred and heated to reflux under nitrogen atmosphere for 24 h. After evaporation of under reduced pressure, the residue was poured into 500 mL vigorously stirred anhydrous ethanol (EtOH). The product then obtained was filtered, washed and freeze dried to obtain a yellow precipitate of aminated β-CD (ACD).

2.3.2. Preparation of OA-g-ACD copolymer

OA-g-ACD was prepared by reacting the primary amine of ACD with the —COOH of OA to form an amide linkage using EDC. The reaction was optimised for 1:2 molar ratio of acid to amine. Briefly, 1.0 g ACD was initiated with (52.16 mmol, 0.1 g EDC in 1 mL water) and was stirred for 4 h. To this solution, 10 g (w/v) OA in 50 mL EtOH was added and stirred for 20 h. The white polymer obtained was washed with EtOH and was dried at 4 °C overnight.

The % yield for the preparation of OA-g-ACD was determined using the following equation:

$$\%\text{Yield} = \frac{m2}{m1} \times 100 \quad (1)$$

Where 'm_1' is the weight of OA and ACD, 'm_2' is the weight of OA-g-ACD obtained. The mean of three repeated experiments is reported.

2.4. Preparation of INS loaded OA-g-ACD

Prior to loading, INS was dissolved in HCl (0.01 N, pH 2.0). pH of the solution was then adjusted to 8.0 using 1 M NaOH. To this, an equal volume of OA-g-ACD in methanol was added dropwise within 20 min at room temperature to yield a white opalescent solution. The resulting suspension was centrifuged at 10,000 rpm for 30 min. The supernatant was used for determining drug loading %, drug loading efficiency %, entrapment efficiency % and theoretical loading % as:

$$\text{Encapsulation efficiency } \% = \frac{\text{total amount of INS} - \text{free INS}}{\text{total amount of INS}} \times 100 \quad (2)$$