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Glutaraldehyde crosslinked and alkaline denaturation induced self association of haemoglobin to design nanocarriers for In vitro release of insulin in simulated gastrointestinal fluids (SGFs)

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ABSTRACT

Adopting a novel synthetic route the haemoglobin nanoparticles were prepared by its simultaneous micro-emulsion mediated crosslinking with glutaraldehyde and alkaline induced denaturation. The as prepared nanoparticles were characterized by techniques like Fourier transform infrared spectroscopy (FTIR), Transmission electron microscopy (TEM), Scanning electron microcopy (SEM), Zeta potential and Dynamic Light Scattering measurements. The particle size analysis revealed that size of the nanoparticles lays in the range 60–150 nm with surface charge of −27.1 mV. The TEM images suggested for aggregated structures which confirm denaturation of haemoglobin macromolecules under alkaline conditions. The nanoparticles were assessed for water intake capacity and the effect of various factors like chemical composition of nanoparticles, pH and temperature of the swelling bath, and simulated biological fluids on water sorption capacity was investigated. The insulin was loaded on to the prepared nanoparticles and investigated for swelling controlled release of insulin in simulated gastrointestinal fluid (SGIFs). The influence of chemical composition of nanoparticles, pH and temperature of the release media, and simulated physiological fluids was studied on the released amount of insulin and an optimized formulation was achieved. The prepared haemoglobin nanoparticles were also investigated for their in-vitro cytotoxicity.

1. Introduction

Nanoparticles are the simplest structures having dimensions in the range of nanometer. These nanosize particles can be used in various fields such as medicine, drug delivery, environmental protection, electronics industry, optics, textiles, cosmetics etc [1]. Due to their unique physico-chemical properties, the nanoparticles are being widely used now-a-days. The nanoparticles used for the purpose of drug delivery need to be biocompatible and adequately stable inside the body and even their degradation products should not be toxic and immunogenic with long term stability. Thus, it is always beneficial to use biopolymer nanoparticles such as chitosan, alginate, albumin, soya protein, gelatin etc for the purpose of oral drug delivery [2].

Among various available routes of drug administration, the oral delivery is the most convenient and preferred method. However, the oral delivery of macromolecular drugs has to face certain challenges such as poor solubility, stability and bioavailability of these drugs. Thus, for achieving oral delivery of these drugs, one must address all these challenges such as acidic gastric environment, intestinal barrier and continuous secretion of mucous which do not allow the drug to reach its target site [3]. Nanoparticles are the right choice for fabricating drug carries that can shield the drugs from degradation and deliver the drug to the targeted sites within the GI tract thus allowing more efficient and sustained drug delivery [4].

There are numerous diseases that have been successfully treated through recent technologies, in which the drugs are administered via means of polymeric nanoparticles. These nanosize particles act as drug carriers and help in achieving controlled delivery of drugs [5]. Thus, the controlled delivery of drugs has become a boon to medical science.

One of the major health issues arising globally now days is Diabetes. Diabetes is a group of metabolic diseases in which blood glucose level becomes too high resulting from failing in insulin secretion, insulin action or both [6]. Insulin is a hormone synthesized by the beta cells of islets of langerhans in the pancreas, and its function is not only to maintain blood sugar level but also to prevent it from going very high (hyperglycemia) or low (hypoglycemia). When blood glucose level becomes high, the pancreas releases insulin through the beta cells that passes to bloodstream and insulin absorbs the excess sugar [7,8].

In type 1 diabetes, the beta cells responsible for insulin release are destroyed by the poor immune system of the body whereas in type 2

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diabetes, the body becomes insulin resistant and it is not able to use the insulin the right way [9]. It is well known that the insulin is quite efficient and effective drug to bring down the glucose levels of blood and, thus is potentially used in the treatment of diabetes mellitus [10]. An early introduction of insulin can also protect islets from apoptosis and increase β-cell regeneration in type 2diabetes. Diabetic patients have to take frequent insulin injections daily which are quite painful and inconvenient way for the insulin administration [11]. Thus, the recent technologies involving nanocarriers for oral delivery of insulin have been used to overcome all the mentioned problems. The nanocarriers used for oral delivery of insulin should be biocompatible and biodegradable in nature and must provide protection to the insulin from harsh environment of the stomach [12]. Thus, natural polymers such as cellulose, chitosan, alginate, gelatin, albumin, haemoglobin etc can be used for the preparation of nanocarriers [13].

Insulin is a hormone secreted by the pancreas which regulates the concentration of blood glucose as per need of the body. Thus, insulin is mandatory for maintaining the normal metabolisms of the body and its deficiency can cause serious trauma in the body [14]. Hence, the artificial insemination of insulin can be done in case of its deficiency, for which nanoparticles of various natural polymers can be used as drug carrier. The present study has focused on the use of nanoparticles of haemoglobin for the oral delivery of insulin. Haeamoglobin is known to be a natural blood protein and has been attempted as carrier for the oral delivery of insulin due to the following reasons:

1. The haemoglobin nanoparticles are totally non-toxic, non-carcinogenic and blood compatible in nature.
2. The haemoglobin nanoparticles serve a dual purpose if employed as insulin carriers. Firstly, they carry and deliver insulin and, secondly being essential hem-protein, the hemoglobin also provides energy and iron to the human body.
3. The synthesis of nanoparticles from hemoglobin is quite easy and their size may also be desirably controlled by adjusting the experimental protocols.

Thus, being motivated by the multi advantages of haemoglobin, the present study aims at preparing haemoglobin nanoparticles and employing them as carriers for insulin for studying the release behavior of insulin under in vitro conditions.

2. Materials and methods

Materials - Insulin (Activity 40 IU/mL) was purchased from Torrent Pharmaceuticals Ltd. Intrad 38272, Mehsana (Gujrat), India. Haemoglobin human lyophilized powder was purchased from Sigma Aldrich and the crosslinker used for crosslinking haemoglobin was glutaraldehyde (Research Lab, Pune, India). Other chemicals used in the study were of high purity grade and for preparing various solutions distilled water was used.

2.1. Simulated gastric fluid (SGF)

In order to mimic stomach acid, the simulated gastric fluid (SGF) was used in the present study. This simulated fluid was prepared by dissolving 3.2 g of purified pepsin (derived from porcine stomach mucosa having an activity of 800–2500 units per mg of protein), and 2.0 g of sodium chloride in 7.0 mL of hydrochloric acid and making up with water to 1 L. The pH of this solution was found to be 1.2.

2.2. Simulated intestinal fluid (SIF)

Simulated Intestinal fluid (SIF) is an artificial physiological fluid (pH = 6.8), which was prepared to perform the insulin release experiments. It was prepared by dissolving 6.8 g of potassium dihydrogen phosphate and 0.89 g of NaOH in 1 L of distilled to achieve the pH 6.8.

2.3. Preparation of haemoglobin nanoparticles

The microemulsion crosslinking method was used to prepare haemoglobin nanoparticles [15]. In this method, the haemoglobin powder was dissolved in N/50 NaOH solution with constant stirring for 15 min so that the protein gets uniformly dispersed into the alkaline solution. Then 10 mL of toluene was added into the dispersed haemoglobin solution with continuous stirring for 45 min so as to form a stable emulsion. After that, pre-calculated amount of crosslinker (glutaraldehyde) was added to the haemoglobin emulsion solution with continuous stirring for 4 h which leads to the formation of tiny droplets formed as a result of crosslinking reaction between haemoglobin and glutaraldehyde. After that, 4–5 drops of N/20 H_2SO_4 were added to the crosslinked haemoglobin microemulsion which results into the precipitation of haemoglobin nanoparticles. Thus, the prepared nanoparticles of haemoglobin were washed thrice with acetone and dried at hot air oven at 40 °C. The dried nanoparticles were stored in air tight polyethylene bags for further studies.

2.4. Detoxification of nanoparticles

The as prepared haeamoglobin nanoparticles were crosslinked with glutaraldehyde which is a five carbon containing bifunctional crosslinker. Although, it can form stable inter and intra covalent bonds with protein molecules, but studies reveal that some uncreative sites of glutaraldehyde can cause severe health issues which need be addressed. Thus, in order to neutralize unreacted sites of glutaraldehyde, the so prepared crosslinked haeamoglobin nanoparticles were repeatedly treated with L-glutanic acid solution at low pH which extracts and detoxify aldehydic groups from the synthesized haeamoglobin nanoparticles. About 80% yield of haemoglobin nanoparticles was obtained.

2.5. Characterization

The physiochemical and biopharmaceutical characterization of haemoglobin nanoparticles were carried out using different analytical techniques. The physiochemical characterization includes FTIR (Fourier Transform Infra Red) spectroscopy, SEM (Scanning Electron Microscopy), TEM (Transmission Electron Microscopy) and, DLS (Dynamic Light Scattering) and Zeta Potential measurements, which help in determining the size, morphology and surface charge of the nanoparticles. On the other hand, biopharmaceutical characterization includes water intake study, BSA adsorption, and hemolysis tests and cytotoxicity evaluation of nanoparticles using L929 cells.

2.5.1. Physiochemical characterization

2.5.1.1. Fourier transform infra red spectroscopy. FTIR spectra of haemoglobin nanoparticles were obtained by FTIR spectrophotometer in the range 400–4000 cm^{-1} (FTIR-8400S, Shimadzu Spectrophotometer). FTIR technique is normally used to determine the functional groups of the biopolymer present in the nanoparticles [16].

2.5.1.2. Transmission electron microscopy. The transmission electron microscopy was used to determine morphology and size of prepared haemoglobin nanoparticles [17]. The instrument used to record transmission electron microscopy was Morgagni-268-D transmission electron microscope with an acceleration voltage of 80 KV.

2.5.1.3. Scanning electron microscopy. The surface morphology of the haemoglobin nanoparticles were investigated by scanning electron microscopy which provides insights into the morphologies of the insulin loaded and unloaded native nanoparticles of haemoglobin (Scanning electron microscope Shimadzu 2011) [18].

2.5.1.4. Dynamic light scattering. The average dimension of the