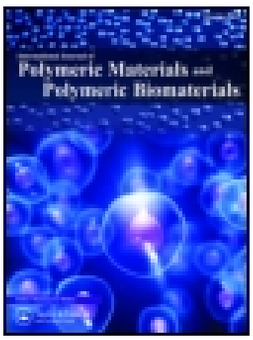

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Soya Protein as Possible Potential Nanocarriers for in Vitro Oral Delivery of Insulin in Simulated Gastrointestinal Fluids (SGFs)

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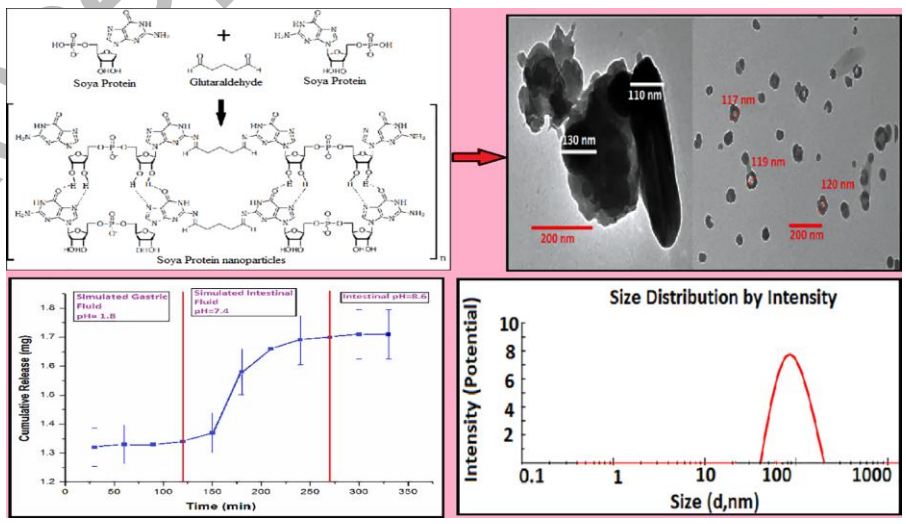
Abstract

The present study aimed to prepare soya protein nanoparticles and employ them as nanocarriers for delivery of insulin. The nanoparticles were characterized by Fourier transform infrared spectroscopy, transmission electron microscopy, scanning electron microcopy, zeta potential and dynamic light scattering measurements. The particle size analysis revealed that the size of the nanoparticles lays in the range 40-200 nm with surface charge of -25.3mV. The insulin loaded nanoparticles were investigated in simulated gastric and simulated intestinal fluids and effect of percent drug loading chemical composition of nanoparticles, pH, temperature of the release media, and simulated physiological fluids was studied on the release of insulin.

Graphical Abstract



KEYWORDS: Soya protein, nanoparticles, insulin, biopolymers.

INTRODUCTION

Nanoparticles are the simplest form of structures having sizes in the range of nanometer. These particles can be useful in diverse range of applications because of their unique properties **[1].** The technologies involved in designing nanoparticles and their subsequent use in drug delivery have grown rapidly in recent years. Nanotechnology has made it possible to create value added new materials having vast range of application, in medicine, electronics, biomaterials, energy production, and consumer products **[2-3].** The nanoparticles used in delivering drugs need to be biocompatible, their possible degradation products should not be toxic or immunogenic and they should also have long term stability. This is the reason why the natural polymers like chitosan, alginate, albumin, soya protein, gelatin etc are being used as biodegradable nanoparticles **[4]** for the purpose of oral drug delivery of a variety of drugs. Numerous synthetic polymers have been investigated for drug delivery applications **[5],** however, in most of the drug releasing systems there are certain shortcomings such as toxicity issues of synthetic polymers and their degradation products, less affinity to biodegradation etc. which demand the use of other materials like naturally occurring polymers like proteins. In the present work, however, we considered soya protein as base material of nanoparticles for the delivery of insulin; the protein nanoparticles will not only act as drug delivery vehicles but also provide energy to the body.

Soybean is known to be the most promising vegetable source of proteins. The increased acceptance of soya protein is due to manifold qualities of soybean such as good functional properties in food applications, high nutritional value, ample availability and low costs **[6].** The major soybean storage proteins referred as glycinin (11S) and ß-conglycinin (7S) are globulins and the functional properties of soya based protein products (such as flour, concentrates and isolates) are reflected on their composition and structures **[7].** Soya proteins are complex macromolecules composed of amino acids, having variety of active sites available for molecular interactions **[8].** These active sites present in amino acids of soybean help in interaction with other groups and may be easily available for achieving desirable properties.

Now days, there are various diseases which can be overcome through nanotechnologies involving the administration of drugs via biopolymer nanoparticles which are the most assuring materials **[9].** At present, diabetes is one of the most common diseases that are present in the persons of almost every age group. Diabetes is a disease that causes the blood glucose (sugar) levels to rise higher than normal **[10].** There are two type of diabetes- Type I and Type II diabetes. Diabetes mellitus is a group of metabolic diseases characterized by elevated blood glucose levels (hyperglycemia) resulting from defects in insulin secretion, insulin action or both. Insulin is a hormone produced by the beta cells of the pancreas, which is required to utilize glucose from digested food as an energy source **[11].** In diabetes, all defects are caused due to insulin impairment. Insulin is the most effective medicine in lowering the glucose level of blood for the treatment of diabetes mellitus [12]. Early introduction of insulin can also protect

islets from apoptosis and increase β-cell regeneration in type 2 diabetes. Subcutaneous injections of insulin remain to be the preferred approach for diabetic patients but often result in poor patient compliance [13]. Unlike many medicines, insulin cannot be taken orally and easily because like nearly all other proteins introduced into the gastrointestinal tract, this drug is also reduced to fragments, whereupon all insulin activity is lost [14]. Therefore, it becomes challenging to deliver insulin to the diabetic patient via oral route although daily administration of insulin through intravenous injection is quite painful for them. Thus, the oral delivery of insulin can resolve the problem of painful insulin injection [15]. The oral delivery of insulin can be achieved using nanoparticles which can efficiently deliver insulin without compromising with its biological activity.

Oral administration of insulin seems to be the most convenient way and it can mimic endogenous productions of insulin [16]. However, a reliable insulin formulation for the oral delivery encounters with some barriers in the intestinal tract that include enzymatic degradation in the GI tract and poor insulin permeability through the GI system. The bioavailability of insulin solution delivered orally is less than 1 % [17]. Thus, such problems can be overcome by delivering insulin loaded nanoparticles which have the property to target beta cells. The targeting property of nanoparticles is based on their ability to deliver a concentrate dose of insulin in the vicinity of the targets via the enhanced permeability and retention effects or active targeting by attaching to the ligands on the surface of nanoparticles [18]. These properties of nanoparticles are based on the hydrophilic nature of particles that allows imbibing of insulin when left in insulin solution and then carrying it to the target without undergoing degradation or losing its

bioactivity [19].The whole scenario of achieving oral delivery of insulin is based on the properties of nanoparticles that do not allow them to swell in acidic pH medium of the stomach while make them swellable in basic region of the GI tract.

MATERIALS AND METHODS

Materials

Insulin (Activity 40 IU/ml) was purchased from Torrent Pharmaceuticals Ltd. Intrad 382 721 Mehsana India. Soya protein acid hydrolysate powder was purchased from Sigma Aldrich and glutaraldehyde (Research Lab, Pune, India) was used for crosslinking soya protein. Other chemicals used were of analytical grade and double distilled water was used throughout the experiments.

Simulated Gastric Fluid (Sgf)

Simulated gastric fluid (SGF) is an artificial dissolution medium that is intended to represent stomach acid. It is prepared by dissolving 2.0 g of sodium chloride and 3.2 g of purified pepsin (derived from porcine stomach mucosa, with an activity of 800 to 2500 units per mg of protein), in 7.0 mL of hydrochloric acid and water up to 1000 mL. This test solution has a pH of about 1.2

Simulated Intestinal Fluid (Sif)

In order to do the release experiments at physiological pH, ie. 6.8, SIF solution was prepared by dissolving 6.8 0 g of potassium dihydrogen phosphate and 0.89 g of NaOH in one liter. The solution showed a pH of 6.8.

Preparation Of Soya Protein Nanoparticles

The method used for the preparation of nanoparticles was a microemulsion crosslinking method as reported in the literature (20). In this method, the soya protein powder was dissolved in0.02 M NaOH solution with constant stirring for 15 minutes so that the protein gets uniformly dispersed into the alkaline solution. Then 10 mL of toluene is added into the dispersed solution with continuous stirring for 45 minutes so as to form a stable emulsion. Now into the soya protein emulsion a pre-calculated amount of crosslinker (glutaraldehyde) was added with continuous stirring. The crosslinking reaction between soya protein and glutaraldehyde is allowed to take place for 3 to 4 h which leads to the formation of tiny droplets. Now to the crosslinked soya protein microemulsion 4-5 drops of 0.05 M H_2SO_4 are added which results in the precipitation of soya protein nanoparticles. The so prepared nanoparticles of soya protein are washed thrice with acetone and dried at hot air oven at 40^oC. The dried nanoparticles were stored in air tight polyethylene bags for further studies.

Characterization

The physiochemical and biopharmaceutical characterization of nanoparticles was done using different analytical techniques. The physiochemical characterization includes FTIR (Fourier Transform Infra Red), SEM (Scanning Electron Microscopy), TEM (Transmission Electron Microscopy), DLS (Dynamic Light Scattering), Zeta Potential etc., which help in determining the size, morphology, surface charge and cytotoxicity of the nanoparticles. On the other hand, biopharmaceutical characterization includes water intake study and cytotoxicity evaluation of nanoparticles using L929 cells. It is worth to

mention here that for all characterization studies a fixed composition of soya protein nanoparticles was taken as a change in the amounts of soya protein and glutaraldehyde may change the size as well as properties of prepared soya nanoparticles and the release properties of insulin as well.

PHYSIOCHEMICAL CHARACTERIZATION

Fourier Transform Infra Red Spectroscopy

FTIR spectra of soya protein nanoparticles were obtained using FTIR spectrophotometer in the range 400-4000 cm^{-1} (FTIR-8400S, Shimadzu Spectrophotometer). The obtained FTIR spectra were used to determine the functional groups of the biopolymer as well as explore the possible interactions between the protein and crosslinking agent (glutaraldehyde)[21].

Transmission Electron Microscopy

The size and morphology of the soya protein nanoparticles were investigated by the transmission electron microscopy [22]. The instrument used to record transmission electron microscopy was Morgagni-268-D transmission electron microscope with an acceleration voltage of 80 KV.

Scanning Electron Microscopy

The surface morphology of the soya protein nanoparticles were investigated by scanning electron microscopy which provides insights into the morphologies of the insulin loaded

and unloaded native nanoparticles of soya protein (Scanning electron microscope Shimadzu 2011) [23].

Dynamic Light Scattering

The average particle size of the crosslinked nanoparticles of soya protein were evaluated with the help of Dynamic Light Scattering measurements [24].The instrument used was Zetasizer Nano ZS90 (Malvern Instruments, UK). The particle size was determined by measuring the rate of fluctuations in laser light intensity scattered by particles as they diffuse through a fluid.

Zeta Potential

The surface charge properties of soya protein nanoparticles were investigated by Zeta Potential Analyzer, which is based on the principle of electrophoretic movement of charged particles under an applied field [25]. The Zeta Potential values were determined using Zetasizer Nano ZS90 (Malvern Instruments, UK).

Biopharmaceutical Characterizations

Water Sorption Capacity

The water intake capacity of soya protein nanoparticles was determined by conventional gravimetric method in which the pre-weighed nanoparticles (0.1 g) were swollen in PBS (phosphate buffer saline) solution at room temperature and then swollen weight of nanoparticles was measured after predetermined time intervals [26-29]. For this purpose the nanoparticles were filtered and then gently pressed between dry filter papers to

remove excess of water. This whole process was repeated at the intervals of half an hour until a constant swelling ratio is obtained. The swelling ratio of nanoparticles was calculated using the following equation,

$$\text{Swelling Ratio} = Wt / Wo$$

Where, *Wo* and *Wt* are the weights of dry and swollen nanoparticles at zero and time t, respectively.

In-Vitro Release Studies

Loading Of Insulin Into The Soya Protein Nanoparticles

Basically there are two general approached loading of drugs into nanocarriers. In the first method the drug (insulin in the present case) is added into the feed mixture of soya protein nanoparticles while in the second method the prepared nanoparticles are allowed to swell in the insulin solution of definite concentration. The second method is superior to the first method in the respect that there is no loss of bioactivity of insulin in the second method, while it may be significantly lost in the first method. Moreover, the percent of insulin is quite high in the second method [30]. Another disadvantage of the first method is that after loading of the drug into nanocarriers, they are purified by swelling them in water for a week. During the swelling process a significant amount of drug is leached out and the obtained percent of drug loading remains quite low. Thus, we adopted the absorption method for loading insulin into the soya protein nanoparticles. For this purpose, a known volume of insulin was diluted with an appropriate amount of phosphate buffer saline (PBS) solution. Then the nanoparticles (0.1 g) were allowed to swell in insulin solution (10 mL) for 24 h. After equilibrium swelling was achieved, the

nanoparticles were filtered and subsequently dried at room temperature for a week. The percent loading of insulin by the nanoparticles was calculated using the following equation,

$$\%\ loading\ = \frac{wd - w0}{w0} \times 100 \qquad (1)$$

Where, W_d and W_o are the dry weights of loaded and unloaded nanoparticles respectively.

Release Experiments

In order to study the release of insulin from the drug loaded soya protein nanoparticles, release experiments were performed in which an appropriate amount of insulin loaded nanoparticles were placed in the definite volume of release media i.e., 10 mL of Simulated Gastrointestinal Fluid (SGF) solution. The release experiments were performed by shaking insulin loaded nanoparticles in a definite volume of SGF at constant speed. After shaking for a fixed period of time intervals, the suspension were centrifuged for 5 min, the supernatants were withdrawn and analyzed for the remaining concentration of insulin using UV-1800, Shimadzu, UV-Visible Spectrophotometer.

Release Kinetics

The kinetics of drug release process is an important part of study as it provides information about the nature of the drug delivery system as well as the mechanistic aspects of the drug delivery process. In the case of insulin, it is always desirable to make the insulin available over an extended period after administration [31]. According to Fick's law, the release kinetics involves the relaxation of biopolymer chains and diffusion

of entrapped insulin molecules into the external release medium. Thus, by using the following equation of Fick's law of diffusion, the mechanism of the insulin release can be easily determined,

$$\frac{W_t}{W_\infty} = kt^n \qquad \textbf{(2)}$$

Where W_t and $W\infty$ are the amounts of insulin released at time t and at infinite time respectively, k is the rate constant; n is the diffusion exponent which is an indicator of the mechanism of insulin transport. When n>0.42, diffusion is non- Fickian diffusion, while n=0.42 represents a Fickian diffusion. The value of n=0.85 represents transport mechanism in which the release of insulin from nanoparticles is zero order. The release of insulin takes place when nanoparticles are placed in the release medium. In order to evaluate the diffusion constant of the insulin, the following equation may be used,

$$\frac{W_t}{W_\infty} = 4\left[\frac{Dt}{\Pi L^2}\right]^{0.5} \qquad \textbf{(3)}$$

Where D is the diffusion constant of the insulin and L is the diameter of the nanoparticles.

Effect Of Different Parameters On The Release Of Insulin

The kinetics of insulin release by soya protein nanoparticles was investigated under different experimental conditions such as simulated physiological fluids, pH, temperature, chemical composition of the nanoparticles etc.

Chemical Stability Of Drug

The chemical stability of insulin in media of different pH was determined by comparing UV spectra of native insulin solution with that of the insulin released at different pH..

Statistical Analysis

All experiments were done at least thrice and the figures and tables were presented along with their respective error bars and standard deviations, respectively.

RESULTS AND DISCUSSION

Reaction Mechanism Of The Preparation Of Soya Protein Nanoparticles

The reaction schemes shown in **Figure 1** presents the crosslinking of two soya protein molecules with one glutaraldehyde molecule, in which amine (NH_2) groups of each soya protein molecules reacts with the carbonyl groups of glutaraldehyde leading to the formation of –N=C- bonds by losing two water molecule. Thus, a linear crosslinked macromolecule is formed in which the two soya protein macromolecules crosslink with one glutaraldehyde molecule. In this way a number of strands of linearly crosslinked soya protein molecules are formed which ultimately take the shape of nanoparticles.

FTIR Spectroscopy

FTIR spectroscopy is the technique used to examine the nature of functional groups present in the soya protein nanoparticles crosslinked with gluteraldehyde. These functional groups help in determining the identity of the particular protein through its participating amino acids. The spectral comparison of soya protein powder and soya

protein nanoparticles can be understood by **Figure 2(a) and (b)**, respectively which confirm the presence of characteristic groups in the nanoparticles. In the FTIR spectra (b) of the nanoparticles of soya protein, the characteristic peaks were observed at 1681 cm^{-1} (-C=O stretching) due to amide I band and 1541 cm^{-1} (C-N stretch with N-H bending mode) due to amide II band of the protein molecule. The absorption by amide I and amide II bands was due to characteristic hydrogen bonding pattern between amide C=O and N-H groups which confirm the presence of secondary structure of protein (32). While, soya protein nanoparticles also show peak at 1070 cm^{-1} due to shifting of C-N band from 1141 cm^{-1} to lower wavenumber (1070 $cm^{-1)}$ because of crosslinking with glutaraldehyde. Similarly some characteristics peaks were shown in soya protein nanoparticles but not in soya protein isolate which also provides an evidence of crosslinking of soya protein with glutaraldehyde [33].

TEM Analysis

TEM is an important analytical technique to determine the size and internal morphology of the nanoparticles. The size of nanoparticles helps to decide the suitability of the carriers for insulin delivery [34]. Moreover, the smaller the size of nanoparticles is, the greater would be the loading of the drug molecules. In the present study the TEM images of native soya protein nanoparticles and insulin loaded nanoparticles are shown in **Figure 3(a)** and (b), respectively**.** Whereas the left image (a) shows aggregated nanoparticles of unloaded soya protein, the right one (b) depicts insulin loaded scattered nanoparticles. Both of the images reveal that the size of nanoparticles varies in the broad range from 40 to 200 nm. It is also clear from the TEM images that the unloaded nanoparticles have an

irregular shape but while after loading of the insulin the soya protein nanoparticles acquire a little regular shape. It is also noteworthy that the state of aggregation is significantly reduced after insulin loading which may be attributed to the fact that the loading of insulin may cause weakening of inter-particle forces.

SEM Analysis

The study of SEM analysis gives information about the external morphology of the nanoparticles i.e., whether they are spherical in shaper or have irregular shapes, or the surfaces are smooth or have cracks and voids. The morphology of a drug carrier is an important feature as it will also determine the extent of drug loading and consequently the drug release profile [35]. The **Figure 3(c)** shows the SEM images of soya protein nanoparticles which reveal that the particles are significantly aggregated and have much irregular shape and sizes. Some of the particles are surprisingly as big as up to 200 µm. However, a higher magnification micrograph shown in the inset of Figure 4 indicates that the individual particles have dimension up to 200 nm.

Dynamic Light Scattering Measurements

The characterization of nanoparticles by dynamic light scattering provides information about the size of nanoparticles in solutions. The principle of dynamic light scattering is based on the passing of a beam of laser through a colloidal solution and then analyzing the modulation of the scattered light intensity as a function of time thus determining the hydrodynamic size of nanoparticles [36]. By the DLS study, cumulative intensity, differential intensity of the nanoparticles and particles size distribution of the

polydisperse system can be determined. The results of particles size distribution of insulin loaded soya protein nanoparticles are presented in **Figure 3(d).** The results clearly show that the size of soya protein nanoparticles fall in the range of 40 to 200 nm with an average value of 109 nm. The polydispersity index (PDI) of the soya protein nanoparticles was 0.289, and it is well known that the value of polydispersity index (PDI) less than 0.3 have good colloidal properties thus showing good colloidal tendency [37]. It was also concluded that the DLS results are consistent with TEM analysis.

Zeta Potential

Zeta potential analysis is a technique through which the surface charge of the nanoparticles is determined. In solution, the thin layer of ions of opposite charge attracts towards the surface charge of nanoparticles and thus the point at which the potential of the system breaks is called zeta potential (38). The zeta potential distribution curve is shown in **Figure 3(e)** which indicates that most of the soya protein nanoparticles have a zeta potential of about -25.3 mV which accounts for a fair stability of the nanoparticles suspension.

Swelling Studies

Effect Of Amount Of Soya Protein

Swelling behavior of the soya protein nanoparticles has been investigated by varying the amount of soya protein in the feed mixture from 1.0g to 5.0g. The results of swelling studies are shown in **Figure 4(a)** which reveals that the swelling of nanoparticles increases with increasing amount of soya protein in the nanoparticles in the studied range.

The observed results may be explained by the fact that on increasing the amount of soya protein, the hydrophilicity of nanospheres also increases and, consequently, more water sorption is observed. However, it is also to be noted that when the amount of soya protein is increased in the studied range, the molar ratio of soya protein to glutaraldehyde also increases and, as a consequence, less glutaraldehyde will be available for crosslinking. This will clearly result in lower degree of crosslinking of soya protein macromolecules thus producing large mesh sizes network of soya protein nanoparticles. This will result in entrance of greater number of water molecules into the soya protein nanoparticles and, therefore, the swelling ration will increase.

Effect Of Crosslinker Variation

The swelling behavior of soya protein nanoparticles depends on the concentration of crosslinker as well. In order to see the effect of crosslinker on the swelling ratio of the nanoparticles, the concentration of glutaraldehyde was varied in the range 2.12 to 15.88 mM in the feed mixture of the nanoparticles while keeping the amount of soya protein constant, i.e., 5.0 g.

The results of swelling studies are shown in **Figure 4(b)**, which indicate that the swelling ratio of nanoparticles increases up to 10.58 mM and then decreases on further addition of crosslinker at fixed amount of soya protein powder. The reason for the observed trend in swelling behavior is due to the fact that the crosslinked network of nanoparticles swells up to some extent with increasing degree of crosslinking and reducing size of nanoparticles. The reduced size of soya protein nanoparticles brings about an increase in

the surface area which results in greater interaction with water molecules thus enhancing the swelling ratio. However, beyond 10.58 mM, of the crosslinker concentration, the swelling ratio of nanoparticles decreases, which is due to the reason that on further addition of crosslinker at constant amount of soya protein, the network inside the nanospheres becomes compact and, therefore, lesser number of water molecules can enter into the nanoparticles which results into the decrease in swelling.

The results, however, may also be explained in terms of the molar ration of the amine and aldehyde of soya protein and glutaraldehyde, respectively. At higher concentration of glutaraldehyde, molar ratio of amine to aldehyde will decrease and less number of amine groups will be available to aldehyde molecules for crosslinking which will result in a greater degree of crosslinking producing small mesh sizes in the network. The reduction in mesh size will clearly result in accommodation of less number of water molecules and bring about a fall in the swelling ration.

Release Studies

The prepared soya protein nanoparticles of definite composition were loaded with insulin as described in the experimental section. For achieving varying percentage of drug loading, a fixed amount of nanoparticles was allowed to swell in insulin solutions of different concentrations and varying degree of insulin loading was obtained.

Effect Of Percent Loading

The extent of insulin released by nanoparticles is greatly affected by percent loading of insulin onto the soya protein nanoparticles. The results are clearly shown in **Figure 5(a)** which reveals that the released amount of insulin is optimum in the highest insulin loaded nanoparticles and it increases significantly when the insulin loading increases from 22 to 46 percent. The reason for the observed results is due to the fact that highly loaded nanoparticles have high rate of diffusion of insulin molecules from the nanoparticles into the release media which results in greater release of insulin with increasing percent loading. The observed results may also be explained by the fact that as the percent loading increases, the solvent front moves much faster into the nanoparticles network and results in rapid relaxation of soya protein chains of the nanoparticles which allows large number of insulin molecules to rush to the release medium [39]. This obviously results in greater release of insulin with increasing percent loading of the drug. Another reason for the observed enhanced release of insulin may be attributed to the fact that with increasing amount of drug loading, the interactions between the biopolymer macromolecules and insulin becomes weak and, therefore, facilitates greater amount of insulin to release from the nanoparticles.

Effect Of Soya Protein Content

The effect of soya protein content in the nanoparticles on the release of insulin has been investigated by the loading 46% of insulin onto all the composition of soya protein nanoparticles varying from 0.5g to 5g. The results of release study are shown in **Figure 5(b)** which reveal that the cumulative release of insulin increases with increasing amount of soya protein in the whole studied range of 0.5 to 5.0g. The reason for the

observed increased release may be attributed to the fact that soya protein is a hydrophilic biopolymer and when the nanoparticles come in contact with water, they absorb larger number of water molecules. Due to absorption of water molecules the macromolecular chains of soya protein undergo rapid relaxation resulting in expulsion of greater number of insulin molecules into the release medium. [40]. Hence, it is concluded that as the amount of soya protein increases in the nanoparticles, the release of insulin also increases.

Effect Of Glutaraldehyde

The effect of glutaraldehyde variation on the released amount of insulin by soya protein nanoparticles has been investigated by varying the concentration of glutaraldehyde in the range of 2.12mM to 15.88 mM while keeping the amount of soya protein constant i.e., 5.0 g. The results are shown in **Figure 5(c)** which show that the cumulative release of insulin increases with increase in the concentration of glutaraldehyde from 2.11mM to 10.58mM while a further increase in concentration of crosslinker results in a decrease in the insulin release. The observed results may be explained by the fact that with increase in concentration of glutaraldehyde, the mesh size of the nanoparticles network increases which results in the increase in the free volume available between the soya protein chains. When the drug loaded nanoparticles contact with the release medium, the water molecules penetrate into the network and occupy the accessible free volume resulting in relaxation of biopolymer chains which brings about an increase in the amount of released insulin. However, beyond 10.58mM of glutaraldehyde, the release of insulin decreases because the network of soya protein nanoparticles becomes denser thus reducing the pore

sizes of the nanoparticles network [41]. The reduced mesh sizes of soya protein nanoparticles clearly results in a decrease in the release of insulin.

Effect Of Simulated Physiological Fluids

The influence of simulated physiological fluids on the released amount of insulin was investigated by conducting the release experiments in biofluids like urea, synthetic urine, saline water, glucose and PBS. The results are shown in **Figure 5(d)** which reveal that the extent of insulin release decreases in these simulated fluids and do not obey any specific order in suppressing the insulin release. The observed results, however, may be explained by the fact that the presence of salt ions in the bulk of the release medium tends to lower the difference between the osmotic pressures of interior of the nanoparticles network and outer medium. The reduced osmotic pressure difference results in a decrease in the released amount of insulin [42].

Effect Of Temperature In Insulin Release

In order to examine the effect of temperature on the release of insulin from the soya protein nanoparticles, the release experiments were conducted at different temperatures. The results are shown in **Figure 6(a)** which indicates that the released amount of insulin increase with increasing temperature from 18^{o}C to 37^{o}C. The reason for the observed increase is due to the fact that at higher temperature the macromolecular chains of the nanoparticles network undergo faster relaxation due to increased kinetic energy. Moreover, at higher temperature the rate of diffusion of water molecules as well as that of the insulin molecules also increase which also results in an enhanced release of insulin. It

may also be added that when the temperature is high, the drug-biopolymer interactions become weak and this also facilitates greater release of insulin molecules.

Effect Of Ph

pH plays a significant role in the release of insulin and it decides whether a particular insulin releasing carrier is suitable for oral administration or not. As per the mechanistic action of insulin delivery, the drug loaded nanoparticles at first reach at the stomach where highly acidic environment accompanied by the presence of proteotic enzymes tends to destroy the bioactivity of insulin. After about 2 h, the insulin passes into intestinal region where the present physiological fluids are alkaline in nature. Thus, the success of an oral insulin formulation rests upon the fact that how safely the insulin molecules may be spared in stomach. The problem has been addressed by encapsulating insulin into soya protein nanoparticles so that in the acidic medium of the stomach the nanoparticles will not swell and insulin molecules will not come out. In order to mimic this situation, the experiments were designed in a specific way as given below:

First of all the insulin loaded nanoparticles were left in simulated gastric fluid for 2 h and then the same nanoparticles were taken out and left in the simulated intestinal fluid. The results are shown in **Figure 6(b)** which clearly shows that in the gastric fluid of pH 1.8, the cumulative release of insulin was too low, whereas in simulated intestinal fluid of alkaline pH the release of insulin was appreciable. The observed results may be explained as below:

At low pH when the drug loaded nanoparticles are put in contact with simulated gastric fluid, the microenvironment within the network changes to acidic and insulin molecules entrapped inside tend to form insoluble amyloid fibers [43]. Thus, reduced solubility of insulin molecules result in transport of fewer molecules into the release medium thus delivering quite less amount of insulin at low pH. However, at higher pH of simulated intestinal fluid, where pH is slightly alkaline, the solubility of insulin increases and insulin molecules show greater tendency to go into the release medium of high pH. It is worth mentioning here that at higher pH the soya protein macromolecules will bear a net negative charge as they are well above their isoelectric point of 4.5 and –COOH groups of amino acids will be present in dissociated anionic forms. This obviously produces an electrostatic repulsion among the protein chains due to carboxylate ions and results in faster relaxation of soya protein chains which produces a greater release of insulin.

It is worth to mention here that apart from the enzymatic barrier encountered by the oral insulin formulation, the problem of poor absorption of insulin is another serious issue. In real life, the body has trouble absorbing insulin from the intestines. The mucus layer in the intestines is thick, and studies have shown that only low levels of insulin pass through this lining and into the bloodstream. Thus, in order to design a successful oral formulation one has to add some appropriate ingredients into the formulation that may enhance the absorption of insulin.

Drug Activity

The activity of insulin was determined by UV spectrophotometer by comparing the UV spectra of native insulin solution and the released insulin. In order to see if the bioactivity of insulin is not lost in harsh acidic environment of stomach, the drug loaded nanoparticles were left in simulated gastric juice pH (2.0) for 2 h and the chemical stability of entrapped insulin was checked by allowing it to release it in a medium pH 7.4 medium. Then the released fraction of insulin was scanned between 200-400 nm and the obtained spectra were compared with that of the native insulin in the same pH of 7.4. The two spectra are shown in **Figure 7 (a) and (b),** respectively which clearly indicate that both the spectra are almost identical to each other and show similar λmax values. This clearly suggests that even after being remained in highly acidic media, the chemical nature of insulin is not altered. Moreover, it was also found that even soya protein nanoparticles do not undergo any cleavage in gastric juice medium. This clearly explains the stability of drug carrier system in highly acidic media and therefore justifies its suitability as a carrier for insulin in controlled release.

Kinetic Analysis Of Release Data

When the insulin loaded nanoparticles come in contact with a solvent, relaxation of biopolymer chains takes place. This allows the solvent to enter into the nanoparticles matrix and as a consequence the insulin molecules diffuse into the external release medium by crossing the swollen polymer network of the nanoparticles. The relaxation of polymer chains and diffusion of insulin molecules determine the type of release mechanism being followed by the releasing insulin molecules. It has been followed by Higuchi equation that if n=0.42, the release is diffusion controlled, when the value of n

lies in between n=0.42-0.85, the release is non Fickian, and when the value comes n=0.85 (case II), the mechanism becomes anomalous in nature. In some cases n has been found to exceed 0.85 and the mechanism is termed as super case II. The values of diffusion coefficients (D) and release exponents (n) have been calculated as described above and are summarized in **Table I**.

CONCLUSIONS

Soya protein nanoparticles are prepared by microemulsion crosslinking of soya protein by glutaraldehyde and the nature of crosslinking is confirmed by the FTIR spectroscopy. The TEM images clearly reveal that the size of nanoparticles lie in the range 40 to 200 nm which are consistent with that determined by dynamic light scattering measurements. The soya protein nanoparticles show a zeta potential of -25.3 mV which suggests for their fair stability. The increasing amount of soya protein content in the nanoparticles results in their enhanced swelling ratio. However, when the concentration of glutaraldehyde is increased, initially the swelling ratio of soya protein nanoparticles increases while at higher concentration of crosslinker the swelling ration fall. The soya protein nanoparticles with greater loading of insulin tend to increase the amount of released insulin. When the amount of soya protein is increased in the nanoparticles, the release of insulin also increases. However, in the case of glutaraldehyde, it is observed that the amount of released insulin increases with increase in the concentration of glutaraldehyde from 2.11mM to 10.58mM, while it further decreases with further increase in crosslinker concentration. In simulated physiological fluids the insulin is released to lower extent. The increase in temperature also brings about an increase in the amount of released

insulin. The soya protein nanoparticles show extremely low release of insulin at low pH and quite appreciable at higher pH. The release experiments also show that the entrapped insulin does not lose its activity when put in harsh simulated acidic environment of stomach.

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Table I Kinetic analysis of release data showing the release exponents and diffusion coefficients under varying experimental conditions

S.N.	Gluteraldehyde (mM)	Soya Protein (gm)	Diffusion Coefficient (cm^2/min)	n	Mechanism	R^2
1	5.29	0.5	0.43×10^{-14}	0.70	Non- Fickian	0.98
2	5.29	1	0.46×10^{-14}	0.75	Non- Fickian	0.99
3	5.29	2	0.32×10^{-14}	0.61	Non- Fickian	0.98
4	5.29	3	0.44×10^{-14}	0.62	Non- Fickian	0.96
5	5.29	4	0.37×10^{-14}	0.64	Non- Fickian	0.99
6	5.29	5	0.34×10^{-14}	0.61	Non- Fickian	0.99
7	2.11	3	0.44×10^{-14}	0.84	Non- Fickian	0.99
8	10.58	3	0.53×10^{-14}	1.0	Anomalous	0.98
9	15.88	3	0.34×10^{-14}	0.5	Fickian	0.98

Figure 1

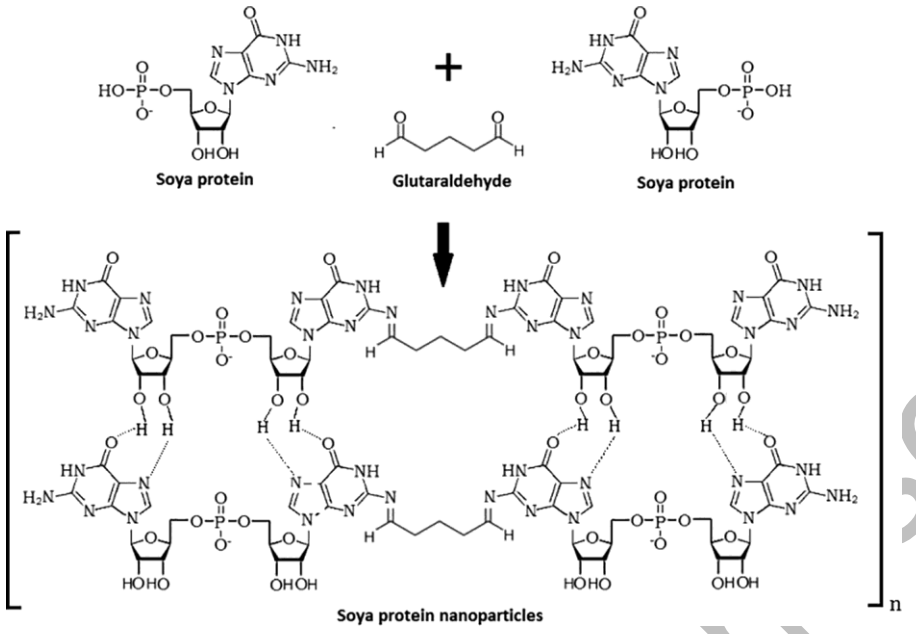


Figure 2

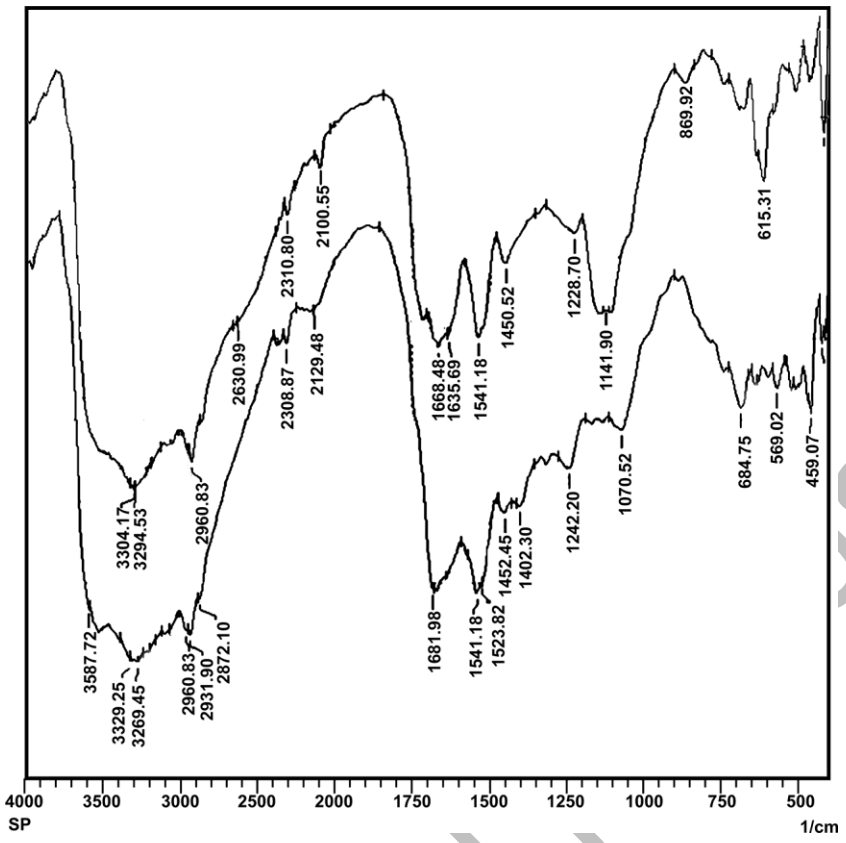


Figure 3

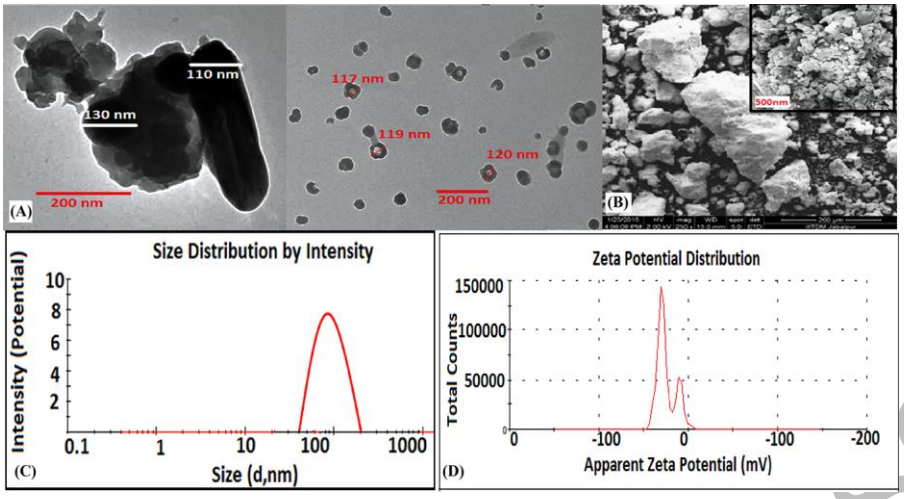


Figure 4

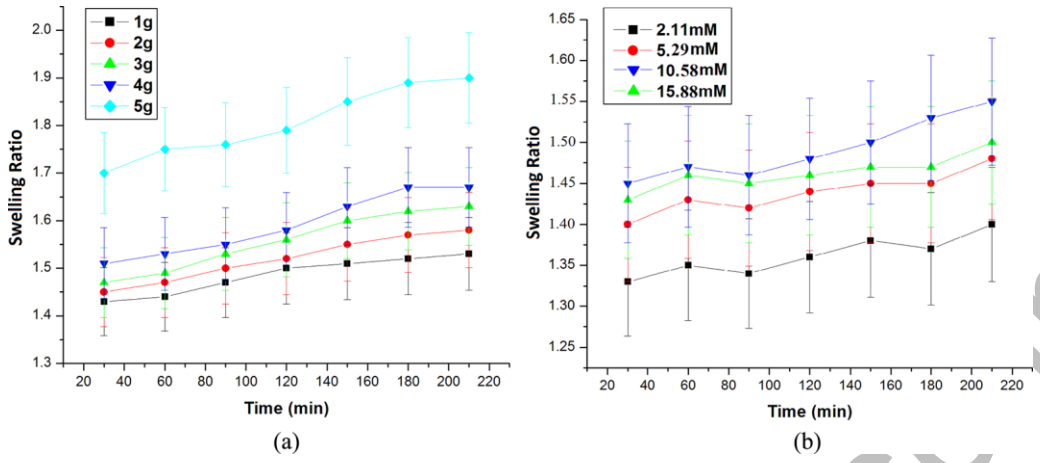


Figure 5

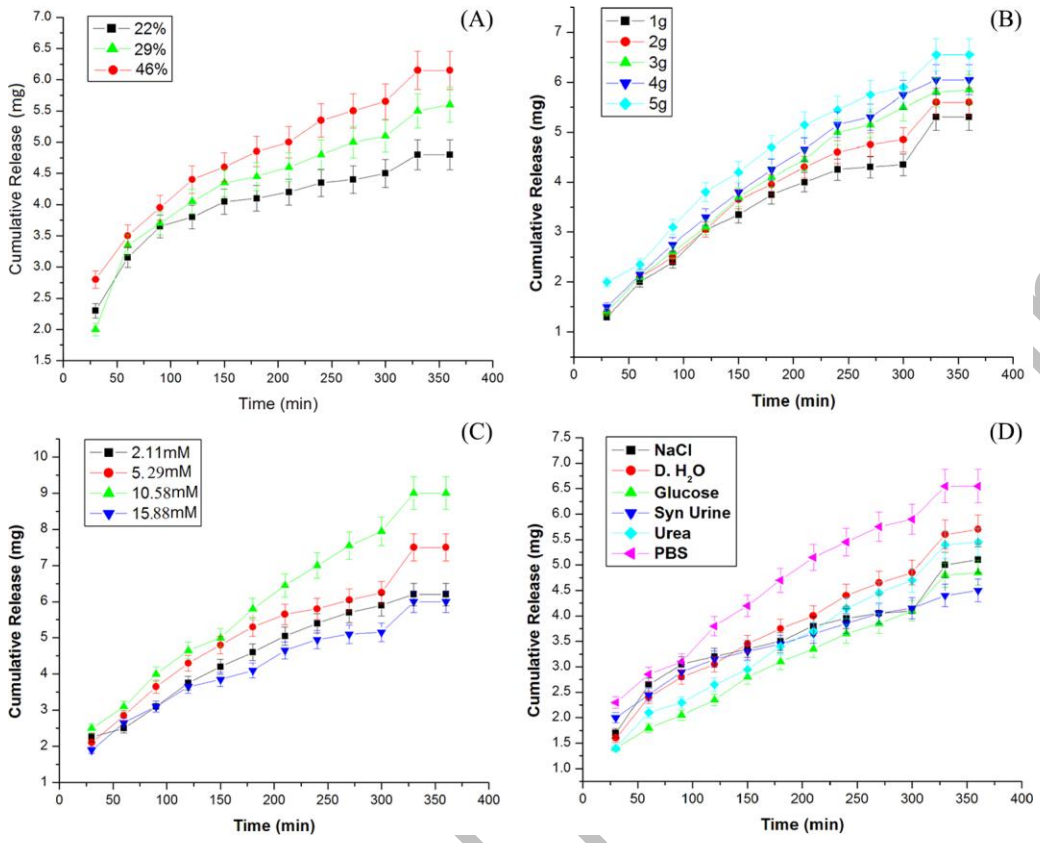
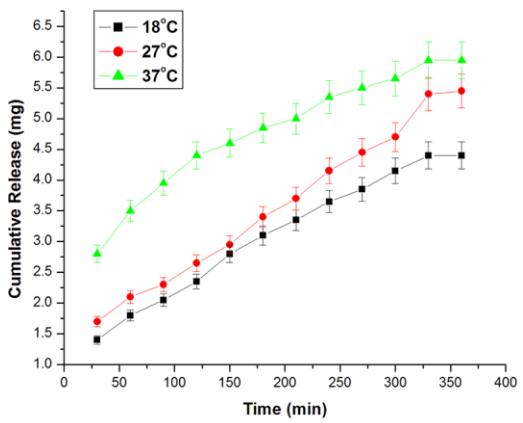
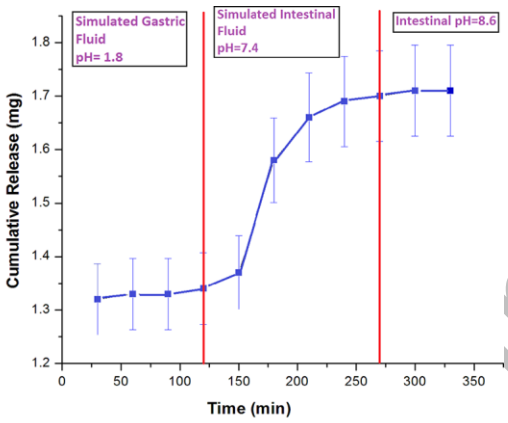


Figure 6



(A)



(B)

Figure 7