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Designing Gelatin Nanocarriers as a Swellable System for Controlled Release of Insulin: An *In-Vitro* Kinetic Study

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In the present investigation gelatin nanoparticles have been synthesized and characterized by various techniques like FTIR, scanning electron microscopy, particle size analysis and surface charge measurements. The particles were allowed to swell in phosphate buffer saline (PBS) and the influence of various factors like chemical composition of nanoparticles, pH and temperature of the swelling bath was investigated on the water intake capacity of the gelatin nanoparticles. The particles were loaded with insulin and the release kinetics of insulin was studied in PBS medium under different conditions. The effects of percent loading of drug, chemical composition of nanoparticles and pH, temperature of the release medium were examined on the release profiles of the drug and the possible mechanisms of drug transport were investigated. The chemical stability of the loaded drug (insulin) was also assessed especially under highly acidic conditions of artificial gastric juice.

Keywords: Biodegradable polymers, controlled release, oral drug delivery, macromolecular drug delivery, nanoparticles, biopolymers, insulin, gelatin

1 Introduction

Diabetes is an increasingly prevalent metabolic disorder in humans and is characterized by hyperglycemia (1, 2). This is either due to a lack of insulin (Fig. 1) or insensitivity of insulin to target cells (3). Diabetes mellitus (DM), the most commonly encountered endocrinopathy, continues to increase dramatically in prevalence (4). Insulin helps to transfer glucose from blood stream to the tissues and cells and it is therefore necessary that the body has the right amount of insulin in order to maintain appropriate glucose levels. So, diabetics must control their blood glucose levels via exogenous administration of insulin (5).

Although there are different routes for delivery of insulin, like ocular, aerosol, nasal, buccal, rectal, pulmonary, the oral routes (6–9), however, is considered to be the most convenient and desired route of drug delivery, especially when repeated administration is necessary because it is the most familiar, easy and patient friendly of all the routes of application. Insulin, if administered orally may be helpful in eliminating the pain caused by infection, psychological barriers associated with multiple daily injections such as needle anxiety (10) and possible infections.

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The act of insulin delivery via oral route, however, is not as simple as it looks. The administrations of insulin, pose several stability and absorption problems that have to be addressed with care. In general, insulin if administered via the oral route suffers rapid enzymatic degradation in the stomach, inactivation and digestion by proteolytic enzymes in the intestinal lumen, and poor permeability across intestinal epithelium because of its high molecular weight and lack of lipophilicity (11). The oral bioavailability of insulin can be enhanced by an appropriate choice and use of any of the novel carrier systems like gels (12), liposomes (13), microspheres (14), nanospheres (15) and nanocapsules (16).

Polymeric nanospheres have come up as an ideal vehicle for many controlled delivery applications due to their ability to encapsulate variety of drugs, biocompatibility, high bioavailability and sustained drug release characteristics. It is most advanced at present, both in scientific knowledge and in commercial (17) knowledge. These have been extensively investigated because of their size-dependent physical and chemical properties. Moreover, in the case of macro drugs like insulin the nanospheres protect the medicine from the harsh environment of the stomach until it can be released and absorbed in the intestine. It is worth mentioning here that the word "macrodrug" implies for a high molecular drug of a few nanometer dimension while nanospheres represent carrier species having a dimension up to 100 nm. The drug release rates depend strongly on

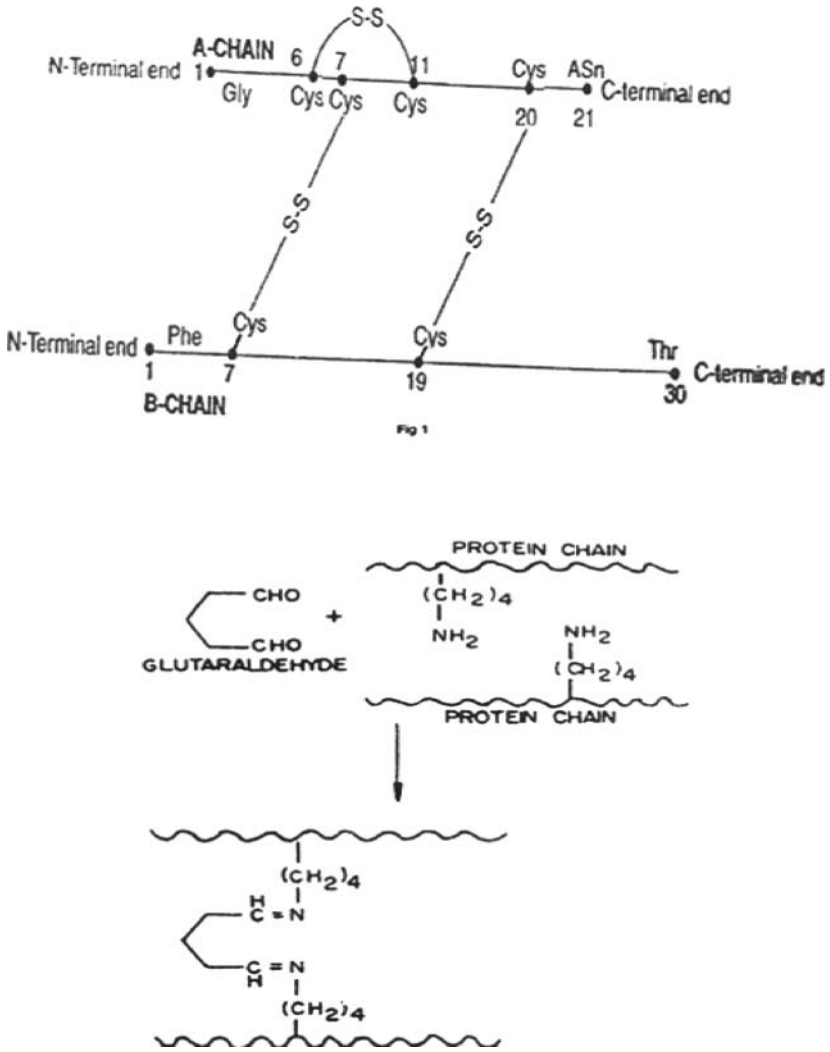


Fig. 1. Schematic representation of insulin structure and reaction between glutaraldehyde and gelatin.

the size of the spheres containing the drug. Smaller particles have larger surface area and, therefore, most of the encapsulated drug would be at or near the particle surface leading to fast drug release whereas larger particles have large cores which allow more drug to be encapsulated and slowly diffuse out (18).

The recent past has witnessed great efforts to develop oral delivery systems for insulin. For example, Aron and Peppas (19) prepared nanospheres of crosslinked networks of methacrylic acid and acrylic acid grafted on to poly(ethylene glycol) as oral insulin delivery devices. Leobandung et al. (20) prepared temperature sensitive nanospheres by a thermally — initiated free redical dispersion polymerization method and employed them for controlled release of insulin Wu and coworkers (21) prepared chitosan-coated insulin liposomes and monitored the blood glucose level using the glucose oxidase method after oral administration of insulin to healthy mice. Now a days, it has become customary to use the naturally occurring polymers as source materials for drug carriers. Many natural polymers used for nanoparticle formulations include gelatin (22), chitosan (23), alginate (24) etc. which are biocompatible, non-toxic, non carcinogenic and biodegradable also. Thus, being motivated by the challenges coming across in designing an oral delivery system for insulin, the present study aims at investigating glutaraldehyde crosslinked gelatin nanoparticles as a swelling controlled release system for insulin. The choice of gelatin rests upon the fact that it is a natural, nontoxic, noncarcinogenic and biodegradable biopolymer of immense biomedical and pharmaceutical utility.

2 Experimental

2.1 Materials

Acid processed gelatin (Type A, Mol. Wt. ca 70 kDa, isoelectric point 7.6) in yellowish granular form, was supplied by Loba Chemie, Mumbai, India and used without any

pretreatment. Type B gelatin (Bloom No. 240, Mol. Wt ca. 65 kDa isoelectric point 4.8) was a kind gift from Shaw Wallace Gelatins Ltd., Jabalpur, India. Glutaraldehyde was employed as a crosslinker of gelatin and obtained from Research Lab, Pune, India. Polymethyl methacrylate (Sigma Aldrich Co., USA, average Mw $\sim 3.5 \times 10^5$) was used for preparing the oil phase. Insulin (activity 40 IU/mL) was purchased from Torrent Pharmaceuticals LTD, Indrad 382 721 Mehshana, India. The artificial gastric juice (pH 2.0) was prepared by taking 0.01 mol L^{-1} HCl, 2.5 g sodium lauryl sulphate and 2.0 g NaCl.

2.2 Preparation of Nanoparticles

Although nanoparticles can be prepared by several methods (25,26), however, in the present study a solvent evaporation technique has been followed as described below in brief.

'Aqueous phase' was prepared by dissolving 2.0 g of gelatin in 15 mL of distilled water while for preparing 'oil phase' 7.0 g of polymethyl methacrylate was dissolved into a 50 mL (1:1, V/V) mixture of chloroform and toluene. The above two solutions were mixed with vigorous shaking (shaking speed 300 RPM, 0.5 HP motor capacity, Toshniwal, India) for 30 min and to this suspension was added, with constant shaking, 1 mL of gluteraldehyde emulsion prepared in toluene (1:1 v/v). The crosslinking reaction, as shown in Figure 1, was allowed to take place for 5 h at 4°C in an ice bath. After the crosslinking reaction is over, the gelatin nanoparticles change from yellowish to a red-brown color which was evidence of the completion of the crosslinking reaction.

The red-brown nanoparticles of gelatin were cleaned by centrifuging and then resuspended four times in toluene and twice in acetone. This results in a fine red-brown powder which was further subjected to detoxification.

2.2.1. *Detoxification of nanoparticles*

The toxicity of glutaraldehyde and its polymeric species pose serious health problems and, therefore, limit its acceptance by the biomedical community. Thus, neutralization of unreacted sites of glutaraldehyde is quite an essential task (27). In the present study, neutralization of toxic effect of glutaraldehyde was done by treating the crosslinked gelatin nanoparticles repeatedly with low pH L- glutamic acid solution, which obviously results in extraction and detoxification of aldehyde groups from prepared nanoparticles. The yield of prepared gelatin nanoparticles was found to be nearly 72%.

2.2.2. *Characterization*

The prepared gelatin nanoparticles were characterized by the following analytical techniques:

2.2.3. *FTIR spectra*

For structural characterizations, FTIR spectra of insulin loaded gelatin nanoparticles, native gelatin, insulin and glutaraldehyde were recorded FTIR-8400 S, Shimadzu spectrophotometer. Prior to analysis, KBr pellets were prepared by mixing 1:10 of sample: KBr (w\w) followed by uniaxillary pressing the powder under vacuum. Spectra were obtained between 4000–400 cm^{-1} at 2 cm^{-1} resolution.

2.2.4. *Scanning electron microscopy*

The morphology of native gelatin nanoparticles was determined by scanning electron microscopy (Philips 515). The powdered sample was coated with gold prior to the microscopic examination using ion sputtering. The accelerating voltage was kept at 20 KV.

2.2.5. *Particle size analysis*

Dynamic light scattering (DLS) measurements for determining the average size and size distribution of the nanoparticles were performed using a Particle Size Analyzer (Malvern Mastersizer, 2000). The intensities of scattered light were detected at 90° to an incident beam. The freeze dried powder was dispersed in aqueous buffer and then measurements were done. All the data analysis was done in automatic mode.

2.2.6. *Surface potential measurements*

Insulin and gelatin nanoparticles are charged species and the charges carried by their molecules are functions of the pH of their solutions. Thus, in order to understand the nature of the drug (insulin) -nanoparticles interactions, surface potential studies are important. In the present study, the surface potential measurements were performed with a digital potentiometer (Model No. 118, EI Product, Mumbai, India). In a typical experiment, 0.2 g insulin loaded nanoparticles were dispersed into 20 mL of respective pH solutions and emf values were recorded using a compound electrode system consisting of Pt and calomel electrodes. Similar experiments were also repeated for respective pH solutions without loaded nanoparticles.

2.2.7. *Chemical stability of drug*

Since the insulin loaded nanoparticles must reside in the harsh environment (pH 2.0) of gastric juice, there may be the possibility of loss of bioactivity of entrapped insulin which needs to be ascertained. Thus, in order to examine the chemical stability of insulin, the loaded nanoparticles were left in simulated gastric juice (pH 2.0) for 2 h and then the loaded nanoparticles were removed, mildly washed, and then transferred to release medium (pH 7.4). The released fraction of insulin was scanned between 200–400 nm and the obtained UV spectra were compared with that of the native insulin in the same pH of 7.4.

2.3 Loading of Insulin

A successful nanoparticulate system should have a high drug loading capacity thereby reducing the quantity of matrix materials for administration. Studies have shown that the use of ionic interaction between the drug and matrix materials can be a very effective way to increase the drug loading (28). The loading of an active agent is normally performed by two general methods. In the first method, the drug is added into the feed mixture during preparation of the nanoparticles, while in the second method the nanoparticles are allowed to swell in the freshly prepared drug solution until equilibrium is reached. The previous method has some disadvantages such as, due to the addition of drug to the reaction mixture of carriers; the chemical and bioactivity of drug may be lost. Moreover, during purification of device that is normally done by allowing it to swell till equilibrium, some drug may be lost due to leaching of the drug along with unreacted chemicals. In the present work, therefore, the later method has been followed.

A varying degree of insulin loaded nanoparticles were prepared by allowing 100 mg of nanoparticles to swell in the drug solution till equilibrium, and then washing and drying them to obtain the release device. The percent loading of drug was calculated by following Equation 1:

$$\%\text{Loading} = \frac{W_d - W_0}{W_0} \times 100 \quad (1)$$

Where W_d and W_0 are the weights of loaded and unloaded nanoparticles, respectively.

2.4 Swelling Measurements

The extent of swelling was determined by a conventional gravimetric procedure (29). In a typical experiment, the dried nanoparticles of known weight (W_d) were placed in a known volume of phosphate buffer saline (PBS, pH 7.4) at room temperature until equilibrium swelling was attained. The swollen particles were pressed gently in between two filter papers to remove excess solvent and then weighed (Ws) accurately. The weight swelling ratio was calculated by the following Equation 2:

$$\text{Weight swelling ratio} = \frac{\text{Weight of swollen nanoparticles}}{\text{Weight of dry nanoparticles}} \quad (2)$$

2.5 *In-vitro* Release Experiment

Before performing *in vitro* drug release experiments, the stability of gelatin nanoparticles was checked in released medium (PBS). For this purpose, a known dry weight of nanoparticles were mildly shaken in PBS for a definite time period and after the shaking is complete, the nanoparticles were centrifuged, dried and weighed. It was found that there was almost no difference in the dry weights of nanoparticles before and after shaking. This provides experimental evidence of the nanocarrier's stability.

In vitro release of insulin from loaded nanospheres was studied in phosphate buffer (pH 7.4) saline. The release of insulin was carried out by placing the dried and loaded nanoparticles (50 mg) in a test tube containing a definite volume (10 mL) of phosphate buffer saline (PBS) as the release medium (pH 7.4, 1.2 mM KH_2PO_4, 1.15 mM Na_2HPO_4, 2.7 mM KCl, 1.38 mM NaCl). The resulting suspension was gently shaken for a predetermined time period (half hour) and after shaking was over, 5 mL of supernatant was withdrawn and replaced by 5 mL fresh PBS. The aliquot was removed and subjected to UV scan which showed absorption maxima at 271 nm corresponding to the insulin present. The sample was analyzed for insulin content by measuring its absorbance in a UV-Vis. Spectrophotometer (Shimandzu 1700 Phama Spec) and the released amount of insulin was determined with the help of a calibration plot.

In order to ensure that gelatin nanoparticles do not lose gelatin due to its dissolution into the release medium, stability of nanoparticles was checked by gently shaking the unloaded nanoparticles for a definite period. After the shaking was complete, the release medium was analyzed for dissolved gelatin spectrophotometrically. It was found that almost no gelatin was dissolved into the release medium.

2.6 Kinetics of Release Process

For monitoring the progress of the release process, 5 mL of aliquots were withdrawn at desired time intervals and instantly replaced by fresh release medium (PBS). In the aliquots withdrawn, the amount of insulin was determined spectrophotometrically as described above.

For achieving mechanistic insights into the release process, the following Equation 3 was used (30):

$$W_t/W_\infty = Kt^n \quad (3)$$

Where $Wt/W\infty$ is the fractional release at time t and equilibrium time, respectively and k

$$\frac{W_t}{W_\infty} = 4\left(\frac{D_t}{\pi l^2}\right)^{0.5} \quad (4)$$

is rate constant. The diffusion constant may be calculated by the above Equation 4 (31) and exponent n is release exponent.

Where l is the diameter of dry nanoparticle, and D is the diffusion coefficient.

In order to calculate the value of n a plot is drawn between logarithm of fractional release and t and n is calculated from the slope of the linear plot. Similarly, the value of D can be calculated from the slope of the plot drawn between fractional release and $t^{0.5}$.

2.7 Modeling of Release Mechanism

A drug (insulin) loaded gelatin nanoparticles may be visualized as a three dimensional network of macromolecular chains of gelatin containing insulin molecules which occupy the space available between the network chains. When such drug loaded nanoparticles are allowed to swell in a release medium, the solvent (normally water) molecules enter into the nanoparticles network as a result of their diffusion into the nanoparticles matrix and subsequent relaxation of polymer chains. The insulin molecules dissolve into water and release out through water channels. The diffusion of insulin molecules and relaxation of gelatin chains determine the type of release mechanism being followed by the drug molecules. It has been laid down by the Higuchi equation (32) that if $n = 0.43$, the release is diffusion controlled (Fickian), when $n = 0.84$ the release is said to be non Fickian (or case II). For n being in between 0.43 and 0.84, the mechanism becomes anamolues.In same cases, however, the value of n has been found to exceed 0.84 and the mechanism is known as super case II (33). The values of D and n have been calculated as described above and the summarized in Table 1.

2.8 Statistical Analysis

In order to test the reliability of the experimental data, all experiments were done at least three times. The data summarized in the Tables have been expressed along with the calculated S.D. values and the curves have been drawn showing the respective error bars.

3 Results and Discussion

3.1 Characterization of Nanoparticles

3.1.1. *FTIR spectral analysis*

Glutaraldehyde is a well known crosslinking agent for proteins and it reacts easily at room temperature with color change characteristics of Schiff base linkage. The color change is due to the formation of aldimine linkage (−CH=N−) between the free amino groups of protein and gluteraldehyde.

The structural characterization of the prepared nanoparticles was done by recording FTIR spectra of insulin-loaded nanoparticles, native gelatin, insulin and glutaraldehyde on a FTIR spectrophotometer, as shown in Figure 2 (a), (b), (c) and (d), respectively. The IR spectra (a) clearly shows strong bands at 3150 cm^{-1} (N−H stretching) and 1650 cm^{-1} (C=O stretching) due to the presence of gelatin (34). The absorption spectra at 1450 cm^{-1} represent aldimine linkage and, thus, provide strong evidence of crosslinking of gelatin due to glutaraldehyde. The bands at 3670 cm^{-1} confirm the presence of N−H stretching while −NH bending/carboxylate ion stretching at 1700 cm^{-1} indicate the presence of insulin (see the peaks of native insulin in spectra (c)) in the drug-loaded nanoparticles (35).

It is worth mentioning here that the IR spectra (a) of well purified and neutralized gelatin nanoparticles do not show any peaks due to glutaraldehyde (spectra d) and this also confirms that the prepared and detoxified gelatin nanoparticles are free from toxic glutaraldehyde and ,therefore, may be employed for oral administration.

Table 1. Data showing the release exponents and diffusion coefficients under varying experimental conditions

Gelatin (g)	*Glutaraldehyde (mM)*	*pH*	*n**	*$D \times 10^{13}$ cm^2 min^{-1}**
1	10.6	7.4	0.42 ± 0.016	2.0 ± 0.092
2	10.6	7.4	0.22 ± 0.009	2.1 ± 0.088
3	10.6	7.4	0.18 ± 0.007	2.4 ± 0.098
4	10.6	7.4	0.51 ± 0.020	1.9 ± 0.078
2	5.3	7.4	0.36 ± 0.015	1.7 ± 0.082
2	15.9	7.4	0.42 ± 0.018	1.7 ± 0.078
2	21.2	7.4	0.30 ± 0.014	1.1 ± 0.062
2	10.6	8.6	0.46 ± 0.020	0.3 ± 0.001
2	10.6	1.2	0.22 ± 0.006	1.9 ± 0.083

*Data have been expressed as Mean ± S.D. of at least three determinations.

3.2 SEM and Particle Size Analysis

The SEM image of nanoparticles is shown in Figure 3, which clearly suggests a smooth morphology of the gelatin nanoparticles. The SEM image also reveals that the size of nanoparticles is not uniform and varies in the range 50 to 200 nm. The image also reveals that no hair cracks and

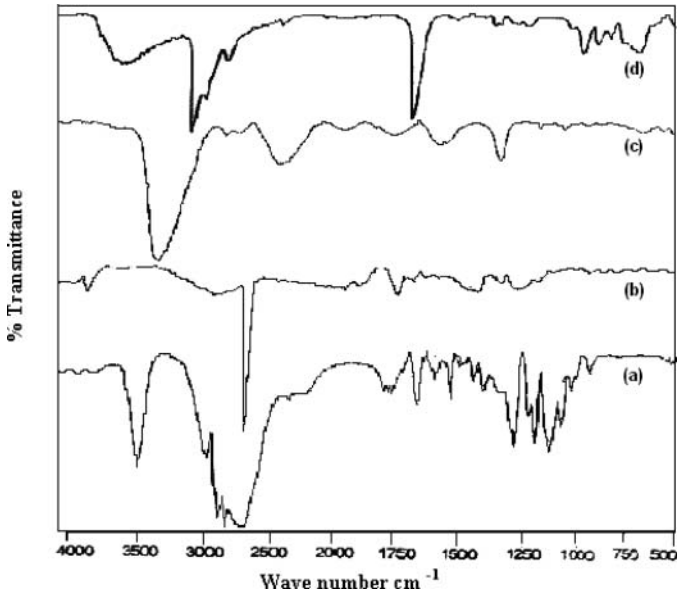
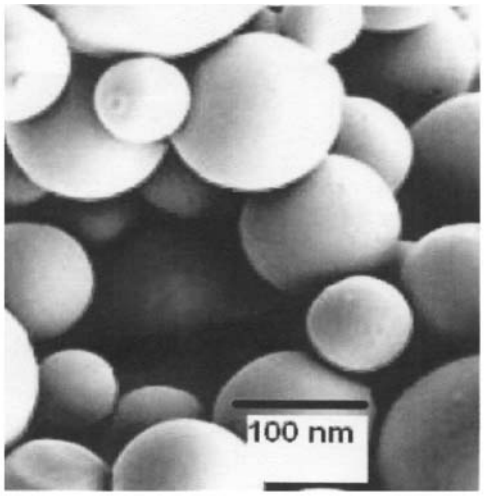


Fig. 2. FTIR spectra of insulin loaded gelatin nanoparticles (a) loaded gelatin nanoparticles; (b) Gelatin; (c) Insulin;(d) Glutaraldehyde.



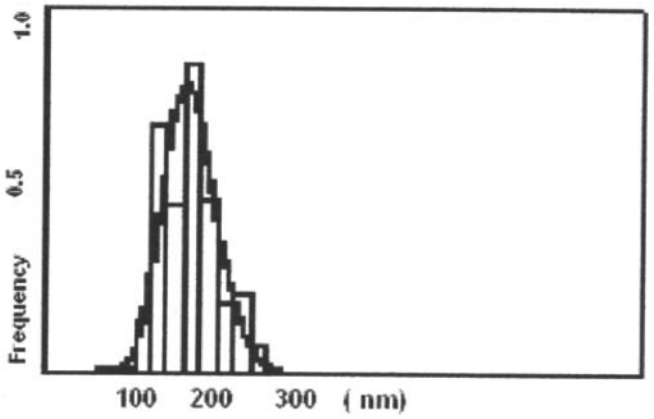


Fig. 3. Scanning electron micrograph (SEM) and particle size distributition of gelatin nanoparticles.

heterogeneities are present on the particles surfaces thus indicating their smooth and even surfaces.

The particle size distribution curve is depicted in Figure 3, which indicate that the size varies in the range 50 to 250 nm. The dimension of the gelatin nanoparticles determined from particle size analysis and SEM study seem to vary to a little extent. It is important to mention here that recent studies indicate that particles between 100 to 200 nanometres in diameter are the optimal size at which nanoparticles are able to be carried through the wall of the human intestine (36).

3.3 Surface Potential Measurements

The surface potential measurements are particularly important in those systems where the particles bear charged functionalities which vary with varying pH of the suspension or solution. In the present system since both the gelatin nanoparticles and insulin molecules are charged species, obviously the suspension of insulin loaded nanoparticles offers a definite charge which to a greater extent depends on factors such as pH of the suspension, isoelectric point of the gelatin, nature of charge carried by the drug molecule and drug- gelatin interaction. In the present work emf values of various buffer solutions and insulin loaded gelatin nanoparticles have been determined and summarized in Table 2. The results may be discussed as below:

Table 2. Data showing surface potentials of loaded gelatin nanoparticles

pH of Release medium	*EMF(mV)* Buffer Solution*	*EMF(mV)* Loaded particles*
1.2	238 ± 9.6	289 ± 13.2
7.4	51 ± 2.2	59 ± 3.7
8.6	−39 ± 1.8	−128 ± 6.8

*EMF data have been expressed as Mean ± S.D. of at least three determinations.

1. The emf of buffer solutions is positive when their pH values are in the acidic range while a negative emf is seen at alkaline pH of the solution. The negative value of emf may be explained on the basis of the well known Nernst theory.
2. When the suspensions of insulin loaded gelatin nanoparticles are measured, it is noticed that at pH 1.2 and 7.4, the positive emf increases significantly while at pH 8.6 the emf acquires a large negative value. The positive and negative potentials may be explained by the fact that since the isoelectric point of gelatin is near 7.8, the macromolecules of gelatin will bear a positive charge below this isoelectric point, while after this value, the gelatin molecule bears a net negative charge as is evident from the negative emf value.
3. It is also believed that due to the loading of insulin molecules, which also bear a small positive charge, the net charge over gelatin macromolecules must have increased to a small extent.

3.4 Chemical Stability of Drug

As discussed previously the insulin 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 pH 7.4 medium and comparing the UV spectra of released insulin with that of the native insulin solution prepared in PBS (pH 7.4). The two spectra are shown in Figure 4 (a) and (b), respectively which clearly indicate that they are almost identical to each other and obviously suggest that remaining in highly acidic media does not change the chemical nature of insulin, Moreover, it was also found that even gelatin nanoparticles do not undergo any cleavage in gastric juice medium. This clearly explains the stability of drug carrier system in highly acidic media (37) and, therefore, justifies its suitability as a carrier for insulin in controlled release.

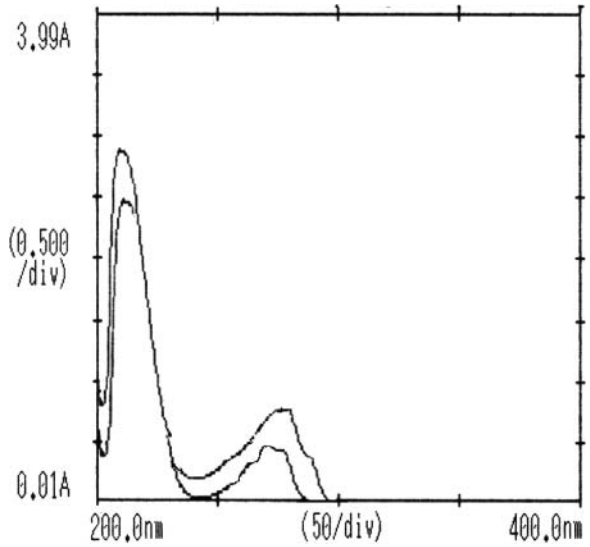


Fig. 4. UV spectra of Pure insulin solution at pH 7.4 (upper curve), and released insulin solution at pH 7.4 (lower curve).

3.5 Results of Swelling Experiments

3.5.1. *Effect of gelatin*

Gelatin is a hydrophilic biopolymer and, therefore, its increasing amount may result in an enhanced hydrophilicity of nanoparticles network when added to the reaction mixture. In the present study, the impact of hydrophilicity of the network on its water sorption property was investigated by varying the amount of gelatin in the range 1–4 g.The results are summarized in Table 3, which clearly indicate that there is gradual fall in the swelling ratio of the particles in the studied range of gelatin concentration.The observed results may be attributed to the fact that as the number of hydrophilic gelatin chains increases, the nanoparticle networks become more and more dense due to the increasing interaction among the gelatin molecules and consequently, a smaller number of water molecules penetrate into the nanoparticles network. This clearly brings about a reduction in the swelling ratio of the nanoparticles. Similar types of results were also reported by other workers (38).

Table 3. Data showing the influence of composition and experimental condition on the equilibrium swelling ratio

Gelatin (g)	*Glutraldehyde (mM)*	*pH*	*temperature (T)*	*Equilibrium Swelling Ratio**
1	10.6	7.4	25	4.5 ± 0.14
2	10.6	7.4	25	4.0 ± 015
3	10.6	7.4	25	3.7 ± 0.11
4	10.6	7.4	25	3.4 ± 0.12
2	5.3	7.4	25	4.5 ± 0.19
2	10.6	7.4	25	4.4 ± 0.18
2	15.9	7.4	25	4.2 ± 0.22
2	21.2	7.4	25	4.0 ± 0.23
2	10.6	1.2	25	3.2 ± 0.16
2	10.6	7.4	25	4.5 ± 0.25
2	10.6	8.6	25	3.1 ± 0.17
2	10.6	7.4	37	3.8 ± 0.22

*Swelling ratios have been expressed as Mean ± S.D. of at least three determinations.

3.5.2. *Effect of crosslinker*

The effect of crosslinker on water sorption capacity of particle has been studied by varying the concentration of glutaraldehyde in the range 5.3–21.2 mM. The results are shown in Table 3, which clearly show that increasing the concentration of crosslinker results in a substantial fall in the swelling ratio of the nanoparticles. The results may be explained by the fact that on increasing the concentration of crosslinker the number of crosslinks in the macromolecular network of nanoparticles increases which results in a reduction in mesh sizes of the network. This obviously leads to a slow diffusion of water molecules into the network and restricts relaxation of network chains thus resulting in a fall in the water sorption capacity. Similar types of results have also been reported elsewhere (39). It has also been reported that with increasing crosslink density of the network, the glass transition temperature (Tg) of biopolymer also increases witch results in stiffening of network chains (40). The enhanced stiffening of chain lowers the relaxation of gelatin chains, which results in lower uptake of water molecules by the gelatin nanoparticles.

3.6 Effect of pH

The role of pH in regulating water sorption behavior of nanoparticles is of grater significance, as a variation in pH of the swelling medium often results in a change in free volumes accessible to penetrant water molecules, which in turn, affects swelling characteristics of the nanoparticles. In the present investigation, the effect of change in pH of the swelling medium was investigated by varying pH of the swelling bath in the range 2.0 to 7.4. The results are shown in Table 3, which indicate that at acidic pH value (2.0) the swelling is low while it attains an optimum value at pH 7.4. The reason for the optimum swelling at pH 7.4 may be attributed to the fact that since this pH is close to the isoelectric point of the gelatin and therefore, at this particular pH, gelatin chains become electrically neutral acquiring equal number of positive and negative charges present along the chains. This obviously results in an increase in ionic concentrations inside the nanoparticles network which causes an increase in osmotic pressure which consequently results in an increase in swelling as predicted by the theory.

3.7 Effect of Temperature

The influence of temperature on the swelling of particles is of great significance as it directly controls the diffusion of water molecules into the nanoparticles network and water-polymer interaction. In the present study, the effect of temperature on the degree of water sorption has been investigated by carrying out water sorption experiments in the range 25–37°C .The results are presented in Table 3, which clearly indicate that the swelling ratio decreases with increasing temperature of the swelling medium. The results may be explained by the fact that at higher temperature, the hydrogen bonds between the water molecules and gelatin macromolecules are broken and this converts bound water into free water (28). Now, because of faster relaxation of polymer chains, water molecules are then forced out, thus resulting in a lower water sorption.

3.8 Effect of Physiological Fluids

It is well established that the equilibrium swelling behavior of a polymer network in a solvent is the result of a balance between osmotic and restoring elastic pressure. The presence of salt ions in the surrounding medium is capable of tilting this balance and may result in either an increase or decrease in swelling. Since drug release carriers often come in contact with various biofluids in the body which essentially contain electrolytes, it is important to study the swelling behaviour of nanoparticles in various simulated biological fluids. In the present study the effect of biological fluids on the extent of swelling has been examined by performing swelling experiments in the presence of D-glucose, urea (5%, w/v), saline water (0.9%) and synthetic urine. The results are summarized in Table 4 which shows a decrease in swelling ratio. The observed decrease may be attributed to a fall in osmotic pressure of the nanoparticles-biofluids system due to increased ionic concentration in the swelling bath. Furthermore, the salt ions-gelatin interaction may also result in a lower water sorption.

Table 4. Data showing the swelling ratio of the particle in various simulated physiological media

Physiological fluids	*Swelling ratio * (after 5 h)*
PBS	4.5 ± 0.14
D-Glucose	4.0 ± 0.13
Saline water	3.8 ± 0.14
Synthetic urine	3.6 ± 0.19
Urea	3.5 ± 0.18

*Swelling ratios have been expressed as Mean ± S.D. of at least three determinations.

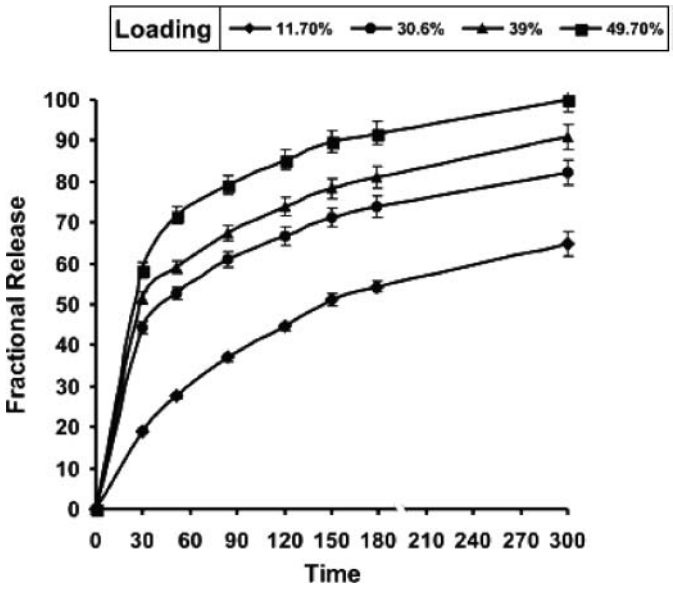


Fig. 5. Effect of % loading of insulin on its release profile for a definite composition of nanoparticles [gelatin] = 2.0 g, [glutaraldehyde] = 10.6 mM, pH = 7.4, Temperature = 25°C.

3.9 Results of Release Experiments

3.9.1. *Effect of percent loading on insulin release*

An important aspect in the use of nanoparticles as drug carrier is the effect of drug loading levels on the rate of drug release. Apart from other factors, the amount of drug delivered depends on the extent of loading and its subsequent release into the release medium. The varying degree of insulin loaded nanoparticles were obtained by equilibrating definite amount of nanoparticles in drug solutions of different concentration varying in the range 11.7–49.7% (v/v).The swollen nanoparticles were washed with distilled water and dried at room temperature for one week.

The release experiments were conducted in PBS medium using varying degrees of insulin loaded nanoparticles. The release results are shown in Figure 5, which reveal that the released amount of insulin increases with increasing percentage loading in the range 11.7–49.7%. The results are quite expected and may be explained by the fact that the larger the initial load, the faster the movement of the solvent front penetrating the surfaces of the loaded nanoparticles. A higher loading of the particle may also facilitate relaxation of macromolecular chains of the nanoparticles, and thus, may result in a greater amount of released insulin. Similar types of results have also been reported by other workers (41).

3.10 Effect of Gelatin on Insulin Release

Drug release profiles are often sensitive to chemical architecture of the carrier, as well as experimental conditions of its fabrication. The size and morphology of nanoparticles

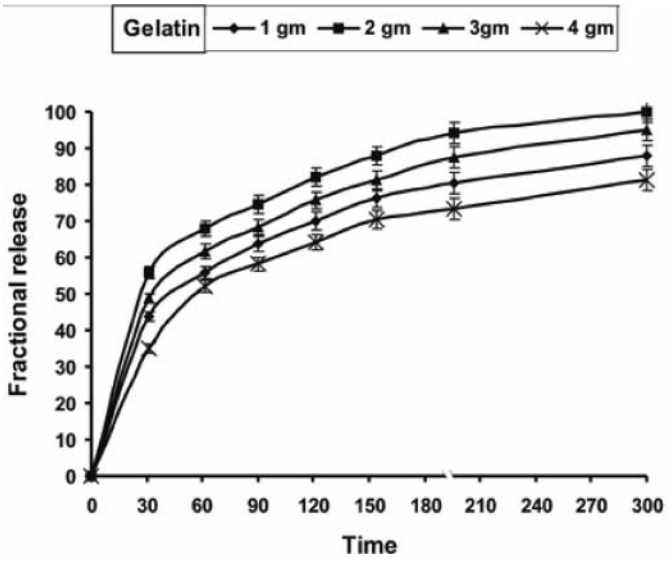


Fig. 6. Effect of varying amount of gelatin in nanoparticles on release profiles of insulin for a definite composition of nanoparticles [glutaraldehyde] = 10.6 mm, pH =7.4, Temperature = 25°C, % loading 49.7%.

may be affected by factors such as the concentrations of gelatin and glutaraldehyde in the feed mixture, molecular weight of polymethyl methacrylate and temperature and shaking time of emulsions. The influence of gelatin on the release profiles of insulin has been investigated by varying the amount of gelatin in the range 1.0 to 4.0 g.The results are shown in Figure 6 which clearly reveals that release of insulin increases up to 2.0 g of gelatin, while beyond it there is a fall in the released amount. The reason for the observed initial increase is apparent because gelatin is a hydrophilic polymer and its increasing amount will increase hydrophilicity of the network. This results in enhanced water sorption, which consequently leads to a larger release of insulin. However, beyond 2.0 g of gelatin content, the network density becomes so high that the network chains hinder the inclusion of incoming water molecules as well as outgoing insulin molecules and, therefore, the release rate decreases. It is also likely that, due to greater volume fraction of polymer in the network, the molecules of water and insulin have to travel a longer path which obviously results in a lower release of insulin.

Although the present system is swelling controlled and the amount of released insulin varies proportionally with the extent of swelling of the nanoparticles, however, there are numerous other factors which may affect the amount of released drug. One of such factors is the interaction between polymer (gelatin) and drug which may also affect the released profile. In the present case, the increases in release up to 2 g of gelatin content may be due to the reason that although swelling is low, however increased, hydrophilicity of the network may result in higher release. But on further increasing the amount of gelatin, the decrease in the released amount may be due to the increasing insulin - gelatin interaction which may result in lower release of the drug. Furthermore, the decreasing swelling ratio also results in a fall in the released amount of insulin.

3.11 Effect of Crosslinker

A large number of crosslinkers are known for gelatin among which genipin, citric acid, adipic acid, proanthocyanidinare and glutaraldehyde (42) are the most common ones. In the present study, however, glutaraldehyde has been used because its reaction is rapid; it is less expensive, readily available and highly soluble in aqueous solutions. Glutaraldehyde is an important reagent in the biomedical field, as it has been used extensively used as a crosslinking agent for the preparation of bioprostheses, such as heart valves, vascular grafts, elastic cartilage, tendon xenografts and artificial skin. Glutaraldehyde is presumed to crosslink by inter and intra molecular covalent bonds (43).

One of the effective means of modifying the release characteristics of a drug carrier is by manipulating the employed amount of crosslinker in the feed mixture. This usually results in a change in the release behavior of the drug in a complex way. In the present investigation, the amount of crosslinker has been varied in the concentration range 10.6–21.2 mM in the feed mixture. The results are shown in Figure 7 which indicate that on increasing glutraldehyde concentration in the studied range the fractional release is appreciably reduced.The decrease observed may be attributed to the fact that on increasing the concentration of glutaraldehyde in the nanoparticle, the number of crosslink points increases which increases the chain density of the nanoparticle network, thus reducing the mesh sizes of the

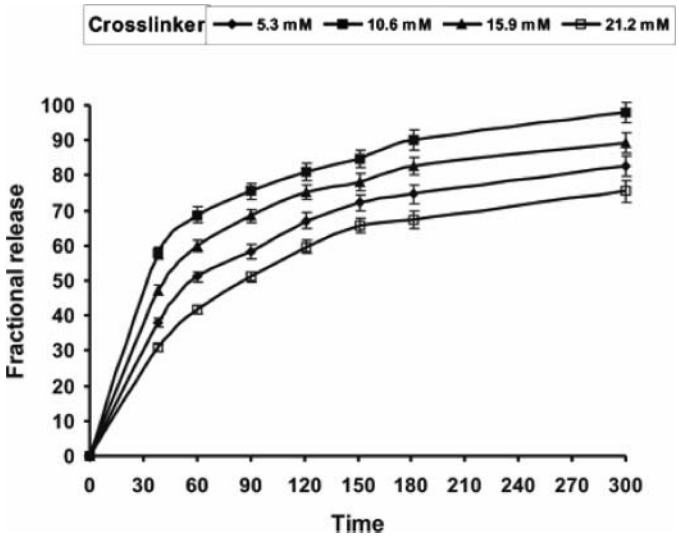


Fig. 7. Effect of varying amounts of glutaraldehyde (crosslinker) on release profiles of insulin for a definite composition of nanoparticles [gelatin] = 2.0 g, pH =7.4, Temperature = 25°C, % loading 49.7%.

free volumes available between the network chains. Thus, an increased crosslink density makes the passage of water molecules difficult from the release medium into the loaded nanoparticle network, consequently, it also hinders the diffusion of larger insulin molecules from loaded nanoparticles into the release medium. This obviously decreases fractional release of insulin. The decrease in fractional release with increasing crosslinker concentration has been widely reported (44).

3.12 Effect of pH

Oral administration of macromolecular drugs remains a significant challenge because peptides and proteins are susceptible to hydrolysis and digestion by the acid and enzymes in the gastrointestinal (GI) tract. Also, the bioavailability of orally delivered peptides and proteins is very low due to membrane permeability. Thus, to improve therapeutic efficiency and reduce or eliminate side effects of orally administered drugs, it is reasonable to deliver drug to specific regions of the GI tract. Several methods of targeting to specific sites have been proposed. Two of these are the utilization of pH change between stomach and the small intestine within the GI tract (45,46) and exploitation of bacterial enzymes localized within the colon (47) are of current interest in controlled drug delivery system.

In order to study the effect of pH on the fractional release of insulin two pH values were selected as 2.0 and 7.4, which are identical to the acidic and alkaline environment of stomach and blood, respectively. When insulin is administered orally, it first goes to stomach where pH is acidic and then passes to the intestine region of alkaline pH value .Thus, in order to mimic these *in vivo* conditions the insulin loaded nanoparticles were first left in pH 2.0 medium for 2 h and then the carriers were transferred to release media of pH 7.4.The results are shown in Figure 8 and may be explained as below:

In acidic pH the gelatin nanoparticles swell to a lesser extent and, therefore, less number of water molecules goes into the nano particles matrices. This obviously causes a lower amount of insulin release. However, in alkaline medium (pH 7.4) the nanoparticles swell to a greater extent and results in a larger fractional release (48).

3.13 Effect of Temperature

The release behavior of insulin is greatly concerned with the temperature of the release medium. In the present study, the temperature of release medium has been varied in the range 25–37°C and its effect on the fractional release of insulin has been investigated. The results are displayed in Figure 9, which indicate that with increasing temperature, the fractional release of insulin decreases. The observed decrease in the fractional release may be attributed to the fact that with increasing temperature the hydrogen bonds between the water molecules and network chains are broken

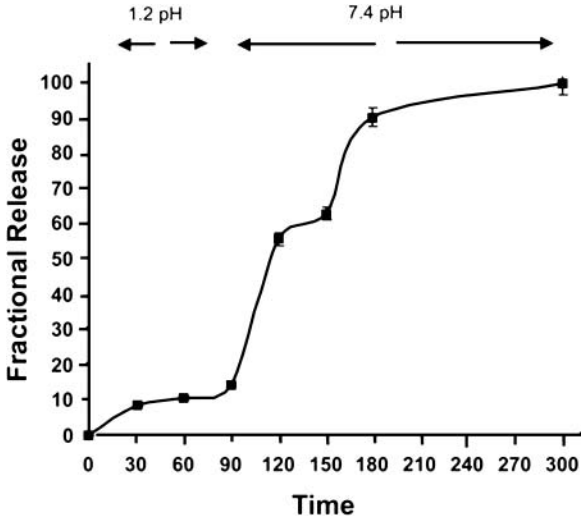


Fig. 8. Variation in released amount of insulin with varying pH of the release medium for a definite composition of nanoparticles [gelatin] = 2.0 g, [glutaraldehyde] = 10.6 mM, Temperature = 25°C, % loading 49.7%.

which results in a lower swelling of the nanoparticles and consequently lower release of the drug.

3.14 Effect of Simulated Physiological Fluids

The effect of nature of the medium on the release kinetics of insulin has been investigated by performing release experiments in various simulated physiological fluids. The results are depicted in Figure 10, which reveal that the fractional release of insulin is significantly suppressed in physiological fluids compared to that in the PBS. The lower release of insulin in these biofluids may be due to the presence of salt ions in the release medium which lower the ion osmotic pressure of the system. This obviously causes lower

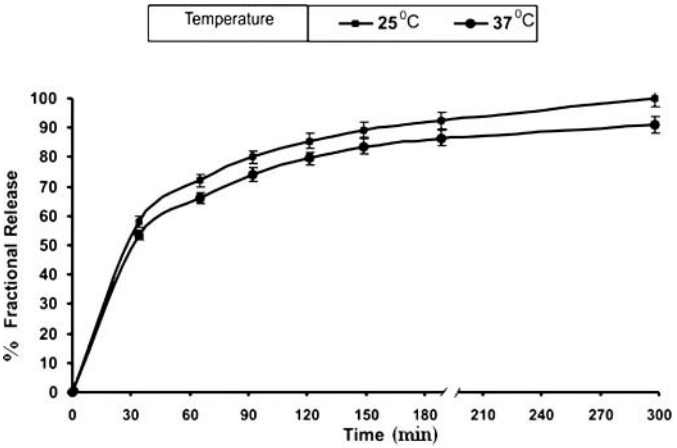


Fig. 9. Effect of temperature on the release amounts of insulin for a definite nanoparticles composition [gelatin] = 2.0 g, [glutaraldehyde] = 10.6 mM, pH = 7.4, % loading = 49.7%.

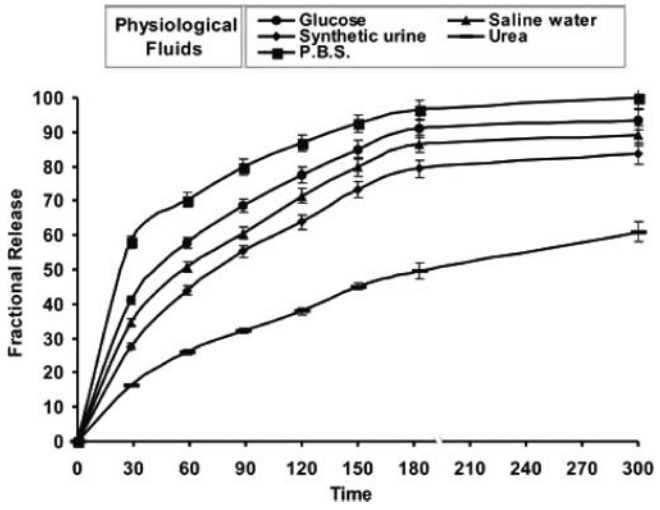


Fig. 10. Effect of physiological fluids on the released amount of insulin for a definite nanoparticles composition [gelatin] = 2.0 g, [glutaraldehyde] = mM, pH = 7.4, % loading = 49.7%.

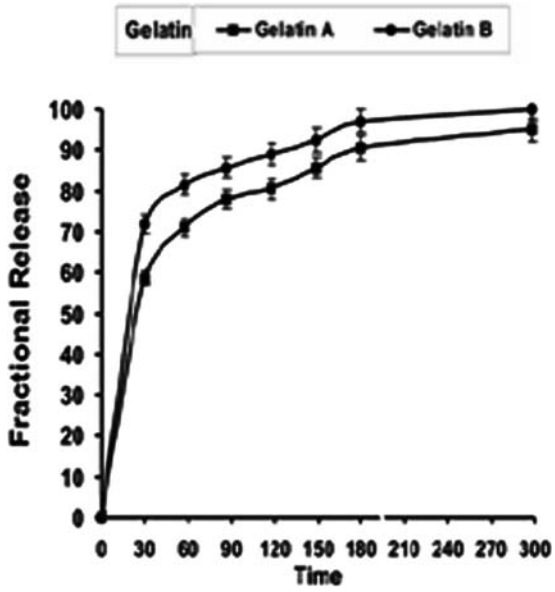


Fig. 11. Effect of type of gelatin on the released amount of insulin for a definite composition of nanoparticles [gelatin] = 2.0 g, [glutaraldehyde] = 10.6 mM, pH = 7.4, % loading = 49.7%.

swelling of loaded nanospheres, which consequently results in a lower release of insulin.

3.15 Effect of the Type of Gelatin

Gelatin is a natural polymer that is extracted from collagen by alkaline or acidic pretreatment and thermal denaturation. Depending on this pretreatment two types of gelatin can be distinguished, A and B. Gelatin A is extracted from bovine skin, and processed by alkaline pretreatment which converts glutamine and aspargine residues into glutamic acid and aspartic acid, respectively and results in a higher carboxylic acid content for gelatin B (118/1000 amino acids) than for gelatin A (77/1000 amino acid). In order to study the influence of type of gelatin on the release profile of insulin the drug was loaded onto nanoparticles of both gelatin A and B and the released amounts of insulin were determined under identical experiment conditions. The results are shown in Figure 11, which clearly indicate that the released amount of drug is quite higher for type B than that for type A .The results may be explained by the fact that at the experimental pH 7.4 the gelatin B molecules possess net negative charge because the isoelectric point of gelatin B (4.8) is less than the experimental pH. Thus, the insulin molecules, which are almost fully ionized at pH 7.4, will attach to these negatively charged centers present along the gelatin molecules and, therefore, results in a greater percent loading. When largely loaded nanoparticles of type B gelatin are placed in the release medium the $-COO^-$ groups present along the gelatin chains repel each other, thus producing a greater repulsion in the nanoparticles. This obviously results in a larger swelling of the loaded nanoparticles, which in turn, produces greater release of insulin with type B gelatin nanoparticles. A Similar type of results has also been published elsewhere (49).

4 Conclusions

By careful adjustment of experimental protocol, the solvent evaporation technique has resulted in fabrication of gelatin nanoparticles in the range 50 to 250 nm for possible oral delivery of insulin. The size of the nanoparticles is further confirmed by SEM and particle size analysis results.

The nanoparticles produced show adequate water sorption capacity which, however, decreases with increasing amount of gelatin and crosslinker (glutaraldehyde). The water intake capacity of gelatin nanoparticles depend significantly on pH and temperature and show optimum swelling at pH 7.4 and temperature 25°C.

When insulin is loaded onto the nanoparticles, their surface potential acquires enhanced positive value thus supporting attachment of insulin molecules to the particle surfaces. The amount of insulin release increases with increasing percent loading of insulin.

The release of insulin also varies with chemical composition of the nanoparticles. It is observed that when gelatin is 2.0 g, the release of insulin is maximum. Similarly with increasing crosslinker concentration the released amount decreases.

The gelatin nanoparticles fulfill the criteria of nanocarriers to be employed for oral delivery of insulin. That is, they offer minimum release of insulin at low pH (simulated gastric pH) while optimum release is noticed at higher pH (intestinal pH). Furthermore, the amount of insulin release

also decreases with increasing temperature. In simulated biofluids also a lower release of insulin is observed. The nature of gelatin also effects drug release and greater release is observed when nanoparticles are expanded from Type B gelatin.

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