Abstract
The present work focuses on designing of genipin crosslinked nanoparticles of gelatin and evaluating water imbibitions capacity of nanoparticles under varying experimental conditions. The gelatin nanoparticles of different composition and sizes were prepared by microemulsion crosslinking method and characterized by different techniques like Fourier Transform Infrared spectroscopy (FTIR), scanning electron microscopy (SEM), transmission electron microscopy (TEM), particle size analysis and surface charge measurements. The effect of composition was studied on the size of gelatin nanoparticles. The nanoparticles were investigated for their water intake capacity under varying experimental conditions like pH, temperature, presence of biological fluids and chemical composition of the nanoparticles.

Key words: Gelatin, nanoparticles, characterization, genipin, swelling.

Introduction
Gelatin is known to be a potential biomacromolecule derived from animal collagen by acidic or alkaline hydrolysis. Looking to its unique non-cytotoxicity, biodegradability and anti-carcinogenicity, it has been recognized as GRAS, ie. generally regarded as safe material by the Food and Drug Administration of the USA. This biopolymer has been exhaustively used in various pharmaceutical, cosmetics and food application [1, 2]. Its use in medicine as intravenously administered plasma expanders is one of the vital biomedical applications. Structurally gelatin is a polypeptide that possesses cationic groups like lysine and arginine, anionic moieties like aspartic and glutamic acid and hydrophobic groups like valine, leucine, isoleucine and methionine [3]. As for as the chemical composition of gelatin is concerned, the gelatin is dominated by amino acids like glycine which forms its 33% portion and orients into the core of the triple-helix, while its 24% is dominated by proline and 4%-hydroxyproline. The rest are other residues. Gly-X-Y represents the continuously repeating amino acid sequence. A typical structure is “-Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro-” as shown in Figure 1 [4]. Depending on the acidic or alkaline conditions of hydrolysis of collagen, the gelatin is available in two types; viz. type A and B, respectively having isoelectric points (IEP) at 6-9 and 4.8-5.0.

Realizing the fascinating properties of gelatin the last several decades have witnessed continuing research efforts in designing gelatin based drug delivery systems [5]. The rational for attempting to fabricate gelatin based drug delivery systems lies in the fact that this biopolymer has extraordinary pharmaceutical and physicochemical properties like biocompatible nature, ease of modification and derivatization, unusual loading capacity, fairly stable shelf life etc. [6, 7]. Although gelatin nanoparticles have largely been investigated for delivering a variety of drugs, however, anti-cancer drugs have been a main focus of investigations [8, 9]. The inclination of researchers towards anticancer drugs using gelatin nanoparticles is mainly due to ability of gelatin nanoparticles to be used for passive targeting because of enhanced permeability and retention effects (EPR effects), that enable drug loaded gelatin nanoparticles to remain at the diseased site for longer duration thus achieving complete removal of the drug [10]. In addition to anticancer drugs, the gelatin nanoparticles have also been employed to deliver proteins and vaccines Moreover, macrodugs like insulin [11], bovine serum albumin (BSA) [12], alkaline phosphatase (ALP) [13] and angiogenic basic fibroblast growth factor (BFGF) [14] have

Figure 1: Basic chemical structure of gelatin
been successfully encapsulated into gelatin nanoparticles with retained in vivo bioactivity. Apart from nanoparticles, the hydrogels of gelatin have also been found to protect the plasmid DNA in the systemic circulation and upon cellular transport [15]. Another novelty of gelatin nanoparticles have been their versatility in different administration routes that include peroral, ocular, pulmonary and parenteral [16]. Looking to the great potential of gelatin nanoparticles in drug delivery applications, the present study aims investigate swelling behaviour of gelatin nanoparticles which is a property of prime importance in designing swelling controlled drug delivery systems. The overall performance of a drug delivery system greatly depends on the strategy adopted to prepare nanoparticles as it is the property that decides the efficacy of a drug delivery system. Although several synthetic routes like coacervation [17-19], solvent extraction-emulsification [20-22], nanoprecipitation [23, 24] and self-assembly [25] are available for preparation of gelatin nanoparticles, however, in the present work emulsion crosslinking method has been used to prepare gelatin nanoparticles.

**Experimental**

**Materials**

Acid processed gelatin (Type A, 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, isoelectric point 4.8) extracted from human bone was purchased from E.Merck, India. Genipin was obtained from Sigma Aldrich, USA and used as received. Polyethyl methacrylate (PMMA) (Sigma Aldrich Co., USA, Average MW ~ 120,000 Da, inherent viscosity 0.20) was used for preparing oil phase. Other chemicals and solvents were of analytical reagent grade. Bi-distilled water was used throughout the experiments.

**Methods**

**Preparation of Nanoparticles**

The preparation methods of nanoparticles for pharmaceutical use are divided broadly into two categories, those based on physicochemical properties such as phase separation [27] and solvent evaporation [28], and these based on chemical reactions such as polymerization and polycondensation. In the present study, emulsion crosslinking technique was followed which may briefly be described as below:

'Aqueous phase' was prepared by dissolving a definite amount of gelatin in distilled water while for oil phase paraffin oil was used. 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, a fixed volume of genipin emulsion prepared in 9mL ethane diol & 1mL paraffin oil. The crosslinking reaction was allowed to take place for predetermined time period. Nanoparticles so prepared were cleaned by centrifuging and re-suspending in toluene three times and then twice in acetone. The final product was dried at room temperature to obtain a fine bluish powder which was stored in air tight polyethylene bags. The whole reaction scheme of crosslinking of gelatin with genipin may be depicted as shown in **Fig. 2**. The reaction between gelatin and genipin is not well characterized but it has been proposed to occur in two distinct steps (Scheme I). In the first step (Scheme IA) rapid nucleophilic attack of a lysine amino group to the ring structure of genipin results in the opening of the dihydropyran ring and the formation of a tertiary amine. A subsequent slower reaction (Scheme IB) then results in the crosslinking process with nucleophilic substitution by a lysine amino group from a second fragment of gelatin. These two independent reactions lead to the crosslinking of gelatin and produce gelatin nanoparticles.

**Swelling Experiments**

It is recognized that the in vivo degradation of gelatin depends on their crosslinking extent, which can be widely regulated by changing the concentration of glutaraldehyde used for nanoparticles preparation [29]. In this study, the water content of gelatin nanoparticles prepared was determined as a measure of their crosslinking extent because it well correlates to the crosslinking extent [30]. The extent of swelling of nanoparticles was determined by a conventional gravimetric procedure. In a typical experiment, pre-weighed nanoparticles were allowed to swell in phosphate buffer saline (PBS, pH 7.4) for a predetermined time period (up to equilibrium swelling), thereafter the particles were taken out from the water and gently pressed in-between the two filter papers to remove excess of water and finally weighed using a sensitive balance (APX–203 Denver, Germany). Twenty-four hours was found to be enough for equilibrium swelling. The swelling ratio was determined by the following equation (1).

**Swelling ratio** = Weight of swollen nanoparticles/Weight of dry nanoparticles

**Characterization of gelatin nanoparticles**

**2.2 Methods**

**2.2.1 Preparation of Nanoparticles**

The preparation methods of nanoparticles for
pharmaceutical use are divided broadly into two categories, those based on physicochemical properties such as phase separation [31] and solvent evaporation [32], and those based on chemical reactions such as polymerization and polycondensation. In the present study, emulsion crosslinking technique was followed for preparation of genipin crosslinked gelatin nanoparticles, which may briefly be described as below: 'Aqueous phase' was prepared by dissolving a definite amount of gelatin in distilled water while for oil phase paraffin oil was used. 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, a fixed volume of genipin emulsion prepared in 9mL ethanediol & 1 mL paraffin oil. The crosslinking reaction was allowed to take place for predetermined time period. Nanoparticles so prepared were cleaned by centrifuging and re-suspending in toluene three times and then twice in acetone. The final product was dried at room temperature to obtain a fine bluish powder which was stored in air tight polyethylene bags.

2.2.2 Characterization:
(i) FTIR Spectra
The IR spectra of genipin cross linked gelatin nanoparticles was recorded on a FTIR Spectrophotometer (Shimadzu 8201 PC).

(ii) Scanning Electron Microscopy
Morphological studies of genipin crosslinked gelatin nanoparticles were performed using SEM, Philips 515, fine coater (Philips, Eindhoven, The Netherlands). Drops of the polymeric nanoparticles suspension were placed on a graphite surface and freeze-dried. The sample was then coated with gold by ion sputter at 20 mA for 4 minutes, and observations were made at 10 kV.

(iii) Transmission Electron Microscopy Study
The morphological features of genipin crosslinked gelatin nanoparticles were investigated by recording transmission electron micrographs (TEM) (Hitachi Hu-11 B), respectively.

(iv) Surface Potential Measurements
In order to understand the nature of the genipin crosslinked gelatin nanoparticle interaction surface potential studies were performed with a digital pH meter (Systronics Model No. Digital pH Meter MK VI, Ahmadabad, India). In a typical experiment 0.2 g nanoparticles were dispersed into 20 mL of respective pH solution and emf was recorded using a compound electrode system. A similar experiment was also repeated for drug loaded nanoparticles.

(v) Particle Size Analysis
GNPs were characterized for mean particle size (mean intensity weighted diameter, z-average) and polydispersity index (width of the size distribution, PI) by photon correlation spectroscopy (PCS), using a Zetasizer Nano ZS (Malvern Instruments, UK). For size determination, all samples were diluted fivefold with Milli-Q water.

3 Results and Discussion
3.1 Characterization
3.2 FTIR Spectra
The FTIR spectra of genipin cross linked gelatin nanoparticles is shown in Figure 3 which confirm the presence of gelatin and genipin in the prepared nanoparticles. The strong band appeared at 2930 cm⁻¹ may be assigned to C-H stretching thus confirming the presence of methyl group, whereas the band seen at 1526 cm⁻¹ may be attributed to the presence of gelatin caused by N-H bending vibrations of primary amine groups present in gelatin. The spectral peak at 1446 cm⁻¹ may be assigned to ring stretching vibration of dihydropyrane ring (heterocyclic ring) of genipin thus ensuring crosslinking of gelatin by genipin. Moreover, the band observed at 1680 cm⁻¹ may be assigned to C=N stretching of amine groups present in the gelatin molecule.

3.3 SEM Analysis
The morphological features of the prepared nanoparticles have been investigated by the scanning electron microscopy (SEM) analysis of gelatin nanoparticles as shown in Figure 4(a). It is clear from the observed image that shape of particles is uniform and the size of the nanoparticles is estimated up to 150 nm.

3.4 TEM Analysis
In order to look into more precise morphology of the gelatin nanoparticles, TEM studies were also performed and the obtained image is shown in Fig.4 (b). It is clear from the image that the particles are almost regular and spherical in shape and have sizes in the range of 50 to 150 nm.

3.5 Particle Size Analysis
The particle size distribution of genipin crosslinked gelatin nanoparticles have been carried out by dynamic light
scattering measurements. The results are depicted in Fig. 5 which clearly reveal that the size of nanoparticles varies between 50 to 150 nm with majority of particles having size of about 90 nm.

Table 1: Surface potentials of native genipin crosslinked gelatin nanoparticles

<table>
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<tr>
<th>Particles</th>
<th>Medium</th>
<th>ξ Potential (mV)</th>
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<tbody>
<tr>
<td>Gelatin Nanoparticles</td>
<td>1.8 Ph</td>
<td>54 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>7.4 pH</td>
<td>-38.4 ± 9</td>
</tr>
<tr>
<td></td>
<td>8.6 Ph</td>
<td>-62.1 ± 2.8</td>
</tr>
</tbody>
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In the present work the values of zeta potentials of genipin crosslinked gelatin nanoparticles were determined at different pH and the obtained results are summarized in Table 1. The results clearly reveal that when the pH varies from 1.8 to 8.6, the zeta potential significantly changes from 54 to -62 mV. Such a high value of zeta potential clearly suggests for highly stable suspension of genipin nanoparticles in aqueous media and it also indicates that the gelatin biomacromolecules have highly charged surfaces.

(v) Particle Size Analysis

It is a well-recognized and widely experimented fact that the size of particles has a great impact on the uptake of nanoparticles. In one of the studies it was demonstrated by Desai and coworkers [35] that when the particle size was 100 nm, the particle uptake was 2.5 times greater than those having a size of 1 μm, while more than 10 times was noticed in comparison to the particles of about 10 μm size in a Caco-2 cell line. So many researchers showed that the size of nanoparticles has severe impact on cellular and tissue uptake, while in some other cell lines only the submicron size nanoparticles were taken up efficiently as opposed to larger nanoparticles [36]. Thus, realizing the importance of nanoparticles size, in the present study the influence of experimental parameters was investigated on the size as well as polydispersity index of genipin crosslinked nanoparticles. It is known that the polydispersity index is a parameter that describes the second moment of the size distribution of the nanoparticles population and a lower polydispersity index implies for narrow size distribution of nanoparticles. In the present work the effect of gelatin, genipin and temperature of crosslinking of gelatin was studied on the size and size distribution of the gelatin nanoparticles.

(i) Effect of gelatin

When the amount of gelatin is varied from 1.0 to 7.0 g in the feed mixture of the nanoparticles, the particle size is found to increase in the range 58 to 170 nm as shown in the Fig. 6(a). The observed increase in the size of gelatin nanoparticles may be attributed to the fact that due to increase in the amount of gelatin the probability of aggregation also increases which brings about an increase in the particle size of the nanoparticles. Similar type of results has also been reported elsewhere [37] where the authors observed a remarkable increase in size of gelatin nanoparticles which were prepared by desolvation method. The authors also attributed their findings due to enhanced flocculation of gelatin nanoparticles. Some workers [38], however, have reported a decrease in the size of gelatin nanoparticles with increasing amount of gelatin in the feed mixture.

(ii) Effect of Crosslinker (genipin)

Genipin is a relatively new crosslinking agent of gelatin and known for its completely nontoxic nature in
comparison to glutaraldehyde. When the concentration of genipin is varied in the range 0.1 to 0.6 wt. percent of gelatin, the size of gelatin nanoparticles is found to decrease from 148 to 80 nm as shown in Fig. 6(b). The observed decrease in size of gelatin nanoparticles may be explained by the fact that with increasing concentration of genipin the gelatin macromolecules are crosslinked at faster rate and acquire relatively compact size thus decreasing the size of gelatin nanoparticles.

**Swelling Results**

Water sorption is a significant phenomenon in drug delivery applications as this physicochemical property forms the very foundation of responsive drug delivery systems. In the present work also the swelling of gelatin nanoparticles has been investigated and found to be largely affected by the experimental conditions and chemical composition of the gelatin nanoparticles.

**Effect of gelatin content**

The effect of gelatin content in the nanoparticles on their swelling behavior has been investigated by varying the amount of gelatin in the range 1.0 to 7.0 g as shown in Fig. 7. The observed results clearly show that the swelling ration initially increases in the range 1.0 to 5.0 g of gelatin, while a decrease is observed beyond 5.0 g which continues up to 7.0 g. The results may be explained by the fact that with increase in gelatin content, the hydrophilicity of the nanoparticles also increase which consequently results in enhanced water sorption. However, beyond 5.0 g, the amount of gelatin increases to so large extent that the intermolecular and intramolecular forces operative results in increased degree of crosslinking which obviously lead to a fall in the swelling ratio. The inter- and intramolecular forces are developed due to the interactions between the functional groups of gelatin macromolecules.

**Effect of genipin**

The crosslinker has a pronounced effect on the overall physicochemical properties of the gelatin nanoparticles. In the present study genipin has been employed as a crosslinker and its influence on swelling ratio of gelatin nanoparticles has been investigated by varying the amount of genipin in the range 0.1 to 0.6 percent of gelatin content in the nanoparticles. The results are depicted in Fig. 8 which clearly show that the equilibrium swelling ratio constantly decrease with increasing content of crosslinking agent. The observed decrease in water sorption capacity may be attributed to the fact that increased degree of crosslinking in the nanoparticles network results in restrained capacity of gelatin chains to undergo relaxation which brings about a fall in the amount of water molecules. Another reason for the observed decrease in swelling ration may be that introduction of greater degree of crosslinking may also increase the glass transition temperature of gelatin network which may also add into restrained mobility of gelatin chains thus causing a depression in the equilibrium water sorption capacity.
Effect of pH of the medium
The pH of the swelling medium is a significant parameter that drastically affects water sorption capacity of gelatin nanoparticles. In the present work the pH of the swelling bath was varied in the range 1.2 to 9.8 and water sorption capacity was monitored at respective pH values. The results are presented in Fig. 9 which clearly indicates that the water sorption capacity decreases with increasing pH up to 7.4 and thereafter it again starts increasing. It is also revealed, therefore, that a minimum swelling is noticed at pH 7.4 which is very close to the isoelectric point of the gelatin (A) used in the present study. The observed results may be explained by the fact that below the isoelectric point of the gelatin there will be net positive charge on the gelatin macromolecules which due to mutual repulsion will produce rapid relaxation of chains thus allowing entrance of water molecules into the nanoparticles network. This will obviously result in greater water sorption which, however, will decrease with increasing pH of the medium. At the isoelectric point, about 7.4 of gelatin A, there will be net zero charge over the molecules of gelatin and thus a minimal repulsion will be produced by the chains. This will clearly lead to a minimum relaxation of gelatin chains and, therefore, water sorption will be minimum. Upon further increasing the pH into alkaline range (i.e. beyond 7.4), the amount of negative charge will increasing on the gelatin molecules and it will again cause an increasing repulsion this enhancing the water sorption potential.

Effect of temperature
The effect of increasing temperature on equilibrium swelling ration of genipin crosslinked nanoparticles has been investigated by carrying out water sorption experiments in the range 15 to 48°C. The results are presented in Fig. 10 which clearly reveal that the water sorption increases up to 42°C and thereafter constantly decreases. The reason for the observed initial increase in equilibrium swelling may be attributed to the fact that with increasing temperature the kinetic energy of gelatin chains also increases which results in rapid relaxation thus permitting greater number of water molecules to penetrate the nanoparticles network. The observed may also be explained by the reason that with increasing temperature the kinetic energy of invading water molecules also increases which also favor greater swelling of gelatin nanoparticles. The results also reveal that, however, beyond 42.50C, the water sorption decreases. The fall in equilibrium water capacity may be attributed to the fact that at much higher
temperature, the forces binding the water molecules and gelatin molecules are broken and this clearly lowers the amount of water sorption.

**Effect of simulated biological fluid**

It is well known that the equilibrium swelling behavior of polymer or biopolymer network in a medium is the result of a balance between the osmotic pressure and the restoring elastic pressure of the network. When inorganic salts or organic molecules are present in the swelling medium, the osmotic pressures as well as restoring capacity of network chains are affected, which may result in either an increase or decrease in the extent of swelling. The effect of biological fluids has been examined by performing swelling experiments in the presence of urea (5% w/v), D-glucose (5% w/v), potassium iodide (15% w/v), saline water (0.9% NaCl), and artificial urine. The results are depicted in Fig. 11 which indicates that in biological fluids a lower

![Fig. 11 Water sorption capacities of gelatin nanoparticles in various simulated biofluids](image)

swelling ratio is obtained, which may be attributed to the fact that the presence of a solute increases the osmotic pressure of the external solution thus decreasing the swelling ratio.

**Conclusions**

The crosslinking of gelatin emulsion by genipin results in nanoparticles of size up to 150 nm while maximum nanoparticles exhibit a size of 80 nm. The FTIR spectral studies clearly confirm the crosslinking of gelatin macromolecules by genipin. The SEM and TEM analysis also confirm nearly spherical shape of nanoparticles of sizes in nanometer range. The size of nanoparticles is found to depend greatly on the amounts of gelatin and genipin in the feed mixture. When the amount of gelatin varies in the range 1.0 to 7.0 g the size of nanoparticles increases from 58 to 170 nm. Whereas increasing the concentration of genipin, the crosslinker in the present study, from 0.1 to 0.6 wt. % of gelatin, the size of nanoparticles decreases from 148 to 80 nm, respectively. The prepared nanoparticles show water sorption capacity which greatly depends on many factors such as chemical composition of the nanoparticles, pH and temperature of the medium. It is noticed that when the amount of gelatin varies from 1.0 to 5.0 g, the water sorption capacity is enhanced by about 6 fold, while beyond 5.0 g of gelatin content the equilibrium water content decreases. The nanoparticles also shown a drop in the water sorption capacity from 5.8 to 1.0 when the genipin concentration is raised from 0.1 to 0.6 wt. % of gelatin content. The nanoparticles show a minimal swelling at their isoelectric point, 7.4, while enhanced level of water sorption is noticed at both the sides of the isoelectric pH. When the temperature increases from 15 to 42.5 °C, the equilibrium water sorption capacity also increases while beyond 42.5°C, it suddenly falls. The nanoparticles also exhibit a suppressed level of water sorption capacity in simulated biological fluids.

**References**


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