Engineering Technology Trends Vol 3 Issue 2 2025

Original Research Article

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Eco-friendly synthesis of gelatin nanoparticles using genipin as the crosslinker

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Abstract: The development of eco-friendly and biocompatible nanoparticle formulations is crucial for advancing sustainable biomedical applications. This study reports the sustainable synthesis of gelatin nanoparticles using the desolvation method, with genipin as a natural crosslinking agent. The effect of genipin concentration on nanoparticle size and polydispersity index (PDI) was investigated, revealing that higher genipin concentrations resulted in reduced particle sizes and improved size uniformity. The optimized formulation exhibited particle size and PDI comparable to gelatin nanoparticles crosslinked with glutaraldehyde, a commonly used but more toxic alternative. Furthermore, transmission electron microscopy (TEM) imaging confirmed relatively spherical morphology of the nanoparticles. The nanoparticles were also shown to be stable in collodial form at 4 °C over 7 days. These findings underscore the potential of genipin-crosslinked gelatin nanoparticles as a sustainable and safer alternative for drug delivery applications.

Keywords: Sustainability; Gelatin nanoparticles; Desolvation; Genipin; Colloidal stability

1. Introduction

Nanotechnology has transformed drug delivery by using nanoparticles to enhance drug bioavailability, control release rates, and enable targeted medication delivery. These improvements have resulted in greater effectiveness with fewer side effects when compared to traditional formulations[1]. Amongst various biomacromolecules, gelatin-based nanoparticles have emerged as a promising option for drug delivery systems. In comparison with other protein-based nanocarriers, gelatin nanoparticles exhibit excellent stability in biological fluids, facilitating the desired controlled release of encapsulated drug molecules. In addition, the unique physicochemical properties of gelatin, its biocompatibility, biodegradability, and chemical versatility, have also positioned these nanoparticles as a valuable platform for advancing nanomedicine and improving the efficacy of drug delivery[2].

1.1. Gelatin and Gelatin Nanoparticles

Gelatin is a naturally derived biopolymer obtained through the partial breakdown of collagen, which is the main biological protein existing in animal tissues, such as skin, bones, and connective tissues. Gelatin

is composed of a mixture of polypeptide chains, with a molecular structure consisting of repeating alanine-glycine-proline units, which provide the characteristic triple-helix structure of collagen[3]. The molecular structure of gelatin is presented in **Figure 1**. Gelatin is available in two main types, gelatin A and gelatin B, which exhibit differences in their isoelectric points and production methods. Specifically, gelatin A is derived through the acidic processing of collagen, while gelatin B is produced via an alkaline treatment of collagen. Gelatin's biocompatibility, biodegradability, and capacity to encapsulate various therapeutic substances have led to its widespread applications as a biomaterial for drug delivery systems[4]. Gelatin has a well-established history of safe application in various sectors, including pharmaceuticals, cosmetics, and food processing. Based on safety assessments, gelatin is categorized as a GRAS (Generally Recognized As Safe) substance by the US FDA. Clinically, it serves roles such as a plasma expander and a stabilizer in various protein-based formulations, vaccines, and hemostatic sponges[5]. Since it originates from collagen, the most prevalent protein in animals, its enzymatic breakdown does not result in harmful by-products. Additionally, gelatin's natural protein structure offers numerous functional groups, enabling a wide range of chemical modifications. This makes it particularly valuable for designing targeted drug delivery systems through cross-linking or conjugation with specific ligands.

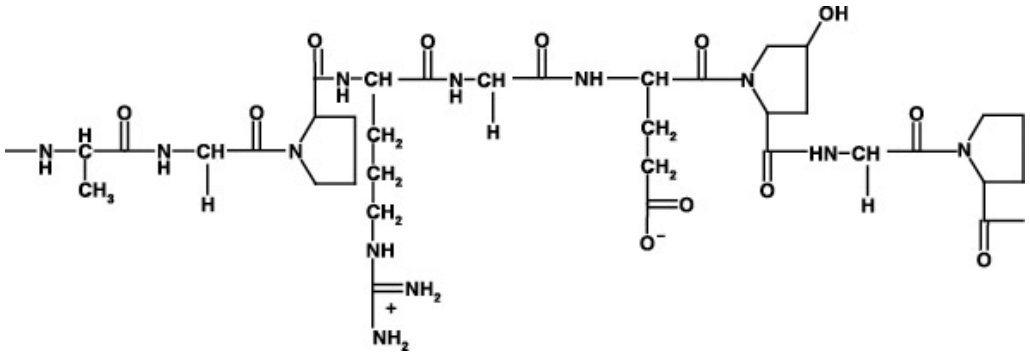


Figure 1. Chemical structure of gelatin.

One of the advanced drug delivery systems developed through the modification of gelatin is the gelatin nanoparticle system. Gelatin nanoparticles have gained significant attention as efficient and versatile drug carriers because of their biocompatibility, biodegradability, and non-immunogenic nature. Gelatin nanoparticles can greatly improve drug bioavailability by shielding therapeutic substances from enzymatic breakdown and boosting their solubility and stability under physiological conditions. Furthermore, gelatin's capacity for thermal and pH-sensitive gelation facilitates the controlled release of drugs, targeting drugs to the intended sites upon drug administration while reducing systemic adverse effects. Gelatin nanoparticles also offer surface functionalization possibilities, enabling ligand-mediated targeting of specific cells or tissues, which is particularly beneficial for cancer therapy and gene delivery[5]. Researchers have successfully utilized gelatin nanoparticles to deliver a diverse array of bioactive compounds, such as hydrophobic drugs, peptides, proteins, or nucleic acids[6].

1.2. Synthesis of Gelatin Nanoparticles

1.2.1. Methods for preparation of gelatin nanoparticles

Several methods exist for the preparation of gelatin nanoparticles, including[5]:

• *Emulsification*: This technique involves the formation of an oil-in-water or water-in-oil emulsion, with gelatin dissolved in the aqueous phase. The emulsion is prepared by adding the gelatin aqueous solution to a surfactant dissolved in a non-polar solvent, followed by the evaporation of the solvent. When the surfactant dissolves in the non-polar solvent, it forms micelles. In these micelles, the hydrophobic tails of the surfactant are arranged towards the bulk of the non-polar solvent, while the hydrophilic heads are directed away from the bulk of the solvent, creating an aqueous core where the gelatin solution is dissolved.

• *Coacervation*: This method relies on the phase separation of a colloidal system, where gelatin is

dissolved in an aqueous solution and a coacervating agent, such as salt or a non-solvent, is added to induce the formation of gelatin-rich coacervate droplets that can then be stabilized.

• *Desolvation*: This widely used technique involves the gradual addition of a desolvating agent, to an aqueous solution of gelatin, causing the gelatin to precipitate and form nanoparticles, which can then be crosslinked to improve their stability.

• *Self-assembly*: The gelatin nanoparticles are formed by self-assembly of modified gelatin, in which the hydrophilic gelatin is chemically conjugated with hydrophobic molecules to form an amphiphilic polymer. When the gelatin is modified with hydrophobic groups, it can undergo conformational changes in water to form self-assembled micelles. In these micelles, the hydrophobic segments aggregate inwardly, creating a hydrophobic core, while the hydrophilic segments form the outer shell.

To further enhance the functionality and targeted delivery capabilities of gelatin nanoparticles, their surface can be modified using a variety of strategies. The attachment of surface-modifying moieties to the nanoparticle surface can be achieved through various chemical mechanisms, including physical adsorption, electrostatic binding, and covalent coupling, and this can occur before or after the nanoparticle is formed. The primary purpose of surface modification is to improve the biological performance of gelatin nanoparticles by enabling specific targeting, prolonging systemic circulation, enhancing cellular uptake, and increasing the therapeutic efficacy of encapsulated agents[7]. For these purposes, a plethora of surface-modifying moieties have been utilized. For instance, folic acid is widely used as a targeting ligand as it is specifically and strongly bound to folate receptors expressed on the surface of many cancer cells[8]. Peptide ligands, such as epidermal growth factor receptor (EGFR)-targeting peptides and cell-penetrating peptides like Tat and SynB, facilitate receptor-specific delivery and enhance penetration across biological barriers like the blood–brain barrier[9]. Polyethylene glycol (PEG) is another commonly used surface-modifying agent, which can reduce immune recognition and prolong blood circulation time for gelatin nanoparticles, also known as the stealth effect[10]. By carefully selecting and applying these modifications, researchers can modulate the properties of gelatin nanoparticles to meet the specific requirements of their intended applications, paving the way for the development of more effective and targeted drug delivery systems.

1.2.2. Desolvation method

The desolvation method is the most popular technique for producing gelatin nanoparticles because of its simplicity, reproducibility, and ability to control particle size. This method involves the dropwise addition of a desolvating agent, such as acetone or ethanol, to an aqueous solution of gelatin to induce nanoparticle formation. It is then followed by the addition of a crosslinker to crosslink the precipitated polymer and enhance the stability of the resulting gelatin nanoparticles[11]. The desolvation method for nanoparticle fabrication is graphically illustrated in **Figure 2**.

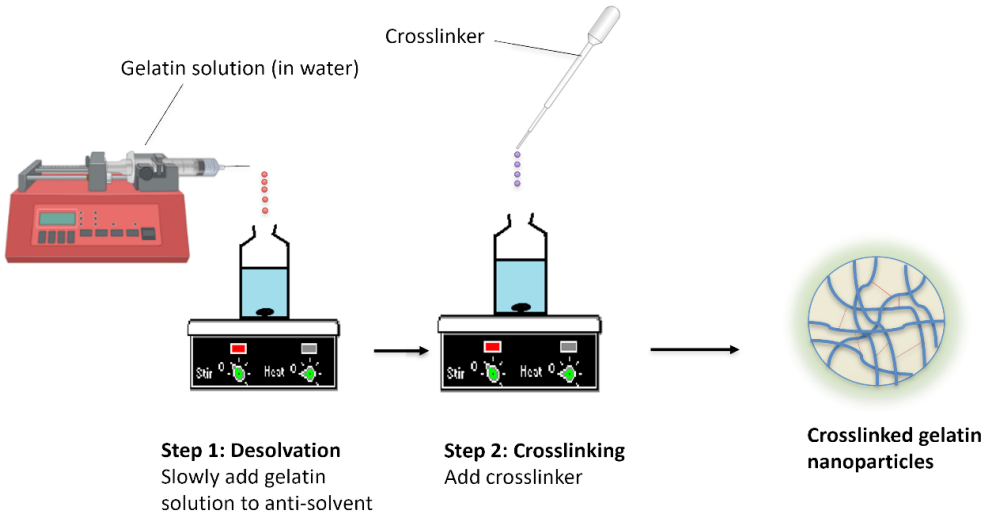


Figure 2. Graphical illustration of the preparation of gelatin nanoparticles by desolvation method.

1.3. Crosslinking Agent: Glutaraldehyde Versus Genipin

Glutaraldehyde is one of the most common crosslinking agents used in the preparation of gelatin nanoparticles via the desolvation method. The crosslinking reaction occurs due to the interaction between the two aldehyde groups of glutaraldehyde and the amino groups of gelatin. Specifically, aldehyde cross-linking primarily involves the aldol condensation reaction between the aldehyde units and the ε-amino groups from lysine and hydroxylysine residues of gelatin chains, leading to the formation of a Schiff-base intermediate. However, concerns have been raised regarding the safety and environmental impact of using glutaraldehyde in the final product[12]. As a volatile organic compound, glutaraldehyde poses a threat to air, water, and soil quality, potentially disrupting ecosystems and harming wildlife. Furthermore, exposure to glutaraldehyde has been associated with respiratory problems and skin irritation among laboratory personnel. These concerns underscore the urgent need for safer, more environmentally responsible alternatives to ensure the sustainable advancement of nanomedicines[13]. Potential alternatives to replace glutaraldehyde as crosslinkers include genipin, carbodiimide/N-hydroxysuccinimide, aldehyde groups, and transglutaminase[14].

Genipin, a naturally sourced crosslinking agent extracted from the fruit of the plant *Gardenia jasminoides*, is a promising candidate to replace glutaraldehyde for improved sustainability and safety. The median lethal dose (LD_{50}) of genipin upon oral administration to mice is approximately 382 mg/kg[15], compared to 100 mg/kg for glutaraldehyde[16]. It offers excellent biocompatibility, diminished cytotoxicity, and efficient chemical crosslinking capability. The crosslinking mechanism of genipin with gelatin involves the establishment of covalent linkages between the amino groups present in gelatin and the reactive functional groups of genipin, resulting in a stable and biocompatible network (**Figure 3**). Genipin, a blue-toned pigment, possesses anti-inflammatory, antioxidant, and antimicrobial characteristics, thereby broadening its scope in biomedical applications. The utilization of genipin in fabricating gelatin nanoparticles not only lessens ecological impact but also strengthens the safety attributes of these nanoscale biomaterials[17].

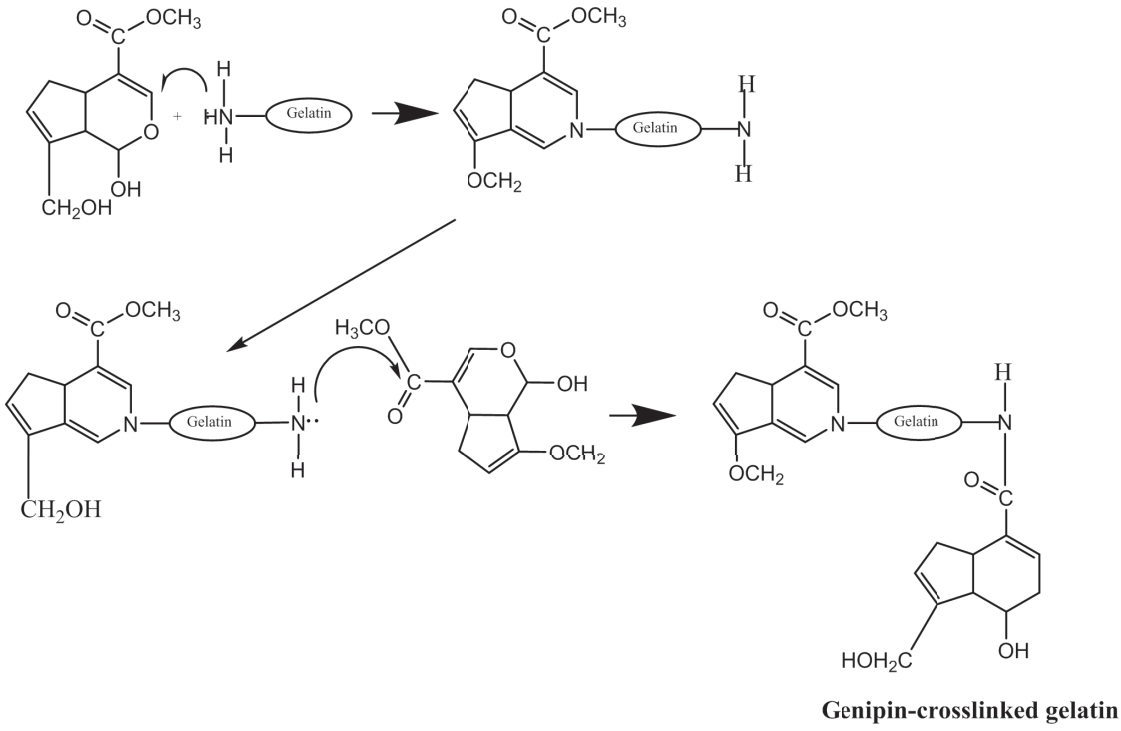


Figure 3. Chemical mechanism of the crosslinking of gelatin by genipin.

The goal of this study was to prepare and characterize genipin-crosslinked gelatin nanoparticles. The impact of the amount of genipin used in the preparation of the nanoparticles on their size was evaluated. Additionally,

the stability of the gelatin nanoparticles in colloidal form was determined by evaluating the hydrodynamic size and polydispersity index (PDI). The findings of this work provide insights into the development of eco-friendly gelatin nanoparticles for potential biomedical applications.

2. Methods

2.1. Preparation of Gelatin Nanoparticles

Gelatin (Sigma Aldrich, USA) was dissolved in 10 mL of dionised water at a concentration of 1% (*w/v*) at 50 °C. The resulting gelatin solution was filtered through a 0.45 µm syringe filter. The filtered solution was then magnetically stirred at 750 rpm, and the pH was adjusted to 4. Afterward, the gelatin solution was added at the flow rate of 0.1 mL/min to 40 mL of acetone using the peristaltic syringe pump; the mixture was then stirred continuously for another 5 min. To this solution, 1 mL of a 0.15% (*w/v*) genipin solution in water was added dropwise. The mixture was further stirred for 30 hours. Finally, the nanoparticle suspension was refrigerated at 4 °C for short-term storage.

2.2. Dynamic Light Scattering (DLS)

The nanoparticles were evaluated for hydrodynamic particle size and PDI using a DLS system (Malvern ZS90, Malvern Panalytical, Worcestershire, UK), the nanosuspension was diluted with 10 mM NaCl with a suitable dilution factor. The samples were measured for particle size in triplicate.

2.3. Transmission Electron Microscopy (TEM)

A 10 µL nanoparticle suspension was placed onto a carbon-coated grid. The media was then blotted, followed by the addition of 10 µL of phosphotungstic acid in PBS buffer (pH 6.8) at 1% (*w/v*), which was promptly removed. The sample was then examined using a JEM-2100 TEM system (JEOL, Akishima, Japan).

3. Results & Discussion

Gelatin nanoparticles were synthesized from gelatin type B using the desolvation method. In this process, gelatin was first dissolved in deionized water and then gradually introduced into acetone. The resulting nanoparticles were stabilized through cross-linking with genipin. Prior studies have demonstrated the particle size and size distribution of gelatin nanoparticles prepared by the desolvation methods has been impacted by differing critical factors, including gelatin solution's concentration and pH[18], type and amount of the desolvating agent[18], type and amount of crosslinker concentration[19], and crosslinking time[20, 21]. For instance, Ahsan *et al*. investigated the effect of different pH levels of the gelatin solution, ranging from 3.5 to 5.0, on the particle size of gelatin nanoparticles and reported that a pH of 4.0 produced the smallest nanoparticle size (**Figure 4**)[18]. **Table 1** summarises critical processing parameters that have been used in recent studies to produce gelatin nanoparticles.

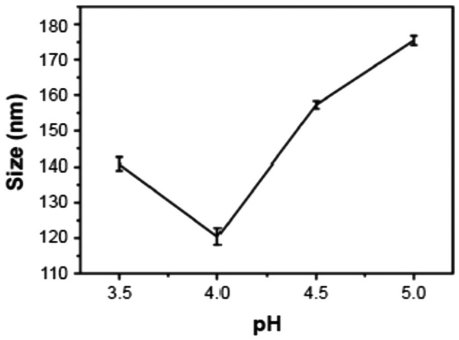


Figure 4. Impact of pH of gelatin solution on the size of gelatin nanoparticles.

Table 1. Summary of critical processing parameters for the preparation of gelatin nanoparticles.

Polymer concentration	Anti-solvent (water-to-antisolvent ratio)	Flow rate	pH	Crosslinker(Crosslinking time)	Reference
5%	Acetone (1:3)	n.a.	3.0	Glutaraldehyde(2 hours)	[22]
2.5%	Acetone (1:1.6)	n.a.	3.0	Glutaraldehyde(12 hours)	[23]

Continuation Table:

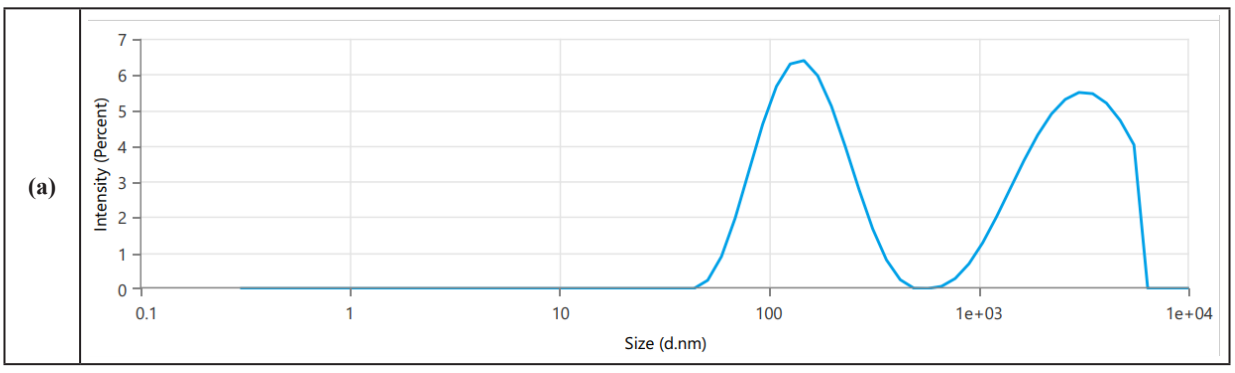
Polymer concentration	Anti-solvent (water-to-antisolvent ratio)	Flow rate	pH	Crosslinker(Crosslinking time)	Reference
5%	Ethanol (1:1.2)	1 mL/min	2.5	Glutaraldehyde(5 hours)	[20]
1%	Acetone (1:0.54)	n.a.	4.0	Glutaraldehyde(12 hours)	[18]
9%	Acetone (1:1.6)	n.a.	2.5	n.a.	[24]
5%	Acetone (1:2.4)	n.a.	2.5	Glutaraldehyde(16 hours)	[21]

One of the critical factors influencing the particle size of the particle is the amount of genipin used. In this study, four concentrations of genipin were evaluated for particle size of the resulting nanoparticles, including 0.05, 0.1, 0.2 and 0.4% (*w/v*). The size and PDI of these nanoparticles are presented in **Table 2**. The other critical processing parameters were chosen based on the optimal conditions reported in previous studies[18, 20].

Table 2. Particle size and polydispersity index (PDI) of gelatin nanoparticles.

Genipin concentration (% *w/v*)	Particle size (nm)	PDI
0.05	257.4 ± 4.3	0.81 ± 0.09
0.1	198.6 ± 2.4	0.36 ± 0.04
0.2	157.2 ± 3.5	0.063 ± 0.02
0.4	145.5 ± 5.1	0.093 ± 0.04

Nanoparticles prepared with increasing concentrations of genipin from 0.05% to 0.2% exhibited a reduction in particle size and PDI. However, further increasing the genipin concentration from 0.2% to 0.4% only gave a slight decrease in size, while increasing the PDI. The size distribution curves of the four genipin-crosslinked gelatin nanoparticle formulations are shown in **Figure 5**. It was also observed that the size distribution of nanoparticles prepared using either 0.05%, 0.1% or 0.4% genipin was bimodal, whereas the size distribution of nanoparticles prepared using 0.2% genipin was unimodal. These results can be explained by the fact that increasing the genipin concentration from 0.05% to 0.2% enhanced the number of crosslinking linkages formed, leading to more compact and uniformly sized nanoparticles. The bimodal distribution observed for the nanoparticles prepared using 0.4% genipin concentration could be due to the excess number of crosslinking linkages formed, leading to aggregation of nanoparticles. Specifically, nanoparticles formulated with 0.2% (*w/v*) genipin had an average particle size of approximately 157 nm and a PDI below 0.1. This small size and narrow distribution are particularly advantageous for nanoparticulate drug delivery systems (DDS), as nanoparticles below 200 nm can more effectively penetrate biological barriers, such as cellular membranes and the endothelial lining of blood vessels. Furthermore, smaller, uniformly sized nanoparticles could be able to evade opsonisation by macrophages, resulting in prolonged circulation times and enhanced accumulation at target sites via the enhanced permeability and retention (EPR) effect[25]. Together, these characteristics can improve the bioavailability and therapeutic efficacy of the encapsulated drugs.



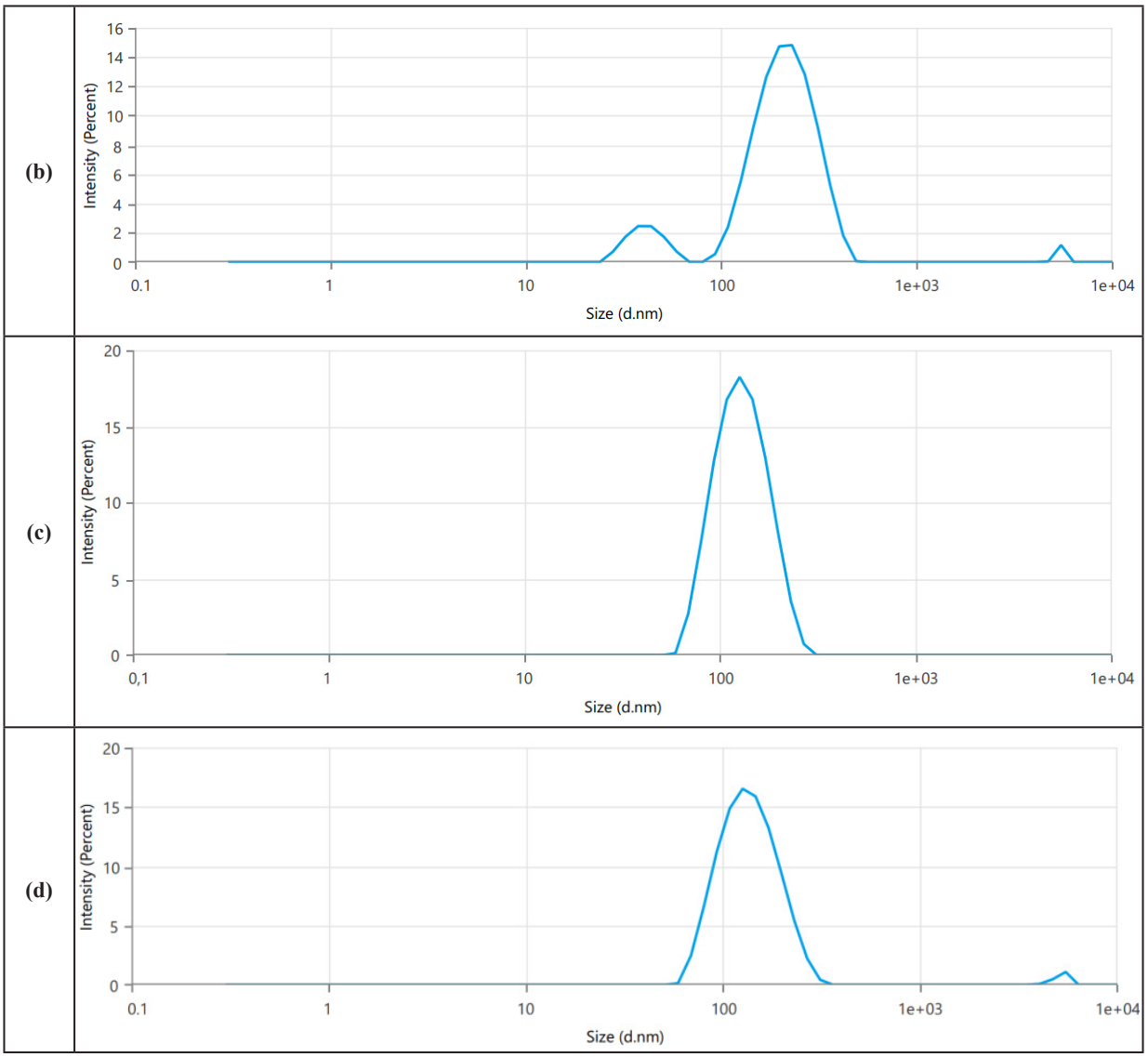


Figure 5. Size distribution curves of the gelatin nanoparticles using genipin at (a) 0.05% (*w/v*), (b) 0.1% (*w/v*), (c) 0.2% (*w/v*) and (d) 0.4% (*w/v*).

One disadvantage of using genipin as the crosslinker compared to glutaraldehyde is that the crosslinking reaction takes longer, at 30 hours compared to around 6 hours when using glutaraldehyde. The time required for the crosslinking reaction in this study was investigated by measuring the particle size and PDI of the produced nanoparticles at various time points. It was found that 30 hours was the time required to produce the smallest nanoparticle size, and further increasing the time did not result in any additional decrease in particle size and PDI. This suggests that the crosslinking reaction was complete after 30 hours. The chemical mechanism of this reaction could be further elucidated through analytical techniques such as Fourier transform infrared spectroscopy (FTIR) or nuclear magnetic resonance (NMR) spectroscopy.

The surface morphology of this gelatin nanoparticle formulation was observed using TEM imaging, as shown in **Figure 6**. The nanoparticles were spherical, with sizes ranging from 150 to 200 nm, in good agreement with the particle size measurements obtained by dynamic light scattering. In addition, the nanoparticles were shown to be uniform in size and shape, with no evidence of aggregation.

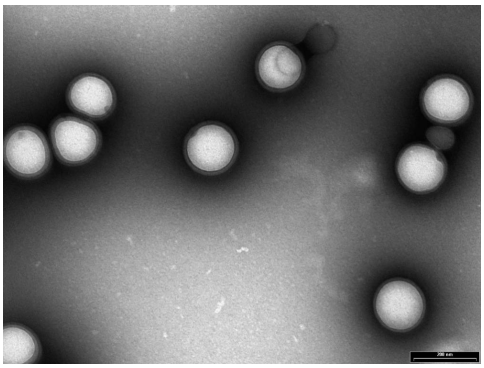


Figure 6. TEM image of gelatin nanoparticles.

Nanoparticle formulations need to exhibit colloidal stability in the short term to allow for administration and preparation. To assess this stability, the hydrodynamic size and size distribution are measured at different time points over seven days at 4 °C. As shown in **Figure 7**, there were no significant changes in particle size or PDI throughout the investigation period, suggesting that the nanoparticles were stable in collodial form. For long-term storage, the nanoparticles may need to be freeze-dried. Freeze-drying is a common method for solidifying nanoparticles. To maintain the particle size and size distribution, cryoprotectants such as sucrose or dextran are typically added to the nanoparticle suspension prior to freeze-drying[26].

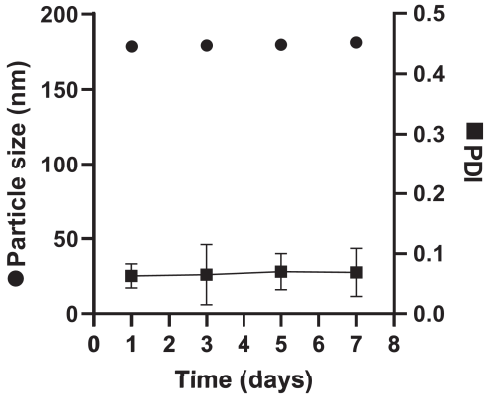


Figure 7. Colloidal stability of gelatin nanoparticles

Conclusion

This study presents the eco-friendly synthesis of gelatin nanoparticles using the desolvation method with genipin as the crosslinking agent. The results demonstrate that genipin concentration significantly influences nanoparticle size and PDI. The optimized formulation exhibited a particle size comparable to that of gelatin nanoparticles crosslinked with the more toxic glutaraldehyde. The produced gelatin nanoparticles exhibited favorable short-term colloidal stability. The environmentally friendly gelatin nanoparticle formulation holds promise for use in future pharmaceutical applications for delivery of bioactive compounds.

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