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Original Article

Optimization of gelatin nanofabrication as a potential biocompatible nanocarrier

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ABSTRACT

Gelatin is a promising natural biomaterial used as a drug delivery system for various indications. It is a cheap, abundant, and safe protein derived from collagen with proven biodegradation, biocompatibility, and non-immunogenicity. According to its source, gelatin can be obtained by acid or alkaline hydrolysis, which affects its physicochemical properties. Gelatin B is more negatively charged than gelatin A, with respective isoelectric points (IEP) of 5 and 8.5. Different gelatin drug delivery systems have been obtained including microparticles, nanoparticles, fibers, and hydrogels. Furthermore, gelatin has multiple functional groups that enable its crosslinking and other modulations to achieve specific targeting, improve and sustain drug action, and control side effects. The scope of this study was to investigate the effect of various parameters affecting the obtainment of a stable gelatin nano-system. Four important factors were scrutinized using the double desolvation method: pH, type, and method of addition of water-miscible antisolvents, and finally the polymer concentration. The obtained nanoparticles were evaluated for their particle size, polydispersity index, and zeta potential.

Keywords: *Acetone; Desolvation; gelatin; nanoparticles; optimization.*

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1. Introduction

Gelatin, a multifunctional natural protein, is cheap to attain by the partial hydrolysis of collagen (whether acidic or alkaline), biocompatible, non-antigenic, temperature-responsive, and biodegradable, all of which are properties that enrich its use as a drug delivery system (DDS). Its capacity for chemical modification, as well as, its balanced hydrophilic and hydrophobic properties, allow enhanced drug loading, controlled drug release profile, and improved therapeutic activity **[1, 2]**. In addition, one of the most prominent properties of gelatin is its versatile structure, caused by its protein nature, which enables ease of modification of its functional groups with various targeting ligands **[3]**.

Several techniques have been used in the fabrication of GNPs, including nanoprecipitation, coacervation phase separation, emulsification-solvent evaporation, reverse phase microemulsion, and desolvation.

Nanoprecipitation, an easy, simple, rapid method, that produces monodisperse particles with diameters not exceeding 200 nm **[4, 5]** does not require drastic conditions like high shear, elevated temperature, or sonication, however, low yield is a con **[6]**. It involves the preparation

of two phases, the solvent phase which comprises gelatin dissolved in deionized water at 50 °C, and the anti-solvent phase which consists of acetone or ethanol with poloxamer as a stabilizer. Cross-linked GNPs are produced upon drop-wise addition of the solvent phase to the anti-solvent phase followed by the addition of a cross-linker. Ultra-filtration is then carried out, to remove any excess stabilizer or unreacted cross-linker, and finally, GNPs are further purified by washing **[7]**. During solvent displacement, an interfacial tension is formed, and due to the mutual solubility of both solvents, violent spreading takes place, releasing nanometric droplets of the solvent from the interface. The stabilizer, in turn, stabilizes these droplets, until complete diffusion of the solvent takes place and protein solidification occurs **[8]**.

Coacervation–liquid phase separation is a process through which a homogeneous single-phase system separates into two phases in equilibrium; a lower polymer-rich phase with a supernatant phase above (Mohanty et al., 2005; Nixon et al., 2011). This method produces particles with sizes larger than 500 nm and with low drug entrapment efficiency **[8]**. In this method, an aqueous solution of gelatin containing a surfactant (e.g., Tween 20) is prepared, then sodium sulfate is slowly added followed by isopropanol to dissolve the formed precipitate. Gelatin aggregates are then created by further addition of sodium sulfate, followed by distilled water and a crosslinker, until a clear solution forms **[11]**.

Emulsification-solvent evaporation is based on the preparation of a single w/o emulsion, making it not suitable for certain routes of drug administration such as the ocular route. This technique involves vigorous mixing of the drug/gelatin aqueous phase with an oily phase (e.g. toluene/chloroform solution of paraffin oil or polymethylmethacrylate) followed by the addition of the cross-linker and subsequent solvent evaporation, to yield particles with diameters ranging from 100 to 400 nm **[12, 13]**. The drawback of this method is the necessity of using large amounts of surfactant to obtain small-sized particles**.**

Reverse phase microemulsion depends on preparing a surfactant solution of sodium bis (2-ethylhexyl) sulfosuccinate (AOT) in normal hexane, to which an aqueous gelatin solution is added, followed by the addition of the crosslinker. Subsequent recovery of GNPs is achieved by evaporation of the organic phase **[14]**. The surfactant AOT is chosen as it forms reversed micelles when dissolved in a non-polar solvent like hexane, where the hydrophobic tails are directed outwards towards the non-polar solvent and the hydrophilic heads are directed inwards, forming a hydrophilic core in which GNPs are formed, with average sizes of 30 to 40 nm **[1, 14, 15]**.

Desolvation is a liquid-liquid phase separation method based on inducing a change in the conformation of gelatin molecules, from stretched to coiled, by dehydrating the molecules of an aqueous gelatin solution by adding a desolvating agent (e.g. alcohol or acetone) **[6, 16]**. The degree of coiling depends on gelatin type, concentration, and ionic strength, as well as, the preparation parameters (e.g. pH, temperature, and type of solvent) **[17]**. The use of gelatin type A or type B produces positively or negatively charged GNPs, respectively **[18]**.

Desolvation could be done in one or two steps. In the single-step desolvation, native gelatin is the precursor for GNP production, while in the double desolvation method; high molecular weight gelatin is obtained first, which is then used as a precursor for the production of the nanoparticles. Therefore, double desolvation is superior because it produces smaller-sized GNPs with narrower unimodal distribution and

higher colloidal stability **[19-21]**, however, batch variation and low yield remain a concern **[22-24]**. Ofokansi et al developed a method that was based on optimizing the pH and temperature of gelatin solution before desolvation, at respective values of 7 and 37 °C. This modification acquired a balance between the charged and uncharged gelatin molecules, thus, retaining the sensitivity of gelatin molecules to desolvation and inhibiting their aggregation **[25]**. On the other hand, a study by Azarmi et al showed that the smallest-sized GNPs, with the narrowest unimodal distribution, were obtained by setting the gelatin solution temperature at 40 °C and desolvating it with acetone at a pH of 2.5 **[26]**. Although extensively used to prepare GNPs, this technique has two main disadvantages; the use of organic solvents and toxic crosslinkers **[8]**.

Gelatin DDS loaded with hydrophilic drugs lose their structural integrity in aqueous environments, leading to undesirable immediate release of the loaded drug. Hence, gelatin crosslinking is a prerequisite to alter the mechanical and biochemical properties and obtain chemically and physically stable gelatin particles with longer circulation times **[1, 27]**. The literature described two main methods of gelatin crosslinking, namely; physical and chemical. Physical methods, such as the use of UV irradiation and de-hydrothermal treatment, are usually inefficient, with difficulty in controlling the crosslinking density of the resultant gelatin matrix. On the other hand, chemical crosslinkers, subclassified into non-zero length and zero-length, act by bridging free carboxylic acid residues and amine groups between adjacent protein molecules, making them far more reliable **[28]**. Many crosslinkers have been exploited in the preparation of gelatin nanoparticles (GNPs) such as glutaraldehyde (GA) **[29],** genipin **[30]**, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide **[31]**, microbial transglutaminase **[32]**, and glyoxal **[33]**. Since GA crosslinked GNPs proved to be stable with no aggregation upon storage at 2-8 °C for nearly a year **[34]**, GA is considered an infamous crosslinker, with toxicological hazards of the residual aldehyde not being a concern due to their minute trace amounts and the ability to purify GNPs by efficient washing **[35]**.

In common practice, optimization of formulation variables is implemented by one factor at a time experiments (OFAT), which is based on sequential investigation of one variable in every experiment, while keeping the rest of the variables constant. Therefore, this study aims to prepare gelatin-type B nanoparticles by the double desolvation technique and optimize their properties by scrutinizing various factors.

2. Materials and Methods

2.1. Materials

Gelatin B bloom225 (from bovine skin) and glutaraldehyde (25% solution) were purchased from Sigma Aldrich (St. Louis, USA). Acetone (liquid chromatography grade) was supplied by Merck (Germany). Absolute Ethanol 99.8% (HPLC grade) was obtained from Fischer Scientific (Loughborough, UK). De-ionized water was used in all experiments.

2.2. Methods

2.2.1. Preparation of gelatin nanoparticles by the double desolvation method

Briefly, 1.25 g gelatin was dissolved in 25 ml deionized water at 50 °C under magnetic stirring at 200 rpm for half an hour, then 25 ml acetone was added at once, to, precipitate the high molecular weight gelatin (first desolvation step). After 30 min, the supernatant was decanted and the precipitate was dissolved in 25 mL water, followed by lyophilization using a freeze dryer (Christ alpha 1-2 LD plus, Martin Christ, Germany) to obtain purified high molecular

weight gelatin. A gelatin solution in de-ionized water at 50 °C was prepared under magnetic stirring for half an hour and then filtered using a 0.45 µm syringe filter. 1 mL of the prepared solution was transferred to a vial and the pH was adjusted to the required value using 5 M NaOH or HCl. The second desolvation step was achieved by adding 5 mL of the antisolvent (either ethanol or acetone) while magnetic stirring (at 50 rpm) for 15 min. The preparation was then left to rest at room temperature for 15 min. Subsequently, the crosslinker (18% w/w glutaraldehyde) was added and allowed to crosslink the preparation for 12 h. Finally, the antisolvent was removed utilizing a rotary evaporator (model Heidolph WB 2000, Germany) and GNPs were purified by triple centrifugation cycles at 15000 rpm, for 40 min at 4 °C using a cooling centrifuge (Hermle Labortechnik, Wehingen, Germany) and re-dispersed in de-ionized water. The resulting GNPs were stored at 4 ºC until needed for further characterization **[36]**. A diagrammatic scheme for the method of preparation is illustrated in **Fig. 1**.

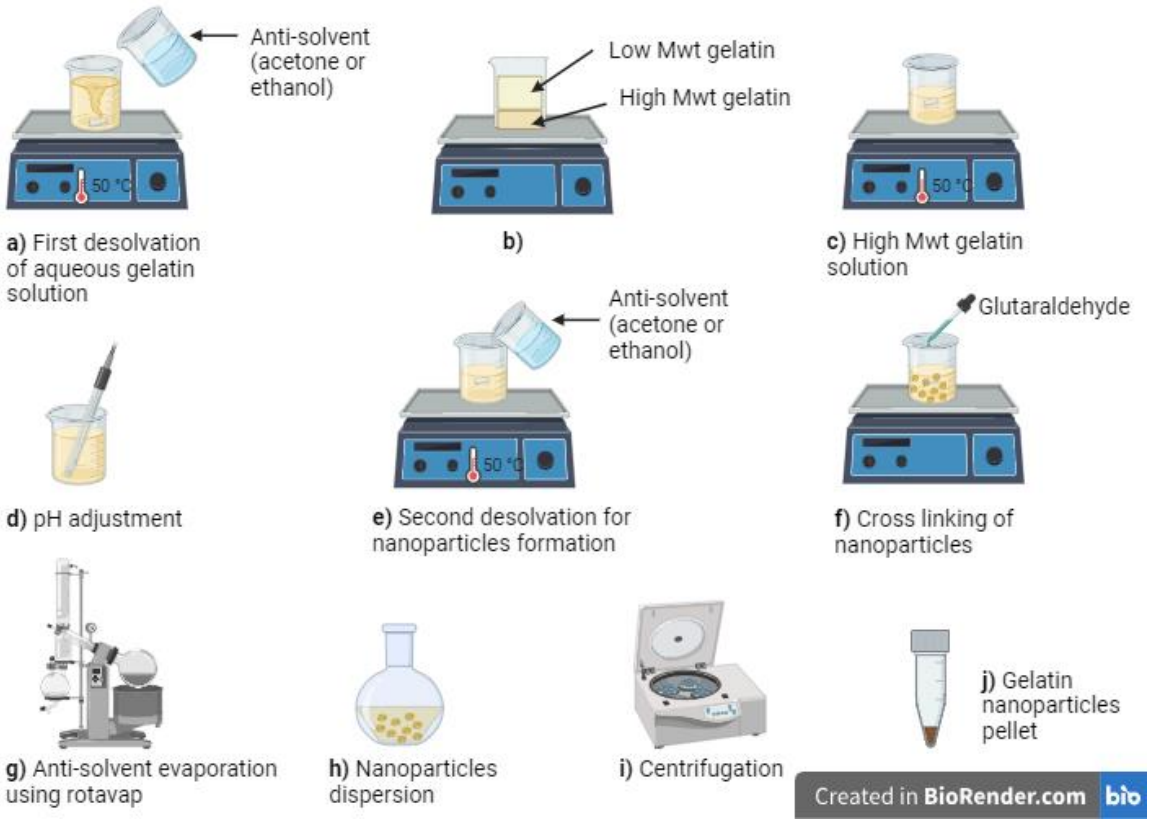

Fig. 1. Method of preparation of GNPs

2.2.2. Optimization of gelatin nanoparticles

In an attempt to optimize the prepared GNPs, different variables were studied, and their respective effects on the particle sizes (PS), zeta potentials (ZP), and polydispersity indices (PDI) of the nanoparticles were determined. Upon studying each variable, all other conditions were fixed.

2.2.2.1. pH screening

Screening the effect of different pH values of the gelatin solution, ranging from 1 to 13, to select the optimum value was done, by adjusting the solution pH using 5 M NaOH/HCL.

2.2.2.2. Antisolvent type

Two water-miscible antisolvents were separately used for the second desolavtion step, namely; acetone and ethanol.

2.2.2.3. Method of antisolvent addition

After selecting the optimal pH and best antisolvent for the fabrication of GNPs, the method of adding the antisolvent was varied, dropwise versus all at once addition, to choose the ideal means of addition.

2.2.2.4. Gelatin Concentration

To analyze the effect of the polymer concentration on the prepared nanoparticles, different concentrations of gelatin solution were utilized in the fabrication of GNPs, *viz*, 0.5, 0.75, 1, 1.25, and 1.5% w/w.

2.2.3. Characterization of GNPs

2.2.3.1. Determination of the particle size, zeta potential, and polydispersity index

The mean PS, ZP, and PDI of the freshly prepared GNPs were determined using a Malvern zetasizer (Nanoseries Malvern Panalytical Instruments Ltd., UK). An aliquot of each formulation was diluted in a ratio of 1:2 with deionized water, then analyzed at 25 °C. All measurements were carried out in triplicates and means and standard deviations were then calculated.

2.2.3.2 Determination of GNP yield

To determine the yield of GNPs, the high molecular weight gelatin fraction was initially precipitated after the first desolvation step, then freeze-dried for 24 h and weighed. Similarly, the resulting GNP suspension was freeze-dried over 24 h and then weighed. Freeze drying was conducted at -60 °C, 0.065 mbar. The following equation was used for yield calculation:

$$\%Yield = \frac{weight\ of\ freeze\ dried\ GNPs}{weight\ of\ high\ Mwt\ gelatin}$$

2.2.3.3. Transmission electron microscopy (TEM)

The morphology of GNPs was visualized by TEM. The dispersion of GNPs was sonicated for 2 minutes on an ultrasonicator (Crest Ultrasonics Corp., New Jersey, USA), then, a few drops were loaded on a carbon-coated copper grid, stained with 1% (w/v) phosphotungstic acid and allowed to air dry at room temperature **[37]**. Afterward, the grid loaded with the sample was examined by HR-TEM (Joel, TEM-2100, Tokyo, Japan), operated at 200 kV.

2.2.4. Statistical analysis

All measurements were performed in triplicates, and the mean values±standard deviation (SD) was calculated. Data analysis was performed using GraphPad Prism v.5.0 and significance was determined using one-way analysis of variance and student-t-tests. Data was considered significant when the *p*-value was less than 0.05.

3. Results and discussion

To optimize the prepared GNPs via the double desolvation method, several factors were examined, including; the pH of the gelatin solution, antisolvent type, method of adding the antisolvent, and finally gelatin solution concentration. The effects of these parameters on the PS, ZP, and PDI of GNPs were determined.

3.1. The effects of pH screening and antisolvent selection

pH is a key factor that can affect the colligative properties of nanoparticles, *viz*, PS, ZP, and PDI, hence, studying the effect of varying the gelatin solution pH was essential, as demonstrated in **Fig. 2a.** Since the isoelectric point (IEP) of gelatin B is 4.8, therefore, formulation of GNPs at a pH close to its IEP results in small net charge on gelatin chains and, hence, small repulsive forces, leading to high

inter-molecular interaction and the formation of larger particles of size ranging from 218.1±8.40 nm to 256.3±6.47 nm and from 157±3.87 nm to 133.6±5.83 nm upon using ethanol and acetone respectively at pH range of 6 to 9. Whereas, at pH values 3 and 10, acetone produced particles of respective sizes 137.7±4.35 nm and 112.7±4.87 nm, while ethanol produced GNPs with sizes of 236.9±6.64 nm and 196±5.29 nm. Since pH values 3 and 10 are away from the IEP, a high net charge is created on GNPs, preventing uncontrolled agglomeration *via* strong electrostatic repulsive forces and, thus, leading to the formation of smaller particles **[23, 38].**

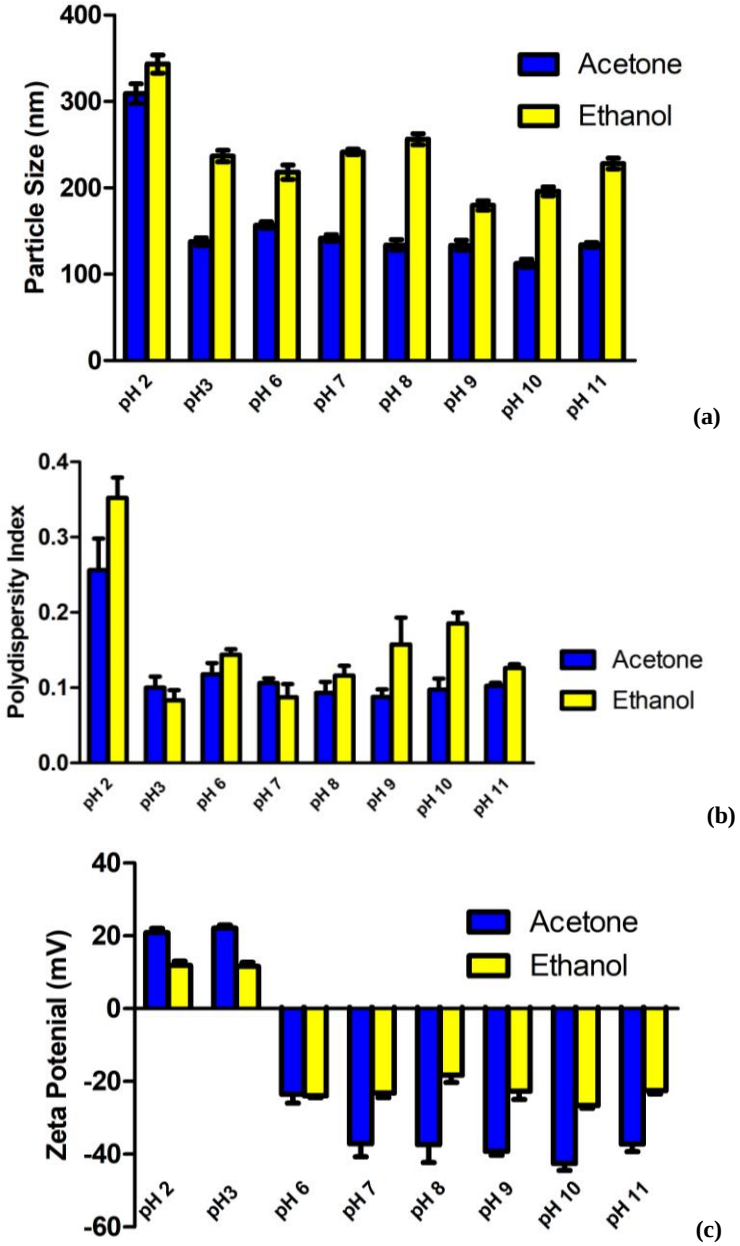

Fig. 2. Effect of pH on **(a)** PS, **(b)** PDI, and **(c)** ZP of GNPs prepared using 1% gelatin solution and desolvated using either acetone or ethanol, added all at once

Conversely, GNPs formulated at pH 2, using either ethanol or acetone, exhibited significantly higher ($p<0.05$) PS of 343.4±10.56 nm and 309±11.34 nm, respectively, compared with those prepared at all other pH values. Reducing the pH to a value of 2 probably increased the water-holding capacity of gelatin molecules, making it more difficult to induce desolvate, thus, resulting in an increase in the PS of the generated GNPs, in addition to, higher PDI values, as shown in **Fig. 2c [39]**.

Regarding the effect of pH on the ZP of the prepared GNPs, extreme pH values that are away from the IEP of gelatin B, ensure high positive/negative charges on the nanoparticles, to keep them suspended, guaranteeing their high physical stability. Lowering the pH to a value of 3 acquires positive charges on GNPs due to the protonation of aspartic acid and glutamic acid moieties in gelatin. As illustrated in **Fig. 2b,** GNPs prepared at pH 3 using acetone had a ZP of 22.13±0.81 mV, while those prepared using ethanol attained a ZP value of 11.63±1.05 mV. Further reduction in pH to a value of 2 led to a non-significant decrease in ZP to 20.88±1.15 mV and 11.87±1.16 mV using acetone and ethanol respectively, as side chains were already completely protonated ($p>0.05$). This reduction in pH value introduced chloride ions in the medium, accounting for the decrease in ZP **[39].** On the other hand, increasing pH above IEP generated NPs with negative charges, reaching a ZP value of -37.1±3.6 mV at pH 7. Afterward, at pHs 8 and 9, the change in ZP was less noticeable reaching -37.4±4.91 mV and -39.23±1.06 mV with the maximum value of -42.57±1.93 mV at pH 10 with acetone as the desolvating agent.

Based on the aforementioned results, pH10 was the selected pH for further optimization, as it produced the smallest particle size.

3.2. Antisolvent selection

The addition of an antisolvent such as acetone, ethanol, methanol, acetonitrile, and others during the desolvation process could reduce the aqueous solubility of gelatin due to conformational changes in the protein structure, leading to its precipitation in the nanometer range. Differences in the properties of desolvating agents like their polarity, dielectric constant, solubility parameters, and hydrogen bonding potential, could impact the physicochemical properties of the produced particles **[40].** As shown in **Table 1** and **Figs. 2 & 3**, there is a significant difference ($p<0.05$) between the properties of GNPs prepared using acetone compared with those prepared with ethanol at all pH values.

Upon comparing GNPs prepared at pH 10, the optimal selected pH for further optimization, acetone resulted in significantly ($p<0.0001$) smaller GNPs with a PS of 112.7±4.87 nm compared to 196±5.29 nm in the case of ethanol, in addition to, a more homogenous dispersion with respective PDI values of 0.098±0.02 and 0.185±0.02. Similar observations were obtained by Azarmi et al **[26]**. Acetone also induced a higher negative ZP and greater yield than ethanol.

The larger PS obtained with ethanol as a desolvating agent could be due to its greater ability to form hydrogen bonds, being a polar protic solvent, thus, can donate and accept hydrogen bonding, favoring the formation of larger lattices and, consequently, larger particles upon desolvation **[40]**. In another light, ethanol might have caused dramatic alterations in the secondary structure of gelatin, reinforcing the formation of larger particles, in a way similar to its effect on the desolvation of albumin in the study conducted by Beigi et al **[40].** As for the higher yield% obtained upon using acetone, it might be related to the ability of acetone to form

NPs in a smaller utilized volume than ethanol, hence, increasing the count of the NPs **[40].**

Accordingly, acetone was the favored desolvating agent for further optimization of GNPs.

Table 1. Physicochemical properties of GNPs prepared at pH 10 using acetone as an antisolvent compared to ethanol

Antisolvent	PS (nm)* ± SD	PDI* ± SD	ZP (mV)* ± SD	Yield (%)* ± SD
Actenoe	112.7 ± 4.87	0.10 ± 0.01	-42.57 ± 1.93	77.3 ± 0.58
Ethanol	196 ± 5.29	0.19 ± 0.01	-26.67 ± 0.68	65.3 ± 1.53

*Results are mean of three determinations ± SD. SD, standard deviation; PS, particle size; PDI, polydispersity index; ZP, zeta potential.

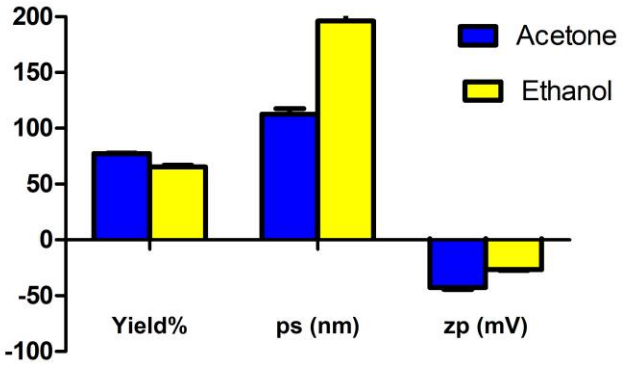

Fig. 3. Physicochemical properties of GNPs prepared using 1% gelatin solution adjusted at pH 10 with acetone as an antisolvent added all at once compared to ethanol

3.3 Method of antisolvent addition

Following the selection of acetone as the anti-solvent, its addition all at once was compared with its dropwise addition, to select the optimum method for the desolvation process. Results in **Table 2** and **Fig 4** displayed a significant difference with a $p<0.05$ between both methods, where the all-at-once addition generated a smaller PS, a more negative ZP, and a lower PDI.

Table 2. Effect of the method of addition of acetone on the physicochemical properties of GNPs

Method of adding acetone	PS (nm)* ± SD	PDI* ± SD	ZP (mV)* ± SD
All at once	112.7 ± 4.87	0.10 ± 0.01	-42.57 ± 1.93
Dropwise	232.5 ± 5.80	0.09 ± 0.04	-35.97 ± 1.17

*Results are mean of three determinations ± SD. SD, standard deviation; PS, particle size; PDI, polydispersity index; ZP, zeta potential.

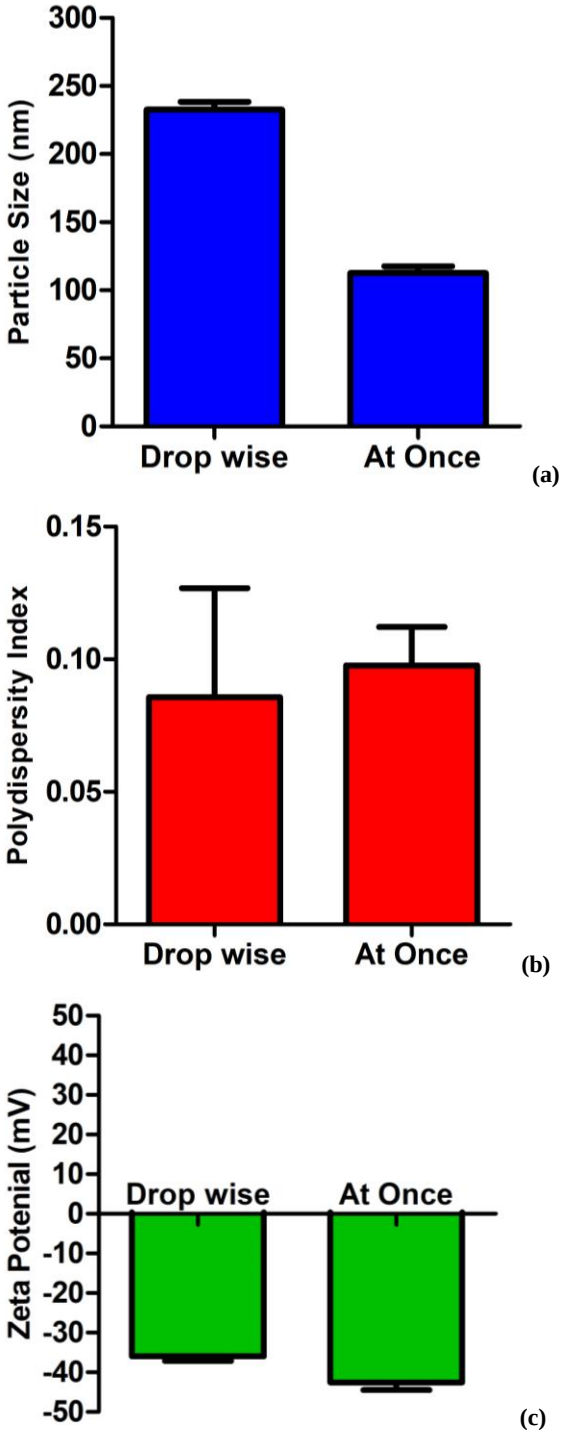

Fig. 4. Effect of the method of addition of acetone on **(a)** PS, **(b)** PDI, and **(c)** ZP of GNPs prepared at pH10 using 1% gelatin solution

The process of synthesis of GNPs encompasses controlled precipitation driven by the removal of water molecules by the desolavting agent, thus, gradual dropwise addition of acetone allows the slow precipitation of gelatin with a higher tendency to aggregate, hence, the larger PS **[39]**. Accordingly, all at once the addition of acetone was preferred over the gradual dropwise addition for further optimization, a conclusion that was similar to that obtained by Khramtsov et al **[41]**.

3.4. Effect of gelatin concentration

Different concentrations of gelatin solution were tested for the preparation of GNPs, ranging from 0.5 to 1.5 %w/w, with 0.25% incremental increases. **Table (3)** and **Fig. (5)** show a significant difference ($p<0.05$) between the lowest two concentrations (0.5 & 0.75 %w/w) and all the higher gelatin concentrations. The smallest PS of 99.44±2.26 nm was obtained at 0.5% gelatin concentration, which was almost doubled, to reach 179.6±4.30 nm when triple the gelatin concentration was used. This anticipated increase in size with higher gelatin concentrations was due to an increase in the viscosity of the solution, in addition to, a vaster number of gelatin strands that can sediment upon desolvation, promoting the formation of larger particles **[23, 39, 42]**.

All the prepared GNPs with different gelatin concentrations exhibited uniform size distributions with PDI values not exceeding 0.14. They also displayed high negative ZP values, with mostly insignificant differences, indicating high physical stability.

Therefore, based on the obtained results, 0.5% was our selected optimum gelatin concentration as it resulted in GNPs with the smallest PS.

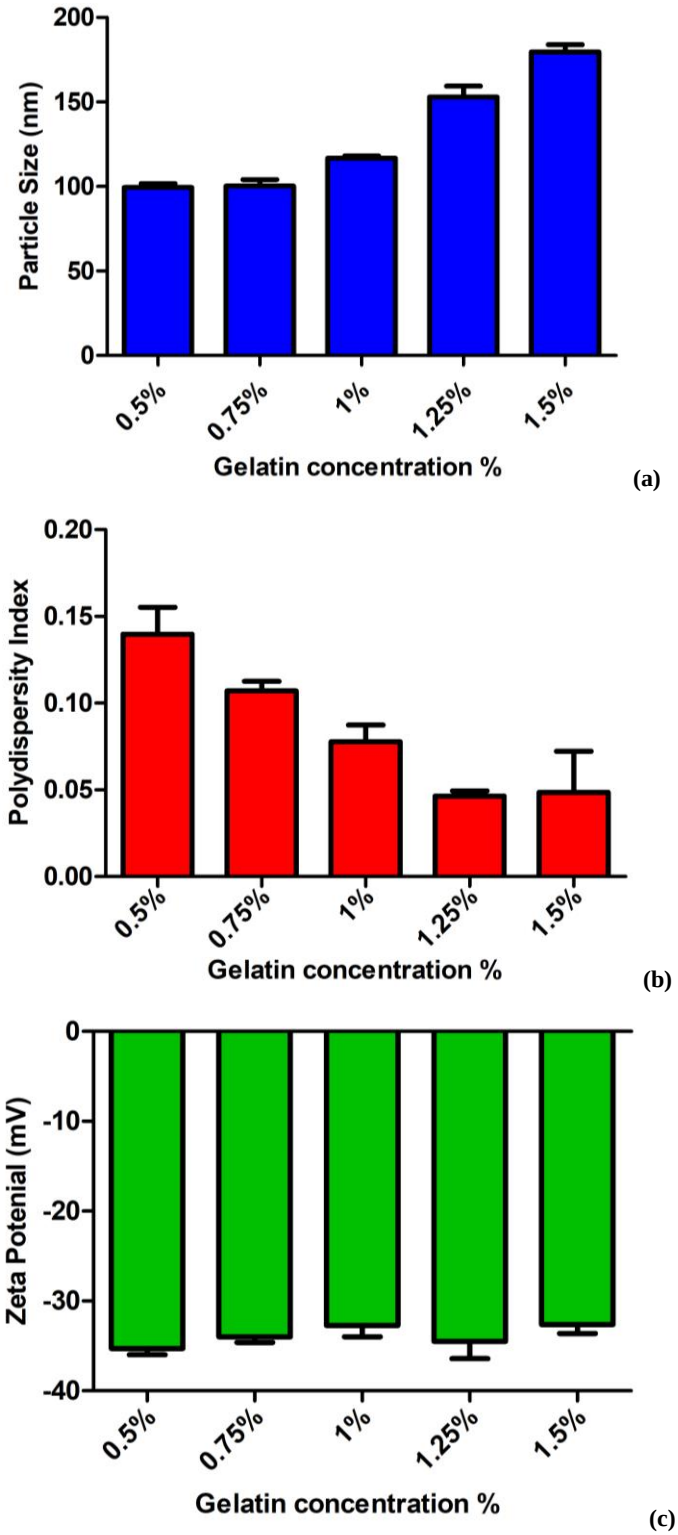

Fig. 5. Effect of different concentrations of gelatin on **(a)** PS, **(b)** PDI, and **(c)** ZP of GNPs prepared at pH 10 using acetone as the antisolvent added all at once

Table 3. Effect of different concentrations of gelatin on the physicochemical properties of GNPs

Gelatin concentration (% w/w)	PS (nm)* ± SD	PDI* ± SD	ZP (mV)* ± SD
0.5	99.44 ± 2.26	0.14 ± 0.02	-35.30 ± 0.69
0.75	100.3 ± 3.81	0.11 ± 0.01	-34.00 ± 0.62
1	116.8 ± 1.25	0.08 ± 0.01	-32.70 ± 1.30
1.25	152.9 ± 6.49	0.05 ± 0.003	-34.47 ± 1.96
1.5	179.6 ± 4.30	0.049 ± 0.02	-32.60 ± 1.02

*Results are mean of three determinations ± SD. SD, standard deviation; PS, particle size; PDI, polydispersity index; ZP, zeta potential.

3.5. Visualization of the selected GNPs by TEM

The selected GNPs, prepared using 0.5%gelatin solution adjusted to pH 10 and desolvated by all at once addition of acetone, were visualized by TEM, as illustrated in **Fig. 6**. TEM images revealed a regular spherical shape of the GNPs with a narrow size distribution. There were no aggregates, validating the suitability of the employed method of preparation, and the selected conditions for preparing GNPs with the required small size and high physical stability.

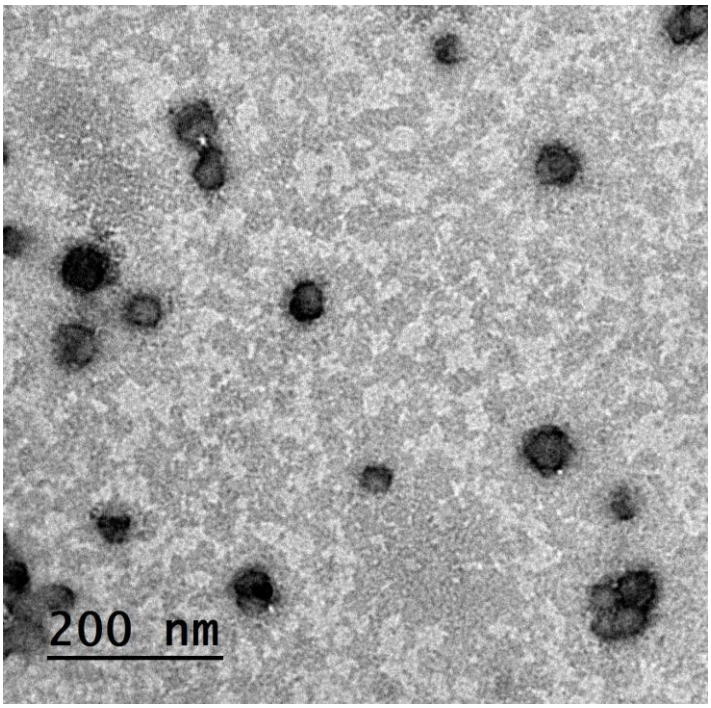

Fig. 6. TEM image of the optimized plain GNPs prepared using 0.5% gelatin solution adjusted to pH 10 and desolvated by all at once addition of acetone

Conclusion

Adjusting the pH of the gelatin solution used in the fabrication of gelatin type B NPs using the double desolvation method at the value of 10 resulted in the smallest particle sizes and highest charges, ensuring stability while utilizing both antisolvents, acetone, and ethanol, but with a superiority in case of acetone. Furthermore, the addition of acetone all at once, while using the lowest gelatin concentration enabled the formation of gelatin NPs with the smallest particle size, which enables its subsequent use as a DDS.

Declarations

Consent to publish

All authors have read and agreed to the published version of the manuscript

Ethics approval and consent to participate

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article in the main manuscript.

Conflict of Interest

The authors assert that there are no conflicts of interest.

Funding Statement

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Authors Contribution

All the authors have made a significant contribution to this manuscript, have seen and approved the final manuscript, and have agreed to its submission.

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