Journal of
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Effect of Some Synthetic Parameters on Size and Polydispersity Index of Gelatin Nanoparticles Cross-Linked by CDI/NHS System

S. Zinatloo-Ajabshir [*a], N. Taheri Qazvini[a, b]

[a] School of Chemistry, University College of Science, University of Tehran, I.R.Iran

[b] Biomaterials Research Center (BRC), University of Tehran, Tehran, I.R.Iran

Article history:
Received 17/04/2015
Accepted 19/05/2015
Published online 01/06/2015

Keywords:
Gelatin
Nanoparticles
Water soluble carbodiimide
Cross-linking agent
Intrinsic viscosity

**Corresponding author:*
E-mail address:
saharzinatlo@khayam.ut.ac.ir

Abstract

In our previous work, the effect of use of a water soluble CDI/NHS system as nontoxic cross-linking agent on fabrication of gelatin nanoparticles was investigated. In this research, the effect of variation in some synthetic parameters of gelatin nanoparticles cross-linked by CDI/NHS system such as type of gelatin and formulation of cross-linking agent on their size and distribution was examined. The conventional two step desolvation method was used for preparation of gelatin nanoparticles. The morphology, mean size and size distribution of the formed nanoparticles were evaluated and compared with each other. In addition, intrinsic viscosities of all the nanoparticles were measured and compared under different conditions. The results showed that the presence of more NHS and absence of NHS catalyst in CDI/NHS system lead to the large particle size and broad size distribution of nanoparticles that were attributed to the fast and slow cross-linking rate, respectively.

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1. Introduction

Gelatin is a biodegradable, biocompatible, non-carcinogenic and inexpensive polymer. Gelatin has variety of potential applications in pharmaceutics and food industry and its novel uses are underway in medicine and in specialized technical areas.

Among many other applications, gelatin nanoparticles could be served as a simple and safe carrier system for controlled drug delivery. Gelatin has numerous available active sites to attach targeting molecules and its phase behaviour in dilute and semi-dilute solutions could be easily tuned by pH and temperature. These properties make this product an interesting colloidal carrier for targeting drug delivery systems. Unlike, the artificial nanoparticles which may have side effects such as cell toxicity and accumulation in the human body, gelatin nanoparticles can be used for transferring large amount of the drug to the target site with minimal side effects [1-5].

Several techniques have been utilized for synthesis of gelatin based nanoparticles such as desolvation [6-11], nanoprecipitation [12-13], coacervation-phase separation [14-16], emulsification- solvent evaporation [17-20] and miniemulsion method [22,23].

Through Desolvation method, a desolvating agent (e.g. alcohol, acetone) is added to an aqueous gelatin solution to dehydrate the gelatin molecules. This results in stretched to coil conformational change. Commonly on the Next step, a cross-linking agent is added to harden the pristine particles. This method was modified by Coester et al [6] which added a desolvation step to separate low molecular weight gelatin molecules.

…Cross-linking of gelatin is very important to attain gelatin nanoparticles with desired characteristics. Unexpectedly, in all reported methods of fabrication of gelatin nanoparticles, glutaraldehyde has been used as cross-linking agent. Even though the use of glutaraldehyde causes to stability of nanoparticles, its high toxicity may restrict the utilizations of the ultimate product. Hence, the use of non-toxic cross-linkingers and evaluation of their effects on the practical characteristics of nanoparticles seems significant. The influence of utilize of a water soluble carbodiimide (CDI) as non-toxic cross-linking agent on synthesis process (via desolvation method) and on the ultimate characteristics of gelatin nanoparticles was studied [24].

The aim of this research is the optimum preparation of gelatin nanoparticles (in presence of non-toxic linking agent) and investigation of the effect of variation in synthetic parameters of nanoparticles such as type of gelatin and cross-linking agent on their size, PDI and intrinsic viscosity.

2. Experimental procedure

2.1. 2.1.1. Materials

All the chemicals were of reagent grade and were used without further purification. Gelatin: type A (Bloom 80-120) & gelatin Bacteriological (LTD Co), HCl, NHS (N-hydroxysuccinimide) and acetone were obtained from Merck. CDI (1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride) was purchased from Aldrich. Double distilled water was used for all the experiments.

2.1.2. Preparation of gelatin nanoparticles

A two-step desolvation method developed by Coester et al. [6] was used. Briefly, 0.5 g gelatin (two different types of gelatin were used) was dissolved in 10 mL distilled water at room temperature. Then, 10 mL acetone was added as desolvating agent to the solution to precipitate the high molecular weight fraction. The high molecular weight gelatin separated and then dissolved again in 10 mL distilled water with a stirring rate of 750 rpm. The pH of the new solution was adjusted at 2.5. Pristine gelatin nanoparticles formed by drop-wise addition of 30 mL acetone. In the next step, to stabilize the gelatin nanoparticles, cross-linking agents (2.5 mL of 1.2% CDI: NHS (5:1) solution, 5 mL of 1.2% CDI: NHS (5:1) solution and 2.5 mL of 1% CDI without NHS solution) were added and the resulted mixture stirred at 750 rpm for 12 hours. After a simple purification process, the acetone was removed using rotary evaporator.

2.2. Characterization of the nanoparticles

2.2.1. Shape and Size

The *Shape and Size:* The morphological study of the unloaded nanoparticles was performed using a digital scanning electron microscopy (SEM) DSM 960 (Carl Zeiss, Jena, Germany). To prepare the samples, 50 micro liters of the nanoparticle dispersions were freeze-dried on a glass surface. The particle size and

the particle size distribution of the nanoparticles was also determined by photon correlation spectroscopy (PCS), Zetasizer 3000 (Malvern Instruments, UK) with a He-Ne laser beam at a wavelength of 633 nm and scattering angle of 90°. To obtain optimum signal intensity all samples were diluted with double distilled water before measurements.

Intrinsic viscosity

The viscosity of nanoparticle dispersions and gelatin solution were determined by measuring the flow time in a capillary Ubbelohde viscometer at controlled temperatures of 25-45 ± 0.1 °C. A minimum of four repetitions were performed for each sample. Intrinsic viscosity [η] determined by extrapolation at zero concentration of the reduced viscosity, $\eta_{sp}/c = [\eta] + k_H[\eta]^2c$ (Huggins equation) [25]. Where, c (g/dl) is solute concentration, η_{sp} is the specific viscosity and represents the incremental viscosity due to the presence of the polymer chains or nanoparticles in the solution and k_H is the Huggins constant.

3. Results and discussion

3.1. Characterization of the nanoparticles

The size of nanoparticles greatly affects their applications [26]. In this research, two-step desolvation method proposed by Coester et al was used for preparation of nanoparticles with a proper size and size distribution [6]. Two-step desolvation method consists of an additional step in which low molecular weight gelatin removed after a desolvation step. This step enhances the stability of particles formed before cross-linking thus reduces the formation of aggregates during cross-linking. Removing of low molecular weight molecules also prevents further irreversible flocculation of particles during storage [6].

The average particle size of the unloaded gelatin nanoparticles cross-linked by CDI without NHS and CDI /NHS and CDI/NHS (twice as much as) and synthesized by another type of Gelatin as determined by photon correlation spectroscopy was found to be 335 (PDI=0.851) and 180 (PDI=0.141) and 240 (PDI=0.558) and 550 (PDI=1), respectively. Table1 and 2 presents the results of nanoparticle size characterization measurements.

Table 1. Effect of type of cross-linking agent on size and PDI of nanoparticles.

Sample no.	Cross-linked by	Nanoparticles size(nm)	PDI	Zeta potential(mV)
G1	CDI/NHS	335	0.851	16.3
G2	CDI/NHS (twice as much as)	184	0.141	-
G3	CDI	240	0.558	-

Table 2. Effect of type of gelatin on size and PDI of nanoparticles.

Sample no.	Gelatin type	Nanoparticles size (nm)	PDI	Zeta potential (mV)
G1	Merck gelatin type A	184	0.141	-
G4	(LTD) Gelatin	550	1	16.3

…SEM was used to characterize the morphology of nanoparticles. As shown in Fig. 1a and b, nanoparticles were spherical and a little non-homogeneous in size distribution, independent of type of used cross-linking agent. The mean size of nanoparticles from SEM micrographs resembles those calculated from dynamic light scattering measurements.

Furthermore the morphological studies from SEM micrographs reveal that apart from smaller size and

PDI, nanoparticles cross-linked by CDI/NHS (Fig 1b) have smoother surface than CDI without NHS (Fig 1a) cross-linked particles.

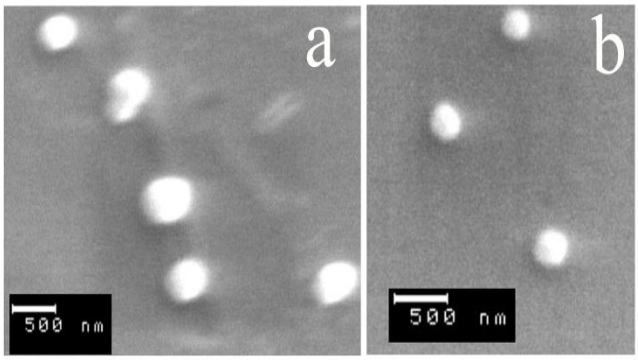

Fig. 1. SEM micrographs nanoparticles cross-linked by CDI (a) and CDI/NHS (b).

Besides, the CDI/NHS-GNPs and CDI without NHS and CDI/NHS (twice as much as)-GNPs were stable under long time storage conditions. The size and PDI of these nanoparticles remained almost unchanged after 3 months storage under refrigerated conditions at 4 ^{0}C.

The rather high PDI indices of all the nanoparticles synthesized using different cross-linking agents, could be related to the rate of mechanism of particle formation in the desolvation method. Through this method, Nanoparticles form through strong interactions of positively and negatively charged segments in or between the gelatin chains.

Because the synthesis conditions are same for all the types of the nanoparticles synthesized here, the difference in particle characteristics may be attributed to the difference in chemistry of cross-linking agents and consequently the difference in the nature of network structures formed by CDI or CDI/NHS.

The network formation mechanism of CDI/NHS cross-linking systems has been described. It has been accepted that [27] CDI activates the carboxylic acid residues of aspartic and glutamic acids on gelatin chains. The presence of NHS is critical in this stage. NHS molecules react with the previously mentioned activated carboxylilc acid groups. In the absence of NHS, the activated carboxylic groups may hydrolyze or rearrange to o-acylisourea residues (Fig 2) [28].

But after reaction with NHS, the activated groups are less likely to rearrange or hydrolyze [29]. The network formation is then launched by the reaction of the unprotoned amino residues of lysine and hydroxylysine with the activated carboxylic acid residues on gelatin molecules. Therefore, NHS/CDI is a zero-length cross-linker because it does not introduce any space between gelatin chains during the formation of amide bonds as chemical cross-linker.

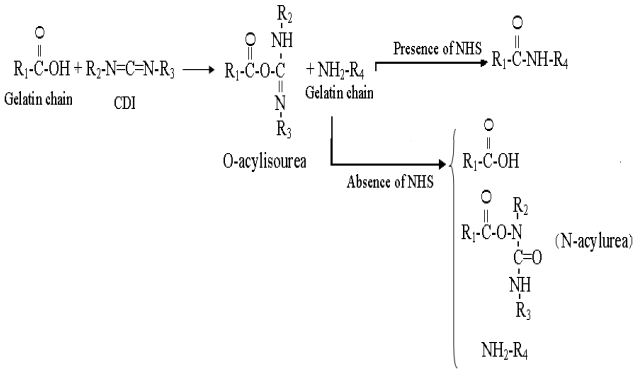

Fig. 2. Cross-linking mechanism of CDI/NHS system.

It seems that for the sake of charge neutralization process during nanoparticles formation, there have been enough free amino groups for both cross-linking agent (CDI and CDI/NHS) to form chemical cross-links. However, in the case of CDI/NHS, the formation of cross-links is more simplified due to charge neutralization considerations that enforce the reactant groups (-NH2, -COOH) to be brought in the proximity of each other and thereupon they can react more easily. This phenomenon leads to faster stabilization of the nanoparticles which inhibit further aggregation that increase the size of the particles. The large particles size and broad size distribution of CDI cross-linked nanoparticles can be attributed to the slow cross-linking rate due to absence of NHS that

acts as catalyst under the investigated conditions. Slow rate of cross-linking causes primary gelatin nanoparticles to agglomerate before cross-linking. The large particle size and broad size distribution of CDI/NHS (twice as much as) cross-linked nanoparticles can be attributed to the fast cross-linking rate due to presence of more NHS catalyst under the investigated conditions. In this case, fast rate of cross-linking leads to reaction between several nanoparticles during cross-linking. Large particles size and broad size distribution of nanoparticles synthesized by using (LTD Co) gelatin can be attributed to the high temperature of its gelling point. In fact, the room temperature is under the gelling point of this type of gelatin and lead to formation of large aggregates. It is noteworthy that any change in the reaction conditions such as pH, temperature, desolvation agent, ionic strength of the solution as well as gelatin type may lead to nanoparticles with other sizes and morphological characteristics.

3.2. Intrinsic viscosity

It is generally known that the main effect of dissolution or dispersion of macromolecules or nanoparticles in a solvent is an increment in viscosity.

This effect is quantitatively expressed by the intrinsic viscosity [η], which is sensitive to the conformation or flexibility of the macromolecules, as well as the size and to the nature of interaction of the solute with the solvent [25]. Thus, exact measurement of [η] can present helpful information about the mentioned before features.

Fig 3a demonstrates the Huggins function, $H=\eta_{sp}/c[\eta]$ as a function of reduced concentration, [η]c, all nanoparticles and a normal gelatin solution prepared at its native pH=5.4 and T=25 ^{0}C. The intrinsic viscosity for G1, G2, G3 and G4 was found to be 15.6, 20.3, 38.1 and 40.7 ml/g respectively, and 25.2 ml/g for gelatin solution at pH =5.4.

The slope of the plot indicates Huggins constant, k_H [30]. For the nanoparticles dispersed in a solvent, k_H provides information about nature of interaction between particles. A large and positive k_H is due to strong repulsive forces between particles.

On the contrary, negative values of Huggins constant represent the attractive forces between particles. As Fig 3 shows, the Huggins constants for the nanoparticles and gelatin solution are positive, which indicate repulsive forces between nanoparticles or gelatin chains in the solution (Table 3). In the case of gelatin solution measured at pH=5.4, very close to its PI (~5), the net charge of gelatin chains in the solution is almost neutral, lead to rather low k_H value.

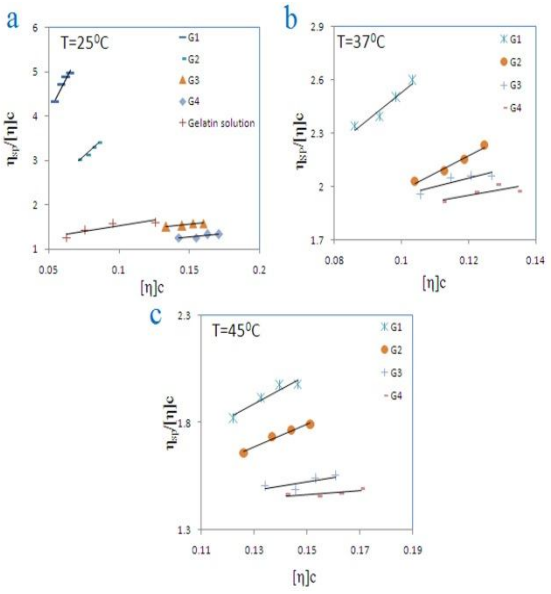

Fig. 3. Huggins function vs. reduced concentration for gelatin nanoparticles at different temperatures, 25 ^{0}C (a), 37 ^{0}C (b) and 45 ^{0}C (c).

Table 3. Huggins constants and intrinsic viscosities of the investigated systems at T=25°C

Sample	G1	G2	G3	G4	Gelatin solution
[η](ml/g)	15.6	20.3	38.1	40.7	25.2
K_H	14.97	11.54	5.51	4.67	3.26

3.2.1. Effect of pH and temperature on nanoparticles

The effect of pH and temperature on the Huggins function of G1, G2, G3 and G4 nanoparticles was depicted in Fig 3-6. The nanoparticles may be used at various temperatures and pHs such as physiological condition (pH=7.4, 37°C). Thus, it is very important to study the effect of pH and temperature on the size and stability of the nanoparticles at desired conditions.

In the case of G1, G2, G3 and G4 nanoparticles, it can be seen that raising temperature at constant pH, did not affect considerably the nature of interparticle interactions and just lead to a little expansion of nanoparticles because improving the solvent quality, so that the intrinsic viscosity increased (Fig 3 and 4; Table 4). It has been shown by Bohidar et al [30] that water becomes a better solvent for gelatin with increasing temperature.

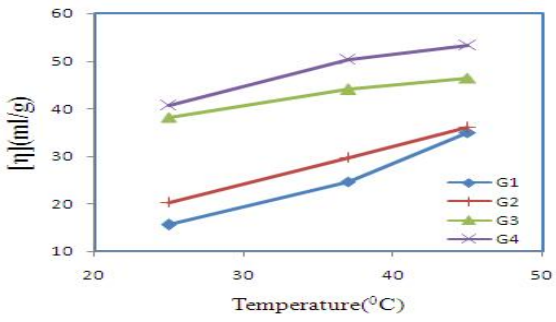

Fig.4. Intrinsic viscositiy vs. temperature for gelatin nanoparticles.

Table 4. Intrinsic viscosities of the investigated systems at different temperatures

Sample	[η](ml/g), T=25°C	[η](ml/g), T=37°C	[η](ml/g), T=45°C
G1	15.6	24.6	34.9
G2	20.3	29.7	36
G3	38.1	44	46.4
G4	40.7	50.4	53.3

Also, in the case of G1, G2, G3 and G4 nanoparticles, two different behaviors can be observed by raising pH from 3.2 to 5.4 and then 7.4 at 25 °C source used in this work was found to be about 5. At pH=3.2, ionization of -NH2 groups lead to net positive charges on the gelatin chains and enhance electrostatic repulsion which represented by high Huggins constant.

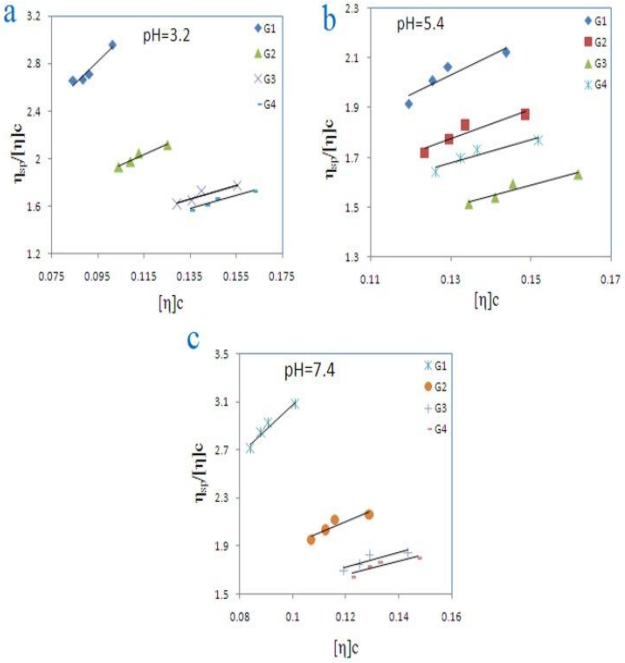

Fig. 5. Huggins function vs. reduced concentration for gelatin nanoparticles at different pHs, pH= 3.2 (a), pH =5.4 (b) and pH =7.4 (c).

Table 5. Huggins constants of the investigated systems at different pHs.

Sample	$K_{H,}$ pH=3.2	$K_{H,}$ pH=5.4	$K_{H,}$ pH=7.4
G1	14.6	8	15.6
G2	9	5.95	9.17
G3	5.9	4.6	6
G4	5.4	4.1	5.51

But, at pH=5.4, the charge balance on the gelatin chain lead to weakness of electrostatic repulsion between nanoparticles, which resulted in smaller k_H. Again, raising pH to7.4 lead to more ionization of –COOH groups and it lead to net negative charge of the gelatin chains which enhanced electrostatic repulsion between chain segments and therefore caused a large k_H at pH=7.4 (Fig 5 and 6; Table 5).

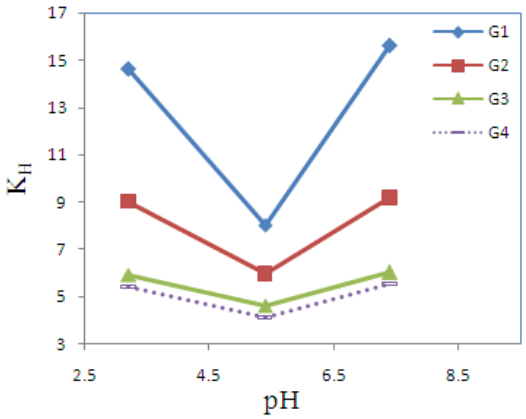

Fig. 6. Huggins constants vs. pH for for gelatin nanoparticles.

4. Conclusion

In this research, the effect of variation in some synthetic parameters of gelatin nanoparticles cross-linked by CDI/NHS system (as a non-toxic cross-linking system) such as type of gelatin and formulation of cross-linking agent on their size and distribution was examined. The conventional two step desolvation method was used for preparation of gelatin nanoparticles. The results showed that the presence of more NHS and absence of NHS catalyst in CDI/NHS system lead to the large particle size and broad size distribution of nanoparticles that were attributed to the fast and slow cross-linking rate, respectively. Fast rate of cross-linking leads to reaction between several nanoparticles during cross-linking and slow rate of cross-linking causes primary gelatin nanoparticles to agglomerate before cross-linking. Large particles size and wide size distribution of nanoparticles synthesized using another type of gelatin were ascribed to the high temperature of its gelling point lead to formation of large aggregates. Moreover, dilute solution viscosimetry experiments confirmed the stability of the nanoparticles under various physicochemical conditions. On the whole, these results suggest that CDI/NHS cross-linked gelatin nanoparticles have high potential to be used for drug delivery applications.

Acknowledgment

Partial financial support from the Iranian Nanotechnology Initiative and vice-president for research and technology of University of Tehran is gratefully appreciated.

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