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Inverse Miniemulsion Method for Synthesis of Gelatin Nanoparticles in Presence of CDI/NHS as a Non-toxic Cross-linking System

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Abstract

In this research, gelatin nanoparticles were synthesized via inverse miniemulsion method by employing a mixture of a water soluble carbodiimide (CDI) and N-hydroxysuccinimide (NHS) as a non-toxic cross-linking system. The gelatin nanoparticles were characterized for their size and size distribution, morphology and stability and were compared with those of nanoparticles cross-linked by glutaraldehyde (GA) as the most commonly utilized cross-linking agent. The results showed the formation of more homogeneous nanoparticles with smaller size when CDI/NHS used as cross-linking agent under the same synthesis condition. Moreover, dilute solution viscosimetry experiments confirmed the stability of the nanoparticles under various physicochemical conditions. The differences in the characteristics of CDI/NHS and GA cross-linked nanoparticles were ascribed to the different nature of network formation using the two cross-linking agents. Generally, these results suggested CDI/NHS cross-linked gelatin nanoprticles as an interesting candidate for drug delivery application.

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 behavior 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 polymer 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 miniemulsion method, a W/O emulsion formed from a mixture including aqueous gelatin droplets and a continuous oil phase by either a emulsifier or a high-speed mechanical stirrer. Next, the forming aqueous droplets of gelatin are hardened with crosslinking agents [21].

Recently, Ethirajan et al. [22] prepared and optimized gelatin nanoparticles using the miniemulsion method. Their experimental results indicated independent of the molecular weight distribution of the used gelatin, stable nanoparticles can be fabricated with a small amount of surfactant.

Cross-linking of gelatin is very important to attain gelatin nanoparticles with desired characteristics. Unexpectedly, in all reportedmethods 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].

In this work, gelatin nanoparticles were fabricated via inverse miniemulsion method by employing water soluble CDI as non-toxic cross-linking agent. The gelatin nanoparticles were characterized for their size and size distribution, morphology and stability and were compared with those of nanoparticles cross-linked by (GA) as the most commonly used cross-linking agent.

2. Experimental procedure

2.1. 2.1.1. Materials

All the chemicals were of reagent grade and were used without further purification. Gelatin type B (Bloom 80-120), glutaraldehyde (GA, 25% aqueous solution), *p*-Xylene, Tween80 (Poly sorbet), HCl, N-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (CDI) were purchased from Merck. Double distilled water was used for all the experiments.

2.1.2. Preparation of D1(cross-linked by CDI/NHS system) and D2 (cross-linked by GA) nanoparticles via miniemulsion process

D1 and D2 nanoparticles were prepared by the inverse miniemulsion method. In the first step for preparation of gelatin nanoparticles, two inverse miniemulsions A and B, that A includes the gelatin droplets, while B includes the GA or CDI/NHS solution as cross-linking agent droplets, were united. For the inverse miniemulsion A, 1 g of 10% gelatin solution was added to 10 g *p*-xylene including 50 mg of the Tween 80 as emulsifier and temperature of mixture was adjusted at 25 °C. After stirring the

mixture at 1000 rpm for 1 h, it was ultrasonicated to achieve a stable miniemulsion by a (LABSONIC® P (B.Braun, Germany) 22 kHz, 400W) at 90% intensity for 3 min under ice cooling.

In a similar manner, miniemulsion B, including droplets of cross-linking agent (1 ml of 1.2% CDI:NHS (5:1) solution or 100 µl of 25% glutaraldehyde solution) in 5 g *p*-xylene stabilized by 25 mg of Tween 80 by stirring the mixture for 1 h at 1000 rpm and ultrasonicating this mixture for 2 min at 90% intensity. In the second step, without delay, the inverse miniemulsion B was added to the inverse miniemulsion A and to fuse the droplets to produce cross-linked gelatin nanoparticles by the fusion and fission process, the mixture was ultrasonicated for 2 min under ice cooling at 75% intensity.

In the final step, after stirring the mixture for 8 min at 25 °C, *p*-Xylene and water were immediately removed by freeze-drying. The dried gelatin nanoparticles were dispersed in water and the dispersed gelatin nanoparticles were analyzed.

2.2. Characterization of the nanoparticles

2.2.1. Shape and Size

The morphology of the nanoparticles was determined by a digital scanning electron microscopy (SEM) DSM 960 (Carl Zeiss, Jena, Germany). To prepare the samples, 50 microliters of the nanoparticle dispersions were freeze-dried on a glass surface. The size and size distribution of the nanoparticles was also determined using photon correlation spectroscopy (PCS), Zetasizer 3000 (Malvern Instruments, UK) with He-Ne laser beam at a wavelength of 633 nm and scattering angle of 90^0. In the case of G1 and G2 nanoparticles, all samples were diluted with double distilled water before measurements, to obtain optimum signal intensity.

2.2.2. Intrinsic viscosity

The viscosity of nanoparticle dispersions and gelatin solution were determined by measuring the flow time in a capillary Ubbelohde viscometer at controlledpHs of 3.2-7.4 and controlled temperatures of 25-45±0.1 °C. For each sample, a minimum of four reiterations were accomplished. 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 dissolved substance concentration, $\eta_{sp}$is the specific viscosity and exhibits the incremental viscosity as a result of the presence of the polymer chains in the solution or nanoparticles in the dispersion 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 study, we used inverse miniemulsion method to fabricate nanoparticles. In inverse miniemulsion process, in the initial step, two separate miniemulsions were produced in *p*-xylene as continuous phase, one containing a gelatin solution (without removal of low molecular weight molecules) as droplet phase and the other one containing GA and CDI/NHS system as droplet phase, and united in the next step. Finally, during fission/fusion process by occurring reaction between the two different droplet species, gelatin nanoparticles were created. The formed cross-linked gelatin nanoparticles could be redispersed into water because of steric stabilization due to surfactant (Tween 80) and also steric and electrostatic stabilization caused by charge and some loose chain ends of gelatin nanoprticles. The average particle size of the D1, D2 nanoparticles as determined by photon correlation spectroscopy was found to be 136 ±10

(PDI=0.4±0.08) and 180±6 (PDI=0.5±0.06), respectively. Table1 presents the results of nanoparticle size characterization measurements.

Table 1. Size and polydispersity index for nanoparticles.

Sample	D1	D2
Cross-linking agent	CDI/NHS	GA
Size(nm)	136±10	180±6
PDI	0.4±0.08	0.5±0.06

In the miniemulsion method, as mentioned before, in the last step, the droplets are formed because of fusion and fission processes when strong shear forces produced by using the ultrasound .This leads to cross-linking agent can be homogeneously distributed inside the gelatin droplets, thereby causing the effective use of the both cross-linking agents. Glutaraldehyde as a non-zero length cross-linker generally bridges between free amino groups of lysine or hydroxylysine [27]. The aldehyde functional groups react with free -NH_2 groups through a nucleophilic addition type reaction. On the contrary, in the case of CDI/NHS cross-linking system, CDI in the network formation mechanism activates the carboxylic acid residues of aspartic and glutamic acids on gelatin chains. NHS molecules react with the previously mentioned activated carboxylilc acid groups [28]. In the absence of NHS, the activated carboxylic groups may hydrolyze or rearrange to o-acylisourea residues [29]. Nonetheless, the activated groups are less probably to rearrange or hydrolyze after reaction with NHS [30]. The network formation is then begun by the reaction of the unprotoned amino residues of lysine and hydroxylysine with the activated carboxylic acid residues on gelatin molecules (Fig 2) Thus, CDI/NHS as a chemical zero-length cross linking system does not introduce any spaces during the formation of amino bonds between gelatin chains Under the investigated conditions, the aldehyde functional groups of GA react with free amino groups via a nucleophilic addition type reaction (Fig 1) an also in the case of CDI/NHS, the network formation is occurred by the reaction of the unprotoned amino residues of lysine and hydroxylysine with the activated carboxylic acid residues on gelatin chains (Fig 2).

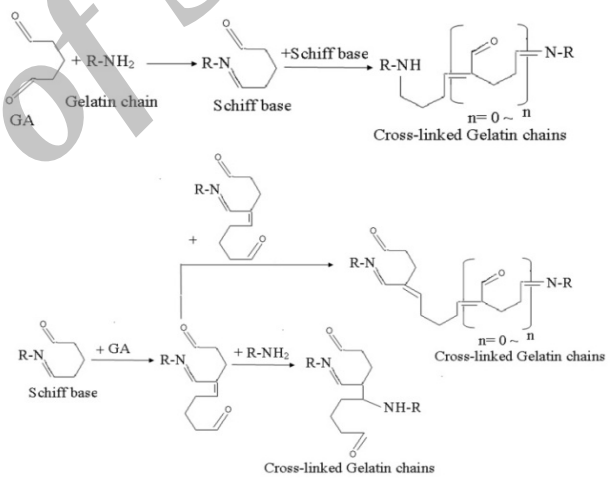

Fig. 1. Cross-linking mechanisms of GA

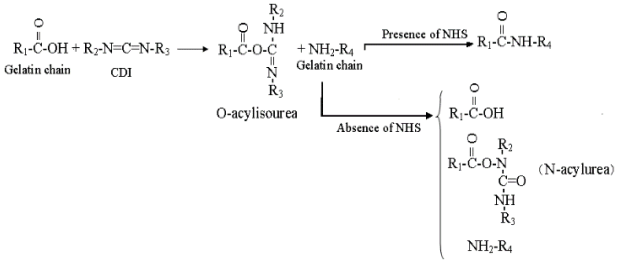

Fig. 2. Cross-linking mechanism of CDI/NHS system.

As already described in experimental section, *p*-Xylene and water were removed by freeze-drying.

The freeze-dried gelatin nanoparticles were dispersed in water and then the dispersed gelatin nanoparticles were analyzed. Nanoparticles tend to agregate during freeze-drying process. If the aggregated particles do not separate during redispersion in water by using the ultrasound, then larger size and wider size distribution will be measured. Thus, large particles size and rather broad size distribution of D1 and D2 nanoparticles can be attributed to not to separate the aggregated particles from each other during redispersion in water.

SEM was used to characterize the morphology of nanoparticles. As shown in Fig 3, nanoparticles were spherical and a little inhomogeneous in size distribution, independent of type of used cross-linking agent. The mean size of D1 and D2 nanoparticles from SEM micrographs resembles those calculated from dynaminc light scattering measurements.

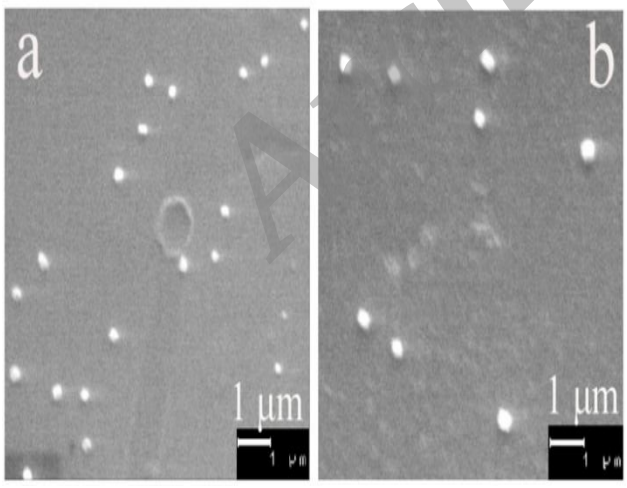

Fig. 3. SEM micrographs of the D1 nanoparticles (a) and D2 nanoparticles (b).

Moreover, the D1 nanoparticles were stable under long time storage conditions. The size and PDI of both nanoparticles remained almost unchanged after 3months storage under refrigerated conditions at 4 °C.

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 4a demonstrates the Huggins function, $H=\eta_{sp}/c[\eta]$ as a function of reduced concentration, [η]c, for D1, D2 nanoparticles and a normal gelatin solution prepared at its native pH=5.4. The intrinsic viscosity was found to be 32.8 and 33.9 ml/g for D1 and D2 nanoparticles respectively, and 25.25 ml/g for gelatin solution at pH =5.4 .

The slope of the plot indicates Huggins constant, k_H [31]. 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 4 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 2). 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. The low k_H values for D1, D2 nanoparticles can be ascribed to the reduction of the surface charge density of particles due to existence of surfactant on the particles surface and also the large size of particles.

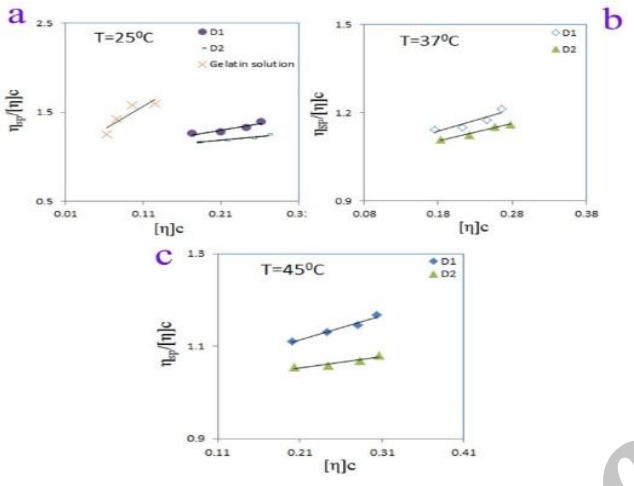

Fig. 4. Huggins function vs. reduced concentration for gelatin nanoparticles at different temperatures, 25 ^{0}C (a), 37 ^{0}C (b) and 45 ^{0}C (c).

Table 2. Huggins constants and intrinsic viscosities of the investigated systems at T=25°C

Sample	D1	D2	Gelatin solution
[η](ml/g)	32.8	33.9	25.2
K_H	1.414	0.884	5

3.2.1. Effect of pH and temperature on nanoparticles

The effect of pH and temperature on the Huggins function of D1 and D2 nanoparticles was depicted in Fig 5a-6c. 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 D1 and D2 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 4; Table 3). It has been shown by Bohidar et al [32] that water becomes a better solvent for gelatin with increasing temperature.

Table 3. Intrinsic viscosities of the investigated systems at different temperatures

Sample	$K_{H,}$ pH=3.2	$K_{H,}$ pH=5.4	$K_{H,}$ pH=7.4
D1	1.872	0.913	1.946
D2	1.433	0.867	1.545

Also, in the case of D1 and D2 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.

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.

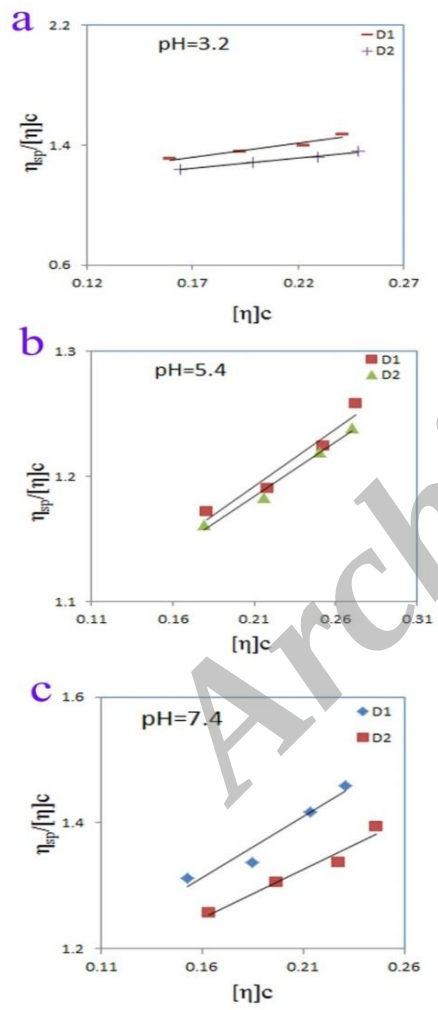

Fig. 5. Huggins function vs. reduced concentration for gelatin nanoparticles at different pHs, pH= 3.2 (a), pH =5.4 (b) and pH =7.4 (c).

Table 4. Huggins constants of the investigated systems at different pHs.

Sample	[η](ml/g), T=25°C	[η](ml/g), T=37°C	[η](ml/g), T=45°C
D1	32.8	33.2	37.9
D2	33.9	34.7	38.3

3. Conclusion

In this research, gelatin nanoparticles with desirable characteristics were synthesized inverse miniemulsion method in presence of CDI/NHS as a non-toxic cross-linking system. Comparison of the size and morphological properties of the nanoparticles cross-linked by CDI/NHS system with those of cross-linked by GA, prepared at the equal condition, showed a smaller mean size, narrower size distribution and more homogeneous morphology. 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.

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