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Synthesis and characterization of gelatin nanoparticles using CDI/NHS as a non-toxic cross-linking system

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Abstract Gelatin nanoparticles, cross-linked by a mixture of a water soluble carbodiimide (CDI) and *N*-hydroxysuccinimide (NHS) as a non-toxic cross-linking system, was prepared. The conventional two step desolvation method with acetone as the non-solvent was used. The mean size and size distribution as well as the morphology of the formed nanoparticles were evaluated and compared with those of nanoparticles cross-linked by glutaraldehyde (GA) as the most commonly used cross-linking agent. Furthermore, intrinsic viscosities of the nanoparticles cross-linked by CDI/NHS and GA were measured and compared under various conditions. The results showed the formation of smoother and more homogeneous nanoparticles with smaller size when CDI/NHS used as cross-linking agent under the same synthesis condition. Moreover, nanoparticles encapsulating paracetamol as a model drug were produced by the two different cross-linking agents and were characterized for drug entrapment and loading efficiencies and in vitro drug release. Both drug entrapment and loading efficiencies was higher in the CDI/NHS cross-linked nanoparticles; however, the release kinetics was comparable to that of nanoparticles cross-linked with GA. The differences in the characteristics of CDI/NHS and GA cross-linked nanoparticles were attributed to the different nature of network structures formed by the two cross-linking agents. On the whole, these results suggested that CDI/NHS cross-linked nanoparticles have high potential to be used for drug delivery application in preference to the nanoparticles synthesized by toxic cross-linking agents.

1 Introduction

Gelatin as a biodegradable, biocompatible and cheap product is becoming a focal point of interest for new uses in health care and in specialized technical areas [1]. It is non-carcinogenic in nature and possesses a relatively low antigenicity [2]. Furthermore, gelatin has numerous available functional groups and therefore is well-suited for attaching targeting molecules. Moreover, due to its unique amino acid sequences, the phase behavior of gelatin in dilute and semi-dilute solutions could be easily controlled and tuned by pH and temperature. All these properties make this product an interesting candidate for colloidal carrier systems. In this regard, gelatin nanoparticles could be served as a simple and safe carrier system for controlled drug delivery. Unlike, the synthetic polymer based nanoparticles which may have side effects such as cell toxicity and accumulation in the human body, gelatin nanoparticles can be used for targeted delivery of the drug with negligible side effects.

Several methods have been employed to synthesis gelatin based nanoparticles. Desolvation [3–12], coacervation-phase separation [13–15] and emulsification-solvent evaporation [16–18] are the main techniques.

Desolvation technique is based on the addition of a desolvating agent (e.g., alcohol and acetone) to an aqueous

Dedicated to the memory of Professor Mohammad-Nabi Sarbolouki (1949–2009).

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gelatin solution in order to dehydrate the gelatin molecules. This results in conformational change from stretched to coil conformation. Next, a cross-linking agent is added to harden the native particles. The method was modified by Coester et al. [5] by addition of a desolvation step to separate low molecular weight gelatin molecules.

The effects of various parameters such as temperature, pH, degree of cross-linking, nature of gelatin and type of desolvating agent on the size of the nanoparticles and release kinetics have been investigated by several groups. Vanderourt and Ludwig [6] studied the effect of some preparation parameters at different pH levels, on particles size, zeta potential value, and drug loading or drug release profile. Their results showed that the effect of investigated parameters depends mainly on the nature of the loaded drug. Later, Saxena et al. [7] investigating the effect of heterogeneity in molecular weight on drug encapsulation efficiency of gelatin nanoparticles, showed that variation made in different physico-chemical parameters (molecular weight of gelatin, temperature, pH) do not bring about any significant change in the size of formed nanoparticles.

Azarmi et al. [8] observed that the nature of gelatin has an obvious effect on particle characteristics. Their results showed that smaller nanoparticles with narrow size distribution can be prepared by gelatin type B if one controls the temperature, the concentration of cross-linking agent and pH and using acetone as desolvating agent. Similarly, Jahanshahi et al. [9] fabricated gelatin nanoparticles as drug carrier and optimized the particle size of the nanoparticles. They showed that gelatin nanoparticles of about 170 nm can be synthesize by optimizing temperature, gelatin concentration, non-solvent (acetone) amount and cross-linking agent and agitating rate.

Recently, by modifying the desolvation method, Won and Kim [10], prepared nanoparticles based on recombinant human gelatin. Saraogi et al. [11] prepared gelatin nano-carriers with uniform size distribution and appropriate release rate by two step desolvation method. They proposed that gelatin nanoparticles may be utilized as potential tool for the delivery of bioactives to the lung tissues with minimized side effects and improved remedial efficiency of the drug. Just recently, using ethanol–water mixture as the non-solvent, Ofokansi et al. [12] prepared gelatin nanoparticles by one step desolvating technique at 37°C and pH of 7.0. Their results showed that there is optimum level of cross-linking agent as well as cross-linking time that yields particles with minimum size and polydispersity index.

To achieve gelatin nanoparticles with desirable properties, cross-linking of gelatin is crucial. Surprisingly, except Won and Kim's work [10] in which genipin was used as cross-linking agent, in all other previously reported methods of synthesis of gelatin nanoparticles, GA has been exploited as cross-linking material. Although the use of GA leads to improvement of mechanical properties and stability of nanoparticles, the high toxicity of this material may limit the applications of the final product. Therefore, the use of non-toxic cross-linking agents and investigation of their effects on the functional properties of nanoparticles seems important.

The main goal of this study is investigating the effect of use of a water soluble carbodiimide (CDI) as nontoxic cross-linking agent on fabrication process and on the final properties of gelatin nanoparticles. The desolvation method was used and the intrinsic viscosity and the drug loading entrapment efficiency of gelatin nanoparticles cross-linked by the new cross-linking system was investigated and compared to those of nanoparticles cross-linked by GA as common cross-linking agent.

2 Experimental

2.1 Materials

All the chemicals were of reagent grade and were used without further purification. Gelatin type B (Bloom 80-120), GA (25% aqueous solution), HCl, NHS, 1-(3-dimethyl-aminopropyl)-3-ethyl-carbodiimide hydrochloride (CDI), and acetone (analytical grade) were obtained from Merck. Double distilled water was used for all the experiments.

2.2 Preparation of gelatin nanoparticles

A two-step desolvation method developed by Coester et al. [5] was used. In summary, 1.25 g gelatin was dissolved in 25 ml distilled water at room temperature. Then to precipitate the high molecular weight fraction, 25 ml acetone as desolvating agent was added to the solution. The high molecular weight gelatin separated and then dissolved again in 25 ml distilled water with a stirring rate of 750 rpm. The pH of the new solution was adjusted at 2.5. Primary gelatin nanoparticles formed by drop-wise addition of 75 ml acetone. Finally, to stabilize the gelatin nanoparticles, the cross-linking agent (250 µl of 25% GA solution or 6.25 ml of 1.2% CDI:NHS (5:1) solution) was added and the system stirred at 750 rpm for 12 h. After common purification process, the acetone was removed using rotary evaporator.

To synthesize the drug loaded nanoparticles, the same procedure was used. Paracetamol (acetaminophen) as a model drug was dissolved in water at a concentration of 20 mg in 5 ml and was added in high molecular weight gelatin solution.

2.3 Characterization of the nanoparticles

Shape and size The morphological characteristics of the unloaded nanoparticles were determined using a digital scanning electron microscopy (SEM) DSM 960 (Carl Zeiss, Jena, Germany). For preparation of the samples 50 µl 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 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 distilled water before measurements.

2.4 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) [19], where, c (g/dl) is solute concentration, η_{sp} is the specific viscosity and expresses the incremental viscosity due to the presence of the polymer chains or nanoparticles in the solution and k_H is the Huggins constant.

2.5 Drug loading

Drug entrapment efficiency (DEE) of the nanoparticles was determined by measuring the absorbance of the drug free and drug loaded nanoparticles by a UV–Vis spectrophotometer (Perkin Elmer, Lambda 800 spectrophotometer) at 242 nm. The DEE was then calculated as:

$$DEE\% = \frac{[\text{drug}]_{NP}}{[\text{drug}]_{total}} \times 100 \quad (1)$$

where $[\text{drug}]_{total}$ and $[\text{drug}]_{NP}$ are the mass of total paracetamol added and the mass of paracetamol in the nanoparticles, respectively.

The drug loading efficiency (DLE) of the nanoparticles was also calculated as follow:

$$DLE\% = \frac{\text{Amount of drug in nanoparticles}}{\text{Nanoparticle concentration}} \times 100 \quad (2)$$

2.6 Drug release kinetics

0.4 ml of drug loaded nanoparticles were placed in a dialysis bag (MWCO 12 kDa) and were dialyzed against 100 ml of phosphate buffered saline (PBS) at pH 7.4, 37°C at a rotation rate of 100 rpm. Then, at definite interval of times, 2 ml samples were taken from the mixture and the concentration of paracetamol was evaluated by the spectrophotometer.

3 Results and discussion

3.1 Characterization of the nanoparticles

It has been shown that particles size has a great impact on the functional properties of nanoparticles [20]. In the present study, to prepare nanoparticles with an appropriate size and size distribution, we used two-step desolvation method proposed by Coester et al. [5]. In this method, low molecular gelatin fraction is removed after first desolvation step. This step enhances stability of particles formed before cross-linking and thus reduces the formation of aggregates. Furthermore, further irreversible aggregation and flocculation of particles during storage may be prevented by removal of low molecular weight molecules [5].

The mean particle size of the unloaded gelatin nanoparticles cross-linked by GA (GA-GNPs) and CDI/NHS (CDI/NHS-GNPs) as determined by photon correlation spectroscopy was found to be 280 ± 11 and 184 ± 7, respectively.

Polydispersity index (PDI) of 0.45 ± 0.09 for GA-GNPs and 0.141 ± 0.07 for CDI/NHS-GNPs, suggested a rather broad size distribution of the nanoparticles.

The results of nanoparticle size characterization as well as drug loading efficiency and DEE are presented in Table 1. The morphological characteristics of the nanoparticles were examined by SEM. As shown in Fig. 1, independent of cross-linking agent, nanoparticles were spherical and slightly inhomogeneous in size distribution. Moreover, the average size of nanoparticles from SEM micrographs is in a relative agreement with those calculated from dynamic light scattering measurements.

The SEM micrographs also show that apart from smaller size and PDI, nanoparticles cross-linked by CDI/NHS (Fig 1b) had smoother surface than GA cross-linked particles.

Furthermore, the CDI/NHS-GNPs were stable under long storage conditions. The size and PDI of these

Table 1 Effect of cross-linking agent on size and drug loading and entrapment efficiencies of the gelatin nanoparticles

Cross-linking agent	Size (nm)[a]	PDI[a]	DLE (%)	DEE (%)
GA	280 ± 11	0.45 ± 0.09	4	10
CDI/NHS	184 ± 7	0.141 ± 0.07	7.3	27

[a] Unloaded nanoparticles

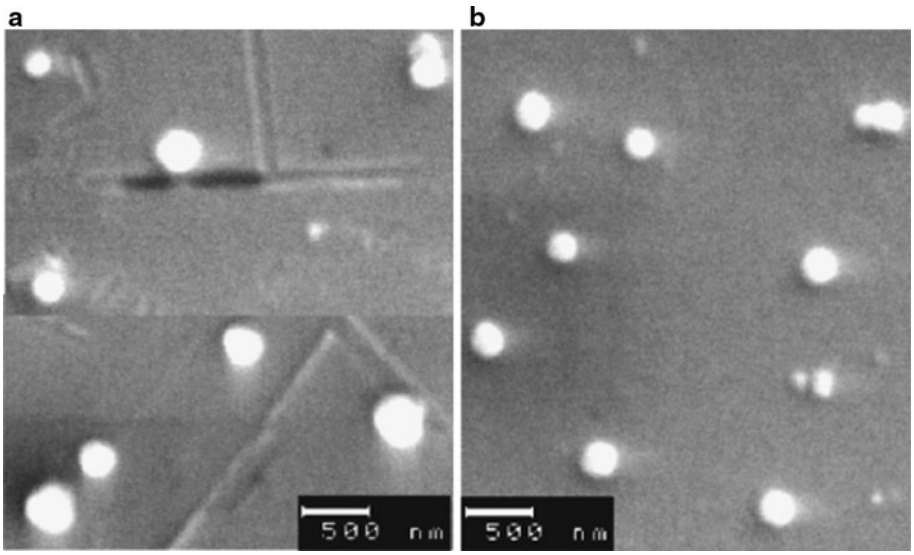

Fig. 1 SEM micrographs of the gelatin nanoparticles cross-linked by GA (**a**) and CDI/NHS (**b**)

nanoparticles remained nearly unchanged after 3 months storage under refrigeration conditions at 4°C.

The relatively large PDI values of both nanoparticles synthesized using different cross-linking agents, may be attributed to the nature of particle formation in the desolvation method. Nanoparticles, in this method, are formed through strong interaction of positively and negatively charged segments in or between the gelatin chains.

This charge neutralization process [21] depends mainly on the charge distribution on the gelatin chains in the solution. Aggregation through random intermolecular association of some other molecules results in particles with different sizes. Therefore, it is likely to form single-folded gelatin chains of ca. 20 nm as well as very large aggregates.

As the synthesis conditions has been kept the same for both types of the nanoparticles synthesized in this work, the difference in particle characteristics may be attributed to the different chemistry of cross-linking agents and thus to the different nature of network structures formed by GA or CDI/NHS.

On the whole, the larger particles size and broader size distribution of GA cross-linked nanoparticles, under the investigated conditions, is a sign of the more inter-particle aggregation during and/or after cross-linking.

It is generally accepted that GA as a non-zero length cross-linker bridges between free amino groups of lysine or hydroxylysine [22]. The aldehyde functional groups react with free –NH2 groups through a nucleophilic addition type reaction. Moreover, the pH at which cross-linking reaction occurs may have a significant effect on the reaction mechanism and therefore on the final properties of the gelatin based products [23]. It is well known that the charge density and charge distribution of gelatin chains in solution directly affected by pH. More precisely, the number and the position of negative charges on the carboxylic groups and the number of free non-protonated ε-amino (–NH2) groups, is determined by the solution pH.

As already mentioned, after addition of desolvating agent, gelatin nanoparticles with different sizes are formed. The particle formation is the result of conformational change of the gelatin molecules from stretched to random coil conformation induced by charge neutralization process [21]. At low pH values, there would be some free –NH2 groups within the formed nanoparticles but the majority of the amino groups are positively charged. Although, these positively charged groups have a major role in stabilizing the particles before cross-linking, they are unavailable for cross-linking reaction with GA.

As described in experimental section, cross-linking reaction in this work, were carried out at pH 2.5. At this pH value, only a few unprotonated amino groups participate in the network formation and GA molecules most likely react with other functional groups in gelatin molecules.

Just recently, Farris et al. [23] have proposed a new mechanism for gelatin cross-linking by GA. Under acidic conditions, they have proved that the carboxyl groups of GA play a major role and the network formation are predominantly governed by the mechanism involving these groups and the hydroxyl groups and the amino acids of hydroxyproline and hydroxylysine, respectively. Therefore, it can be rationalized that under the investigated conditions, two simultaneous cross-linking mechanisms, one inside the nanoparticles and the other on the surface, may occur. The latter mechanism may result in particle aggregation.

On the contrary, the network formation mechanism is totally different in the case of CDI/NHS cross-linking systems. It has been approved that [24] the main role of CDI is to activate the carboxylic acid residues of aspartic and glutamic acids on gelatin chains. At the same time,

NHS molecules react with the previously activated carboxylilc acid groups. The presence of NHS is critical in this stage. In the absence of NHS, the activated carboxylic groups may hydrolyze or rearrange to o-acylisourea residues [25]. However, after reaction with NHS, the activated groups are less likely to rearrange or hydrolyze [26]. The network formation is then started 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 and do not introduce any spaces during the formation of amino bonds between gelatin chains as chemical cross-linkers.

It seems that because of charge neutralization process during nanoparticles formation, there have been enough free amino groups for both cross-linking agent (GA and CDI/NHS) to form chemical cross-links. However, for CDI/NHS the formation of cross-links are facilitated due charge neutralization because the reactant groups (–NH2, –COOH) have been brought close together and they can react easily. This leads to faster stabilization of the nanoparticles and inhibit further aggregation which enlarge the particles. However, it should be noted that the any change in the conditions such as pH, temperature, desolvation agent, and ionic strength of the solution as well as gelatin type may cause to different sizes and morphological characteristics for the nanoparticles. More detailed studies on the CDI/NHS cross-linked gelatin nanoparticles using a broad range of the aforementioned parameters are being carried out.

3.2 Intrinsic viscosity

It is well known that the most important effect of dissolution or dispersion of macromolecules or nanoparticles in a solvent is a considerable increment in viscosity. The effect is usually quantified by the intrinsic viscosity, $[\eta]$. This property is sensitive to the conformation or flexibility of the macromolecules, as well as their size and also to the nature of interaction of the solute with the solvent [19]. Therefore, precise determination of $[\eta]$ can provide useful information about the aforementioned characteristics.

Figure 2 shows the Huggins function, $H = \eta_{sp}/c[\eta]$ as a function of reduced concentration, $[\eta]c$, for the nanoparticles cross-linked by GA and CDI/NHS at pH 2.5 as well as a normal gelatin solution prepared at its native pH 5.4. The intrinsic viscosity was found to be 40.51 and 20.56 ml/g for GA-GNPs and CDI/NHS-GNPs, respectively, and 25.58 ml/g for gelatin solution at pH 5.4.

The slope of the plots shows the Huggins constant, k_H, and is dependent on the solvent quality and is related to the second viral coefficient, A_2, [27]. In the case of

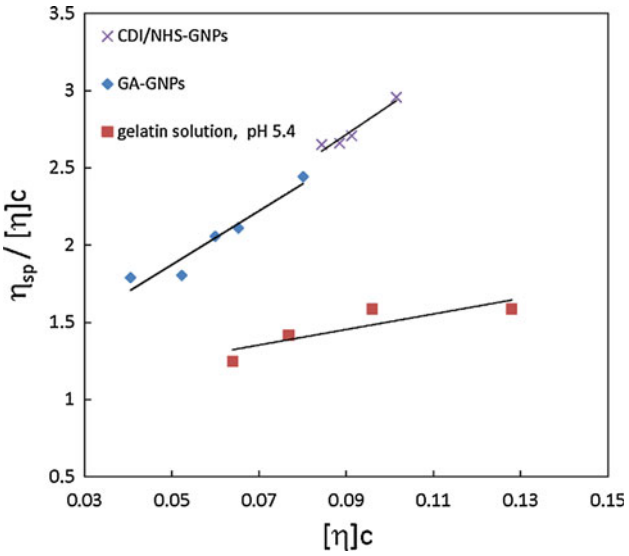

Fig. 2 Huggins function versus reduced concentration for gelatin solution and the gelatin nanoparticles

nanoparticles in a solvent, k_H provides information about nature of interaction between particles. A large and positive k_H represents strong repulsive forces between particles which is common for flexible chain polyelectrolyte in low ionic strength solutions. In contrast, negative values of Huggins constant means that the interactive force between particles is attractive. Huggins constants for both nanoparticles and gelatin solution are positive, representing repulsive forces between particles or gelatin chains (Fig. 2; Table 2). However, the k_H values are much higher for nanoparticles than the normal gelatin solution. This can be attributed to the polyampholytic nature of gelatin.

As described in previous subsection at low pH values in which the nanoparticles were synthesized, most of amino groups of lysine and hydroxylysine had been protonated and regardless of the cross-linking agent, they did not participate in the network formation. Therefore, they have remained on the surface and lead to strong repulsive interactions between nanoparticles.

However, in the case of normal gelatin solution measured at pH 5.4, quite close to its pI ($\sim$5), the net charge of gelatin macromolecules in the solution is nearly neutral, resulted in low k_H value.

Effect of pH and temperature on CDI/NHS-GNPs Figure 3 shows the effect of pH and temperature on the Huggins function of CDI/NHS-GNPs. The nanoparticles have been synthesized at room temperature and pH 2.5. However, they may be used at various temperatures and pHs for example at 37°C and/or pH 7.4. Therefore, it is important to investigate the effect of pH and temperature on the size and stability of the nanoparticles at the desired conditions. The effect of various conditions during and after synthesis on the stability and size of GA-GNPs is well

Table 2 Huggins constants of the investigated systems at different conditions

System	K_H (pH 3.2 and 25°C)	K_H (pH 5.4 and 25°C)	K_H (pH 7.4 and 25°C)	K_H (pH 3.2 and 37°C)
GA-GNP	19.8	–	–	–
CDI/NHS-GNP	17.4	7.9	20.7	15.3
Gelatin solution	–	5.0	–	–

documented in the literature [7, 12]. Thus, for the first time, in this section the results are presented for CDI/NHS cross-linked gelatin nanoparticles.

As Fig. 3 shows, by increasing pH from 3.2 to 5.4 and then 7.4 at 25°C, two different behaviors can be observed. Isoelectric point of the gelatin source used in this work was determined about five. At pH 3.2, ionization of –NH2 groups caused net positive charges on the gelatin chains and enhanced electrostatic repulsion, reflected by a high Huggins constant and $[\eta] \approx 0.16$ dl/g.

However, because of charge balance on the gelatin chain at pH 5.4, the electrostatic repulsions weakened and resulted in smaller k_H. Again, increasing pH to 7.4 caused to more ionization of –COOH groups and net negative charge of the gelatin chains. This enhanced electrostatic repulsion between chain segments and therefore resulted in a large k_H and particle expansions at pH 7.4.

Figure 3 also shows that increasing temperature at constant pH, did not considerably change the nature of interparticle interactions and just caused to a little expansions of nanoparticles because increasing the solvent quality, in order that the intrinsic viscosity increased from 0.16 dl/g at 25°C to 0.25 dl/g at 37°C. It has been shown by Bohidar and co-workers [28] that gelatin–water system shows UCST phase behavior which means that water becomes a better solvent for gelatin molecules with increasing temperature.

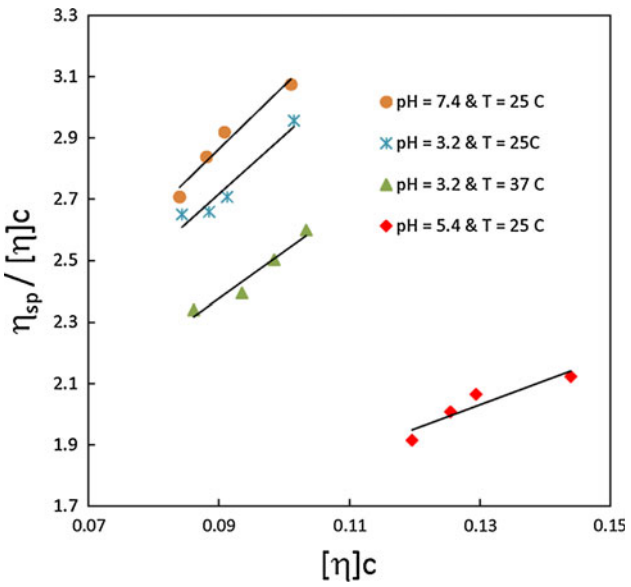

Fig. 3 Huggins function versus reduced concentration for CDI/NHS-GNPs at different conditions

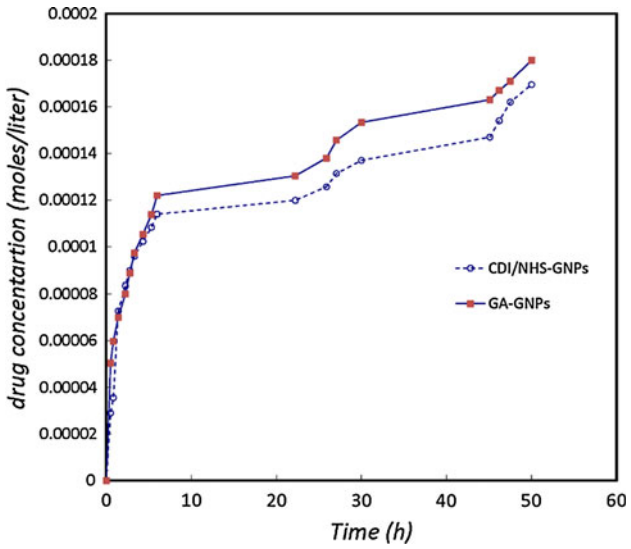

Fig. 4 Paracetamol in vitro release profiles from drug loaded nanoparticles at pH 7.4

3.3 Drug loading and drug release

Drug encapsulation efficiency of the loaded nanoparticles was found to be 10 and 27% in GA-GNPs and CDI/NHS-GNPs, respectively. Furthermore, drug loading efficiency was also affected by cross-linking system and was higher in the nanoparticles cross-linked by CDI/NHS (Table 1).

Figure 4 shows the in vitro drug release kinetics of the loaded nanoparticles measured in 60 h at pH 7.4 by using a dialysis membrane. Although the release kinetics of paracetamol as a model drug from GA-GNPs was a bit faster, both nanoparticles showed approximately comparable release trends at pH 7.4. This similar trend in drug release may be attributed to the similar intensities of the interactions between the drug and both types of nanoparticles.

4 Conclusions

In this work, gelatin nanoparticles with favorable properties were successfully prepared by using a non-toxic cross-linking system based on a water soluble CDI. Comparison

of the size and morphological characteristics of the formed nanoparticles with those of cross-linked by GA, synthesized at the same condition, showed a smaller mean size, narrower size distribution, and more homogeneous morphology. These differences were ascribed to the different mechanism of cross-linking reactions in the presence of each of the cross-linking agents. Furthermore, dilute solution viscosimetry experiments confirmed the stability of the nanoparticles under different physico-chemical conditions. These results beside the preliminary in vitro drug release experiments suggest CDI/NHS cross-linked gelatin nanoparticles as an interesting candidate for drug delivery applications.

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References

1. Schrieber R, Gareis H. Gelatine handbook. Weinheim: Wiley-VCH; 2007.
2. Schwick HG, Heide K. Immunochemistry and immunology of collagen and gelatin. Bibl Haematol. 1969;33:111–25.
3. Zwiorek K, Kloeckner J, Wagner E, Coester C. Gelatin nanoparticles as a new and simple gene delivery system. J Pharm Pharmaceut Sci. 2004;7:22–8.
4. Balthasar S, Michaelis K, Dinauer N, von Briesen H, Kreuter J, Langer K. Preparation and characterisation of antibody modified gelatin nanoparticles as drug carrier system for uptake in lymphocytes. Biomaterials. 2005;26:2723–32.
5. Coester CJ, Langer K, von Brisen H, Kreuter J. Gelatin nanoparticles by two-step desolvation—a new preparation method, surface modifications and cell uptake. J Microencapsul. 2000;17:187–93.
6. Vandervoort J, Ludwig A. Preparation and evaluation of drug-loaded gelatin nanoparticles for topical ophthalmic use. Eur J Pharm Biopharm. 2004;57:251–61.
7. Saxena A, Sachina K, Bohidar HB, Kamra Verma A. Effect of molecular weight heterogeneity on drug encapsulation efficiency of gelatin nano-particles. Colloids Surf B: Biointerfaces. 2005;45:42–8.
8. Azarmi Sh, Huang Y, Chen H, McQuarrie S, Abrams D, Roa W, Finlay WH, Miller GG, Löbenberg R. Optimization of a two-step desolvation method for preparing gelatin nanoparticles and cell uptake studies in 143B osteosarcoma cancer cells. J Pharm Pharmaceut Sci. 2006;9:124–32.
9. Jahanshahi M, Sanati MH, Hajizadeh S, Babaei Z. Gelatin nanoparticle fabrication and optimization of the particle size. Phys Status Solidi (a). 2008;205:2898–902.
10. Won YW, Kim YH. Preparation and cytotoxicity comparison of type A gelatin nanoparticles with recombinant human gelatin nanoparticles. Macromol Res. 2009;17:464–8.
11. Saraogi GK, Gupta P, Gupta UD, Jain NK, Agrawal GP. Gelatin nanocarriers as potential vectors for effective management of tuberculosis. Int J Pharmaceut. 2010;385:143–9.
12. Ofokansi K, Winter G, Fricker G, Coester C. Matrix-loaded biodegradable gelatin nanoparticles as new approach to improve drug loading and delivery. Eur J Pharmaceut Biopharmaceut. 2010;76:1–9.
13. Oppenheim RC, Stewart NF. The manufacture and tumour cell uptake of nanoparticles labelled with fluorescein isothiocyanate. Drug Dev Ind Pharm. 1979;5:563–71.
14. Leo E, Vandelli MA, Cameroni R, Forni F. Doxorubicin-loaded gelatin nanoparticles stabilized by glutaraldehyde: involved of the drug in the cross-linking process. Int J Pharmaceut. 1997;155:75–82.
15. Leong KW, Mao HQ, Truong VL, Roy K, Walsh SM, August JT. DNA-polycation nanospheres as non-viral gene delivery vehicles. J Control Release. 1998;53:183–93.
16. Cascone MG, Lazzeri L, Carmignani C, Zhu Z. Gelatin nanoparticles produced by a simple W/O emulsion as delivery system for methotrexate. J Mater Sci Mater Med. 2002;13:523–6.
17. Bajpai AK, Choubey J. Design of gelatin nanoparticles as swelling controlled delivery system for chloroquine phosphate. J Mater Sci Mater Med. 2006;17:345–58.
18. Ethirajan A, Schoeller K, Musyanovych A, Ziener U, Landfester K. Synthesis and optimization of gelatin nanoparticles using the miniemulsion process. Biomacromolecules. 2008;9:2383–9.
19. Kulicke WM, Clasen C. Viscosimetry of polymers and polyelectrolytes. Berlin: Springer; 2004.
20. Vauthier C, Bouchemal K. Methods for the preparation and manufacture of polymeric nanoparticles. Pharmaceut Res. 2009;26:1025–58.
21. Mohanty B, Bohidar HB. Systematic of alcohol-induced simple coacervation in aqueous gelatin solutions. Biomacromolecules. 2003;4:1080–6.
22. Olde Damink LHH, Dijkstra PJ, van Luyn MJA, van Wachem PB, Nieuwenhuis P, Feijen J. Glutaraldehyde as crosslinking agent for collagen based biomaterials. J Mater Sci Mater Med. 1995;6:460–72.
23. Farris S, Song J, Huang Q. Alternative reaction mechanism for the cross-linking of gelatin with glutaraldehyde. J Agric Food Chem. 2010;58:998–1003.
24. Khor E. Methods for treatment of collagenous tissue for bioprostheses. Biomaterials. 1997;18:95–105.
25. Kuijpers AJ, Engbers GHM, Feijen J, De Smedt SC, Meyvis TKL, Demeester J, Krijgsveld J, Zaat SAJ, Dankert J. Characterization of the network structure of carbodiimide cross-linked gelatin gels. Macromolecules. 1999;32:3325–33.
26. Timkovicz R. Detection of the stable addition of carbodiimide to proteins. Anal Biochem. 1997;79:135–43.
27. Yamakawa H. Concentration dependence of polymer chain configurations in solution. J Chem Phys. 1961;34:1360–72.
28. Gupta A, Mohanty B, Bohidar HB. Flory temperature and upper critical solution temperature of gelatin solutions. Biomacromolecules. 2005;6:1623–7.