Asian Journal of Chemistry Vol. 21, No. 8 (2009), 6277-6284

Preparation of Cisplatin Loaded Gelatin Nanoparticles by Glutaraldehyde Crosslinking; Evaluation of Mechanism of Crosslinking and Involvement of Cisplatin in the Crosslinking Process

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Cisplatin (*cis*-dichlorodiammineplatinum(II), CDDP) is a commonly used chemotherapeutic agent for treatment of various cancers, including testicular cancer, ovarian cancer, lymphoma and glioma. To counteract resistance, clinical dosing is increased which leads to renal toxicity, late neurotoxicity as well as ototoxicity, nausea and vomiting. Gelatin nanoparticles encapsulating cisplatin were produced using a two-step desolvation technique using glutaraldehyde as the crosslinking agent to increase drug concentration in cancer tissues with fewer side-effects. Glutaraldehyde is a highly reactive and efficient crosslinking agent whose mechanism of crosslinking is based on intra-particulate bridging of residual amino groups present in gelatin. Also the possible involvement of primary amino groups of cisplatin in the crosslinking process was investigated. The size of nanoparticles was around 223 to 324 nm and the entrapment efficiency was found to be 35.6 %. Crosslinking studies revealed that cisplatin loaded nanoparticles showed a greater number of free amino groups than plain gelatin nanoparticles indicating possible competition between the amino groups of cisplatin and amino groups of gelatin polymer during the crosslinking process. The possibility of binding of amino groups of cisplatin with the amino groups of gelatin *via* glutaraldehyde was confirmed by thin-layer chromatography. *In vitro* studies showed an initial burst release of cisplatin (22.42 %) followed by gradual and incomplete drug release up to 72 h in PBS pH 7.4. Enzymatic disruption of protein matrix with trypsin further released 9.7 % of the drug and remaining drug corresponds to cisplatin which is covalently linked to amino groups of gelatin.

Key Words: Cisplatin, Gelatin nanoparticles, Glutaraldehyde, Crosslinking.

INTRODUCTION

Cisplatin (*cis*-dichlorodiammineplatinum(II), CDDP) is a commonly used chemotherapeutic agent for treatment of various cancers including testicular cancer, ovarian cancer, lymphoma and glioma[1,2] and has a poor activity in colorectal cancer. After

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both passive and active cellular uptake, cisplatin coordinates with the N7 atom of guanine in DNA to form adducts and causes cellular apoptosis[3]. The resistance mechanism is not fully understood, although the reason is probably multifactorial[4]. Therefore, to counteract resistance, clinical dosing is increased which in turn is associated with serious side effects including renal toxicity and late neurotoxicity as well as ototoxicity, nausea and vomiting[5,6]. For the reduction of side effects, specific drug delivery systems have been widely investigated. Encapsulated anti-cancer drugs in nanoparticles can protect not only the integrity of drugs during their transport in blood circulation but also the normal tissues from the toxicity[7].

Nanoparticles loaded with cisplatin can successfully increase drug concentration in cancer tissues and also act at cellular levels, enhancing antitumor efficacy. Nanoparticles made out of gelatin, a biodegradable polymer can provide controlled and targeted delivery of the drug with better efficacy and fewer side-effects. Gelatin nanoparticles encapsulating cisplatin were produced using a two-step desolvation technique using glutaraldehyde as the crosslinking agent. Glutaraldehyde is the agent of choice for crosslinking gelatin nanoparticles since it is a highly reactive and efficient crosslinking agent. The mechanism of crosslinking is based on intra-particulate bridging of residual amino groups present in gelatin. Also the possible involvement of primary groups of cisplatin in the crosslinking process was investigated. The objective of the study was to investigate the possible interference of cisplatin on the crosslinking process (*i.e.* on the crosslinking degree) of gelatin nanoparticles stabilized by glutaraldehyde. Since the amino groups are involved in the reticulation process, the number of free amino groups of the loaded and plain gelatin nanoparticles was measured. To determine the free amino group content, the method proposed by Hermanson[8] was adapted.

EXPERIMENTAL

Preparation of cisplatin loaded gelatin nanoparticles by two-step desolvation: Gelatin nanoparticles were prepared by a two-step desolvation method developed by Coester *et al.*[9]. 25 mL of 5 % gelatin type A (Bloom 175) solution was prepared at room temperature (25 °C) with 25 mg of drug in it. Gelatin was desolvated by drop wise addition of an equal volume of acetone, a non-solvent for gelatin and kept for sedimentation. The supernatant was discarded and the sediment was dissolved in water and redesolvated at pH 2.5 with 50 mL of acetone under stirring (500 rpm). Gelatin particles were then cross-linked with 200 µL of 25 % glutaraldehyde, the excess of which was neutralized by adding cysteine (500 mg) and finally purification was done by a three-fold centrifugation (16000 g for 20 min) and redispersion in acetone/water mixture (30/70). The purified nanoparticles were stored as dispersion in highly purified water (conductivity < 0.04 µs/cm) at 4-8 °C.

Drug loading: Nanoparticle suspension obtained after centrifugation step (16,000 g for 2 h) was used for determining drug loading. The supernatant containing free drug (obtained after filtering the drug loaded nanoparticle dispersion through

0.2 µm sterile filter) was analyzed by a validated HPLC method[10]. The entrapment efficiency (E.E)[11] was calculated as:

$$\text{E.E. \%} = \frac{[\text{Cisplatin}]_{\text{total}} - [\text{Cisplatin}]_{\text{free}}}{[\text{Cisplatin}]_{\text{total}}} \times 100$$

where $[\text{cisplatin}]_{\text{total}}$ and $[\text{cisplatin}]_{\text{free}}$ are the amount of total drug added and free drug, respectively, in the nanoparticle dispersion. The drug loading efficiency of gelatin nanoparticles was also calculated.

Particle size determination: Particle size was determined using photon correlation spectroscopy (PCS) using a Zetasizer 3000 (Malvern Instruments, Malvern, UK). The samples were measured in suspension after particle preparation. The nanoparticles were diluted in sterile, filtered, highly purified water and measured in concentrations between 30 and 100 µg/mL. Each sample was measured three times, after which the average value was used for further calculations.

Scanning electron microscopy: The morphology of the nanoparticles was observed by means of a scanning electron microscope. Dry nanoparticles were finely spread over a slide double-sided sticky tape mounted on aluminum SEM stubs. Loose particles were blown off the stub using a pressurized air duster. The samples were coated with a gold layer using the JEOL JFC 100E ion sputtering device (sputtering time 2.0 min). The surface morphology of the nanoparticles was observed using a Jeol SEM-6400 scanning electron microscope (Jeol, Tokyo, Japan).

Enzymatic degradation: The enzymatic degradation of both empty and loaded nanoparticles were carried out according to the method proposed by Roser and Kissel[12]. Briefly, 10 mL of deionized water containing 0.4 mg/mL of trypsin was added to 15 mg of empty nanoparticles. The turbidity was determined at room temperature by absorbance at 546 nm using a spectrophotometer (UV-1700, Pharmaspec, Shimadzu, Japan). In a preliminary study, it had been shown that at 546 nm the absorbance was a linear function of the nanoparticle concentration in the range 0.5-3.0 mg/mL.

Determination of degree of crosslinking: Free amino group content in native gelatin type A (Bloom 175), empty and loaded nanoparticles was measured using the 2,4,6-trinitrobenzene sulfonic acid (TNBS)[13] method according to the procedure adapted by Edwards-Levy *et al.*[14]. This procedure consists in the incubation of the material with an excess of TNBS and the back titration of the unreacted amount of the reagent.

Determination of free amino group content in gelatin and in nanoparticles: The free amino groups[15] content in gelatin and in nanoparticles were estimated by Hermanson's method[8] based on TNBSA assay. In order to avoid interference due to the amino groups of free cisplatin molecules, the free drug in the loaded-nanoparticles was eliminated before the determination. Thus, 0.15 g of polysorbate 20 was added to 10 mL DMSO suspension of cisplatin loaded nanoparticles (30 mg/mL) and shaken for 15 min in a cyclomixer (CM101 Remi, India). The suspension was

centrifuged at 16000 g for 20 min and the supernatant discarded and finally purified by repeated washing with acetone/water mixture (30/70) and the nanoparticles devoid of free drug was freeze dried using MoDULYOD-230 freeze drier (Thermo-Electron Corporation, Milford, MA, USA). Native gelatin type A (Bloom 175) (5 mg) or nanoparticles (after free drug depletion and enzymatic degradation) were dissolved in 0.1 M sodium bicarbonate, pH 8.5 (reaction buffer) at a concentration of 20 µg/mL. 0.25 mL of the 0.01 % (w/v) of freshly prepared solution of TNBSA was added to 0.5 mL of sample solution, mixed and incubated at 37 °C for 2 h. 0.25 mL of 10 % SDS and 0.125 mL of 1 N HCl was added to sample solution and measured the absorbance at 335 nm against a blank prepared as described above. The number of amines contained within a sample was accomplished through comparison to a standard curve generated by the use of native gelatin dissolved in a series of known concentrations. The standard was dissolved into the reaction buffer and was assayed under reaction conditions identical to those utilized for the samples.

Thin-layer chromatography (TLC) experiment: Thin-layer chromatography (I.P. 1996, Appendix 4.6) was carried out by using microcrystalline cellulose as the coating substance, activating the plate by heating at 150 °C for 1 h and using a mixture of 90 volumes of dimethylformamide and 10 volumes of acetone as the mobile phase. The samples were applied separately to the plate 5 µL (0.2 % w/v) of each of solutions (a) free cisplatin, (b) cisplatin loaded nanoparticles; (c) cisplatin loaded nanoparticles after enzymatic degradation; (d) physical mixture of plain nanoparticles and cisplatin; (e) physical mixture of plain nanoparticles and cisplatin after enzymatic degradation. After developing the plate was allowed to dry in air and sprayed with a 5 % w/v solution of stannous chloride in 1 M HCl. The chromatogram was obtained after 1 h.

Determination of free cisplatin content in nanoparticles: A weighed quantity of cisplatin loaded nanoparticles were dispersed in normal saline (15 mL) and stirred for 72 h using a magnetic stirrer and were separated by centrifuging at 16,000 rpm for 20 min. The supernatant was collected and analyzed spectrophotometrically (PharmaSpec UV-1700, Shimadzu Corporation, Japan) at 286 nm for the drug content.

***In vitro* release study:** The kinetics of drug release from dispersion of drug loaded gelatin nanoparticles (DLNP) was evaluated using the equilibrium dialysis technique, a method for quantifying drug transport across a dialysis membrane[16]. Cisplatin nanoparticles (≡ 1.25 mg of drug) were placed in the dialysis membrane (cut off size 12000 to 14000, Dialysis Membrane-50, HiMedia, Mumbai, India) and were dialyzed against 10 mL of phosphate buffered saline (PBS) at pH 7.4 at a rotation speed of 50 rpm. After a known time period a sample of 500 µL was collected from the solution. The drug concentration in the sample was analyzed by a validated HPLC method[10].

RESULTS AND DISCUSSION

Gelatin nanoparticles were prepared using Type A gelatin of Bloom number 175 with a molecular weight between 40,000-50,000 Da. The nanoparticles were characterized for their particle size, drug content, enzymatic degradation and degree

of crosslinking. An excess amount of glutaraldehyde (62.5 µg/mg of gelatin) was used to produce plain gelatin and drug loaded gelatin nanoparticles. Glutaraldehyde is the agent of choice since it is highly reactive and an efficient crosslinking agent which produces rapid and reproducible crosslinking action. It is a non-zero length crosslinker as it operates by intra-particulate bridging of residual amino groups. Hardening process was carried out for 2 h since a shorter period was not sufficient for reticulation and longer period produced too many large particles. At the end of 2 h, the excess of glutaraldehyde was neutralized by adding cysteine thereby preventing the formation of aggregates. The particle size was measured by photon correlation spectroscopy. The morphological assessment of the nanoparticles was carried out by scanning electron microscopy. Experimental results showed that the particles formed are in the range 223-324 nm (Fig. 1). The results are in agreement with the result of Vandervoort and Ludwig[17].

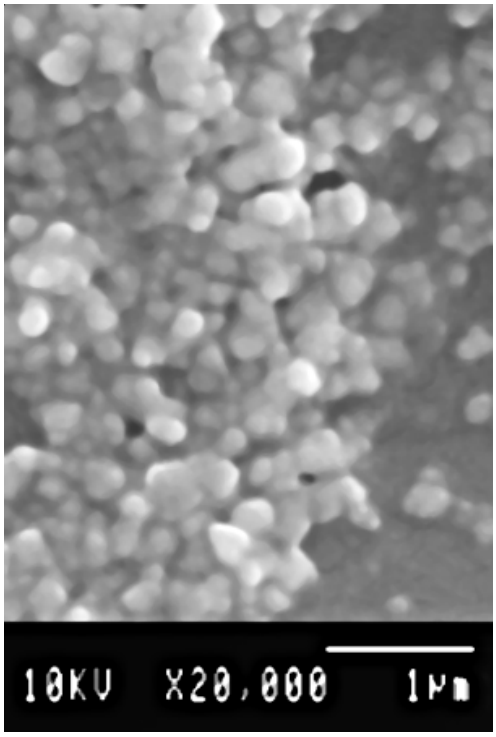

Fig. 1. Scanning electron micrograph of plain gelatin nanoparticles

After the first desolvation step the pH was adjusted to 2.5 using 0.1 M HCl followed by second desolvation step and crosslinking with glutaraldehyde. At this pH the overall net charge carried by gelatin molecules is positive with $-NH_3^+$ groups becoming predominant to facilitate the cross-linking with the -CHO groups of glutaraldehyde. Both inter and intra molecular electrostatic interactions are involved in

the formation of gelatin nanoparticles. The entrapment efficiency was found to be 35.6 %. The amount of free amino groups in both plain and drug loaded gelatin nanoparticles was determined by TNBSA method in order to investigate the involvement of the drug in the crosslinking process. Owing to the reticulation process, the number of free amino groups in plain and drug loaded gelatin nanoparticles were less than the native gelatin whose amino groups were assumed to be 100 %. The results showed that the crosslinking process involved *ca.* 30 % of amino groups in the case of plain gelatin nanoparticles and about 15 % of amino groups were involved in the case of cisplatin loaded gelatin nanoparticles (Table-1). Lesser crosslinking was observed in drug loaded nanoparticles compared to plain nanoparticles prepared under identical conditions. The difference in degree of crosslinking between the two may be related to the presence of cisplatin involved in the crosslinking process and can be explained in two different ways. Firstly, cisplatin may be competing with gelatin and glutaraldehyde may be crosslinking two molecules of cisplatin resulting in lesser quantity of crosslinking agent available for gelatin reticulation. Since an excess amount of gelatin is present compared to the drug content, the extent of such a competition would expectedly be small. The second possibility is that there may be bridging of amino groups of gelatin with the amino groups of cisplatin rather than between two protein chains of gelatin. As a consequence, the number of free amino groups in the drug loaded nanoparticles is more important. To demonstrate present case of cisplatin covalently linked to protein matrix, a TLC analysis was performed on enzyme degraded nanoparticles.

TABLE-1
FREE AMINO GROUP CONTENT PRESENT IN GELATIN, PLAIN NANOPARTICLES AND CISPLATIN LOADED GELATIN NANOPARTICLES

Substrate	Free-NH_2/g of substrate (?mol)	Free-NH_2 (%)
Gelatin	1458±36	100.00
Plain nanoparticles	1021±49	70.03
Cisplatin loaded nanoparticles	1233±14	84.57

Enzyme degradation was achieved using trypsin which degraded 100 % of gelatin nanoparticles and was most suitable for gelatin nanoparticle degradation[18]. Gelatin nanoparticles and other delivery systems based on this polymer are biocompatible and biodegradable without toxic degradation products[19] and that it will be completely biodegradable *in vivo*[20]. Fig. 2 shows that the spots resulting from TLC studies. Free drug: (a) migrates from the origin in the solvent system used (R_f = 0.61). For the cisplatin loaded nanoparticles (b) two orange spots were recovered: a spot with an R_f = 0.61, suggesting that free cisplatin was present in the loaded nanoparticles and a spot on the origin attributable to drug associated to the carrier. For cisplatin loaded nanoparticles (c) two orange spots were also recovered. A spot with an R_f = 0.61 attributable to the free drug and another one on the origin. The spot on the origin may be attributed to cisplatin covalently bound to peptide

fragments resulting from trypsin digestion. In order to confirm this hypothesis, the physical mixtures (d) and (e) (plain nanoparticles: cisplatin and degraded nanoparticles-plain nanoparticles: cisplatin, respectively) were assayed. In both the cases no spots were detected on the origin. Therefore, the orange spot on the origin of the degraded cisplatin loaded nanoparticles was due to cisplatin covalently linked to peptide fragments.

The amount of free drug available was evaluated carrying out *in vitro* studies. The cisplatin loaded nanoparticles (≡ 1.25 mg of drug) were placed in the dialysis membrane (cut off size 12000 to 14000) and were dialyzed against 10 mL of phosphate buffered saline (PBS) at pH 7.4 (Fig. 2). Under these conditions only the free drug would be released, allowing this amount to be recognized from that of covalently linked cisplatin to peptide fragments. *In vitro* studies showed an initial burst release of cisplatin (22.42 %) and followed by gradual and incomplete drug release up to 72 h. Subjecting the protein matrix to enzymatic disruption with trypsin, showed further release of 9.7 % of the drug. The remaining part of the drug corresponds to cisplatin which is covalently linked to amino groups of gelatin. The results did not show any increase in the percentage of the free drug with respect to the value reported (*ca.* 32.12 %). The incomplete release of cisplatin from glutaraldehyde crosslinked microspheres was previously reported by Willmott *et al.*[21]. According to these authors a complete release would occur by degradation of the protein matrix. The remaining part of the drug (*ca.* 68 %) should correspond to the fraction linked to the protein fragments produced by the nanoparticle digestion, as cisplatin bound to the protein matrix should not be cleaved by proteolytic enzymes.

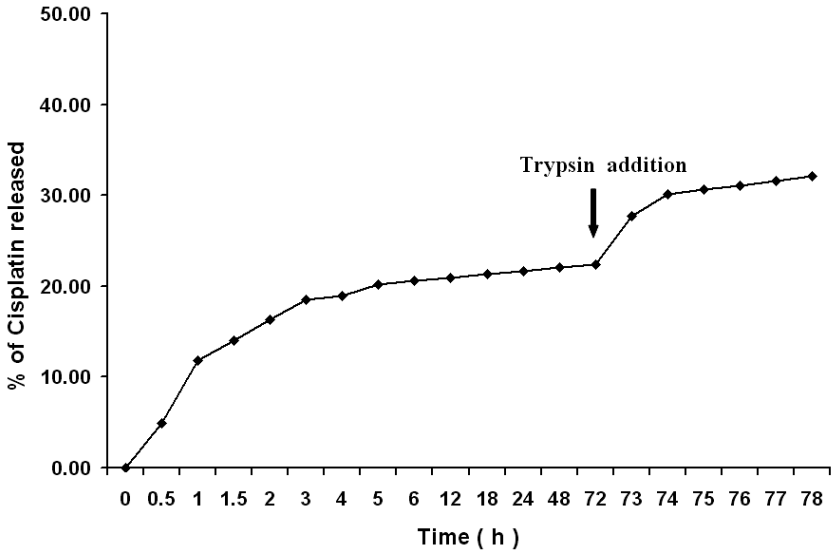

Fig. 2. Cisplatin *in vitro* release study from drug loaded nanoparticles before and after treatment with trypsin enzyme

Conclusion

Crosslinking of gelatin nanoparticles with glutaraldehyde resulted in a biodegradable nanoparticle drug delivery system in which the free drug was present at the surface of the nanoparticles and also entrapped within the matrix of nanoparticle system both of which constituted about 32 %. The rest of the major part of the drug being conjugated to the biodegradable carrier system-gelatin. Cisplatin-gelatin conjugates improve the cytotoxic activity of the drug, allowing cisplatin to escape from the multidrug resistance mechanism[22].

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(*Received*: 4 December 2008; *Accepted*: 26 May 2009) AJC-7608