Cytotoxicity and Cellular Uptake Evaluation of Mitoxantrone-Loaded Poly(lactic acid-*co*-lysine) Arginine–Glycine–Aspartic Acid Nanoparticles

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ABSTRACT: The purpose of this study was to evaluate the *in vitro* characteristics of poly(lactic acid-*co*-lysine) arginine–glycine–aspartic acid (PLA–PLL–RGD) nanoparticles (NPs) loaded with mitoxantrone. PLA–PLL–RGD NPs with a particle size of 200 nm were prepared with a modified emulsification solvent-diffusion method. The encapsulation efficiency of the mitoxantrone-loaded NPs was 85%. *In vitro* release experiments showed that the release of the drug was prolonged and sustained, and approximately 60.2% of the mitoxantrone was released in the first week. The released drug was integrated to achieve desired drug-release profiles and still possessed bioactivity according to a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2h-tetrazolium bromide assay, which indicated that mitoxantrone-loaded NPs were more cytotoxic against Michigan Cancer Foundation 7 (MCF-7) breast cancer cells than mitoxantrone. Furthermore, the association processes of NPs with MCF-7 cells, including binding and effective internalization, were investigated *in vitro*. The cellular uptake of the NPs was qualitatively studied with confocal laser scanning microscopy and was confirmed with flow cytometry analysis. These experimental results indicated that PLA–PLL–RGD NPs could be used as drug carriers for mitoxantrone. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 119: 1011–1015, 2011

Key words: biodegradable; biological applications of polymers; biomaterials

INTRODUCTION

Cancer chemotherapy is associated with many challenges, such as normal tissue cytotoxicity, poor site-selective delivery, low concentrations in the blood, quick metabolism, and poor drug solubility and stability. Therefore, the prospect of improved cancer chemotherapy with nanomaterials as drug-delivery systems is promising.[1–3]

Currently, research on nanoparticle (NP) drug-delivery systems is focused on developing new carrier materials to increase the drug-delivery capability and obtain suitable drug-release rates and perfecting preparation or surface modification methods to improve the targeting ability.

In this study, mitoxantrone, the most efficient anticancer drug for breast and liver cancer, was used as a model drug. However, because of its high toxicity against the heart and extreme hydrophobicity in clinical use, an NP system was developed. Poly(lactic acid) (PLA) was used to reduce the frequency of administration through sustained degradation.[4–6] To achieve active targeting of the tumor, specific recognition ligands [arginine–glycine–aspartic acid (RGD)] with a specific affinity to substrates overexpressed on the tumor cell surface were grafted to the polymer forming the NPs.[7,8] Furthermore, a cationic material based on poly(lactic lysine) (PLL) was expected to be helpful for conjugating more RGD to PLA.[9]

NPs with a diameter of 200 nm were prepared by the nanoprecipitation method. The physical properties, drug encapsulation efficiency, and drug release of NPs were investigated. More importantly, cytotoxicity research on mitoxantrone-loaded NPs for use against Michigan Cancer Foundation 7 (MCF-7) breast cancer cells was compared with research on free mitoxantrone. Confocal laser scanning microscopy (CLSM) was used to illustrate the cellular uptake of NPs.

This research provides much important preparative information for further studies of NPs *in vivo*.

EXPERIMENTAL

Preparation of blank NPs

The copolymer poly(lactic acid-*co*-lysine) arginine–glycine–aspartic acid (PLA–PLL–RGD; molecular

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weight = 11,000; 25 mg)[10] was dissolved in 1 mL of dichloromethane. The solution was then added to a 10-mL aqueous F-68 solution (3% w/v) exposed to ultrasound (JY92-II ultrasonic processor, Ningbo, Jiangsu Province, China) in an ice bath. The resulting emulsion was maintained under magnetic stirring. After 4 h of stirring, the organic solvent was thoroughly removed to obtain an NP aqueous suspension. The NPs were collected by ultracentrifugation and washed twice with distilled water.

Preparation of mitoxantrone-loaded NPs

NPs were prepared with the emulsion method.[11] Briefly, a mitoxantrone solution was emulsified in 1 mL of a dichloromethane solution containing the material (PLA–PLL–RGD) by sonication. An aqueous F-68 solution (3% w/v) was added to the first emulsion (exposed to ultrasound) in an ice bath. The resultant water-in-oil-in-water emulsion was stirred at room temperature for 4 h to evaporate the dichloromethane. The resultant NP solution was purified and obtained by centrifugation.[11–14]

Morphology observation and size distribution

A copper grid, coated with a carbon film, was immersed into the NP solution. After being stained with a 2% phosphor tungstic acid solution, the sample was dried at room temperature and examined with transmission electron microscopy (TEM; JEM-1200). The size distribution of the NPs was determined with a dynamic laser light scattering technique.

Determination of the drug encapsulation efficiency

The supernatants obtained during NP washing (centrifugation) were assayed spectrometrically at 610 nm with an ultraviolet–visible spectrophotometer (TU-1901, Beijing, China). The encapsulation efficiency was expressed as the percentage of mitoxantrone encapsulated in NPs versus the total amount initially used.

Determination of the drug release

For the *in vitro* drug-release study, a dialysis tube (molecular weight cutoff = 10,000) containing a 5-mL NP solution was directly immersed into 15 mL of phosphate-buffered saline (PBS). Aliquots of 1 mL were withdrawn from the solution at predetermined time intervals[15] and replaced with 1 mL of fresh PBS. The amount of mitoxantrone released from NPs was measured with an ultraviolet–visible spectrophotometer at 610 nm.

In vitro cytotoxicity evaluation

The cytotoxicity evaluation of PLA–PLL–RGD NPs and mitoxantrone-loaded PLA–PLL–RGD NPs was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2h-tetrazolium bromide (MTT) colorimetric assay. Viable MCF-7 cells were seeded into 96-well plates and cultured. Samples were added to each well, and the plates were incubated at 37°C. The mitoxantrone concentration at each point was 0.4, 4, 12, and 40 μg/mL. The concentration of the PLA–PLL–RGD NPs without mitoxantrone was adjusted to be the same as that of the mitoxantrone-loaded PLA–PLL–RGD NPs at each point. A control was realized with the culture medium. After incubation, 200 μL of MTT (0.5 mg/mL; Sigma, Santa Clara, St. Louis) was added to the wells and incubated for 4 h at 37°C. The culture medium was removed from the wells and replaced with 150 μL of dimethyl sulfoxide. The absorbance of each well was measured at 570 nm with a Bio-Rad automated ultraviolet-visible spectrophotometer analyzer after the plates were incubated for 1 h at 37°C.

Cellular uptake of NPs

The internalization of the mitoxantrone-loaded NPs by MCF-7 cells was visualized with CLSM. Trypsinized cells were harvested, washed with PBS, resuspended in Dulbecco's modified Eagle medium (Santa Clara, St. Louis) supplemented with 20% v/v fetal bovine serum (Invitrogen, Carlsbad, CA), and seeded in each well of six-well plates at a density of 5000 cells per well. After incubation for 24 h at 37°C with 5% CO_2, mitoxantrone-loaded PLA–PLL–RGD NPs were added to the wells. After removal of the supernatant and two washes with PBS 4 h later, the cells were fixed with 4% paraformaldehyde for 20 min. The cellular uptake and adhesion were analyzed by simultaneous laser light stimulation and imaging (FV1000, Tokyo, Japan) at the defined time.

Quantitative analysis of NP uptake

The cellular uptake was further quantitatively investigated. MCF-7 breast cancer cells (10,000) were placed onto the plates. After incubation for 24 h at 37°C with 5% CO_2, mitoxantrone-loaded PLA–PLL–RGD NPs were added to the plates. The cellular uptake and adhesion were analyzed by flow cytometry 4 h later.

RESULTS

Morphology observation and size distribution of NPs

NPs were prepared with a modified double-emulsion solvent-diffusion method. A TEM photograph

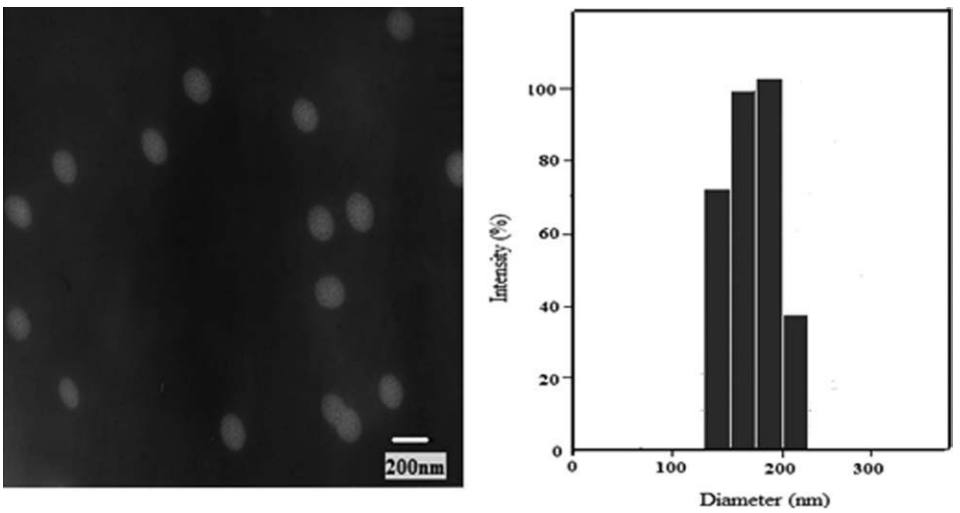

Figure 1 TEM image of NPs (left) and particle size distribution measured by dynamic light scattering (right).

indicated that the PLA–PLL–RGD NPs had a smooth, spherical morphology (Fig. 1). The size distribution, observed by dynamic light scattering, showed that the NPs had a mean diameter of 200 nm.

In vitro drug encapsulation efficiency and release of NPs

The drug encapsulation efficiency (%) is an important index for drug-delivery systems. The encapsulation efficiency of the mitoxantrone-loaded PLA–PLL–RGD NPs was as high as 85%. The *in vitro* cumulative drug-release profile of the NPs is shown in Figure 2. An initial burst release was observed within 6 h, and 36% of the drug was released; this was followed by a sustained release profile for about 1 week. This sustained release mainly resulted from the degradation of the components of the NPs.

Cytotoxicity activity of mitoxantrone-loaded PLA–PLL–RGD NPs

Figure 3 shows the *in vitro* antitumor activity of free mitoxantrone and mitoxantrone-loaded PLA–PLL–RGD NPs on cancer cells; this activity is expressed as the percent reduction of cell viability. The figure shows that the MCF-7 cells treated with the mitoxantrone-loaded NPs had lower or comparable viability in comparison with those treated with mitoxantrone. However, there was no significant difference between the treatment of mitoxantrone-loaded NPs and free mitoxantrone. The cell mortality had a dose and time dependence.

Cellular uptake of fluorescent NPs

The cellular uptake of NPs was evaluated with CLSM and flow cytometry. The concentration of the mitoxantrone suspension that we selected was 4 μg/mL because of cell viability and fluorescence intensity factors. Figure 4(a) shows that in the MCF-7 cancer cells, the nucleus exhibited a strong red layer of fluorescence after 4 h of culture with the mitoxantrone-loaded NPs. This finding was in agreement with the results of Figure 4(b) (obtained for quantitative measurements), which showed a 96.6% uptake ratio. These quantitative and qualitative results for cellular uptake provided strong evidence for the effect of cellular uptake of mitoxantrone-loaded NPs by MCF-7 cells.

DISCUSSION

Characteristics of NPs

Mitoxantrone is one of the most useful drugs currently available for cancer therapy. Nevertheless, a suitable carrier system for this drug is considered to

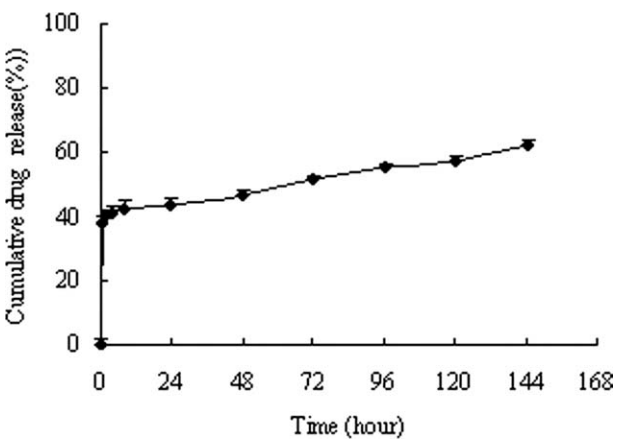

Figure 2 *In vitro* drug-release profile of mitoxantrone-loaded PLA–PLL–RGD NPs. Data represent means and standard deviations (n = 3). The drug-release studies were carried out in PBS at 37°C for 7 days.

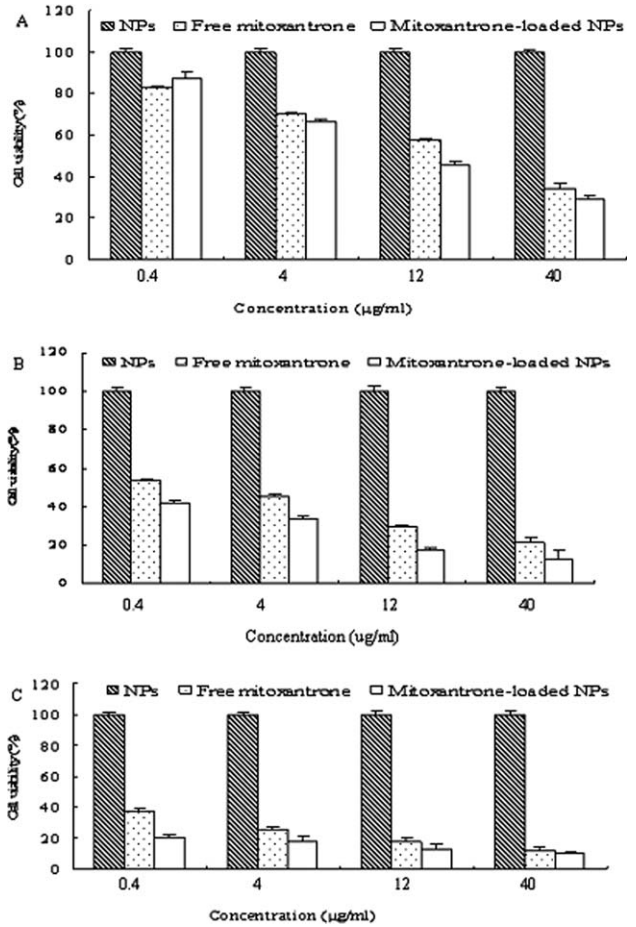

Figure 3 MCF-7 cell viability after (A) 24, (B) 48, and (C) 72 h of treatment by NPs, mitoxantrone, and mitoxantrone-loaded NPs at different concentrations. Each condition was tested in six replicates. The standard deviations are presented as error bars.

be of prime importance because of the heart toxicity problem. The PLA–PLL–RGD copolymer easily forms core–shell NPs in a solvent with a hydrophobic core and a hydrophilic shell. The hydrophobic mitoxantrone is wrapped into the core structure; this reduces the toxicity and also increases the solubility via solubilization within the hydrophobic core of the NPs. The drug-release mechanism is a combination of drug diffusion and polymer degradation. Therefore, the core–shell structure and slow degradation rate of the polymer influence the drug release.

Figure 2 shows an initial burst release within 6 h, which was followed by a sustained release profile for about 1 week. The fast release of the drug was probably caused by the large surface area of the NPs and the short diffusional distance. The *in vitro* fast release may also be attributed to the mitoxantrone adsorbed to the surface of the NPs by electrostatic interactions. The amount of drug released from the PLA–PLL–RGD NPs was also related to the NP size. It has been reported that larger particles have more controlled release in comparison with particles of a smaller size. The molecular weights of polymers also inhibit drug diffusion and hence lower the rate of drug release.

Cellular uptake and cytotoxicity activity of NPs

Drug-loaded NPs must be absorbed into cells at a sufficiently high rate and to a sufficient extent for cancer chemotherapy. Mitoxantrone internalization is significantly efficient, as shown in Figure 4. First, the internalization of cells to NPs is influenced by the physical and biochemical properties of the NPs, such as the particle size, nature of the polymer and drug, and surface biochemical properties. The small size of NPs makes them suitable for endocytotic cellular uptake. NPs that are 100–200 nm in size offer the best properties for cellular uptake.[16] Furthermore, the NP surface presents a proper balance between hydrophilicity (PLL) and lipophilicity

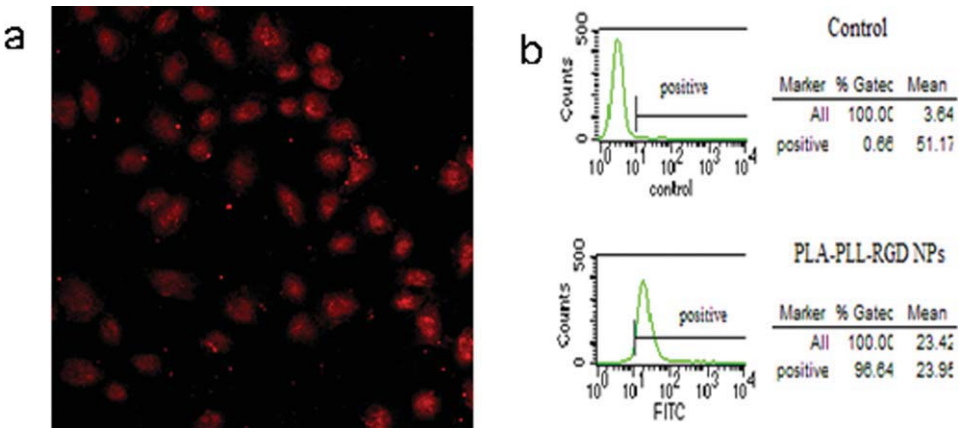

Figure 4 (a) CLSM image of MCF-7 breast cancer cells after 4 h of culture with NPs and (b) flow cytometry analyses of cancer cells after 4 h of culture with mitoxantrone-loaded NPs (FITC = fluorescein isothiocyanate). The PLA–PLL–RGD NP internalized rate was 96.64% versus 0.66% for the control (×200). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(PLA). A particle surface with excessive lipophilicity or hydrophilicity could prevent the internalization of NPs into cells. Therefore, the prepared PLA–PLL–RGD NPs might be more beneficial for uptake by cancer cells.

Second, the RGD sequence is widely used in adhesion research. RGD is known to interact with cell membranes by the integrin ligand, which has been reported to exist in cell membranes.[17–19] The interactions between integrin receptors and ligands regulate the functions of cells, including adhesion, growth, and differentiation. Therefore, RGD-mediated endocytotoxicity may have an important function. The mechanism will be investigated in detail in our future work.

Research on NPs is focused on providing a new mode of cancer drug delivery with high anticancer efficiency, so *in vivo* use is most important. The characteristics of NPs are not clear because of the complicated circumstances *in vivo*. A toxicity assay *in vitro* has shown that NPs are slightly more cytotoxic against cancer cells than the corresponding free drug, although the difference is rather small. Mitoxantrone, released from NPs, inhibits cell growth. Moreover, it has recently been demonstrated in lymphocytes and MCF-7 cells that induction of apoptosis by RGD peptides results from the direct activation of caspase-3.[20] These data offer a potential use for further study.

CONCLUSIONS

Novel anticancer drug carriers were prepared with a double-emulsion solvent-diffusion method. The NPs presented a mean particle size of 200 nm. They showed an initial burst, which was followed by a sustained drug-release profile. MCF-7 breast cancer cell viability studies showed that mitoxantrone-loaded NPs could produce higher cytotoxicity than free mitoxantrone. The cellular uptake of NPs was increased rapidly with an incubation time greater than 4 h. Furthermore, we are now investigating *in vivo* tumor-targeting and antitumor abilities in tumor-bearing mice. The primary results show that mitoxantrone-loaded NPs have a prolonged circulation time and good tumor-targeting ability. All these data suggest that this new delivery system is promising for applications in antitumor therapy *in vivo*.

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