Characterization and *In Vivo* Biodistribution Studies with Poly(Ethylene Glycol)-Modified Thiolated Gelatin Nanoparticles

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1. ABSTRACT

In order to enhance the circulating time and target solid tumors, we have modified thiolated gelatin nanoparticles with poly(ethylene glycol) (PEG). The non-crosslinked and crosslinked thiolated gelatin nanoparticles had size of 150-250 nm and were found to preferentially release their content in high glutathione media. Thiolated gelatin nanoparticles were also found to provide the highest transfection efficiency in cell culture. In this study, the biodistribution and the long circulating potential of PEG-modified thiolated gelatin nanoparticles was evaluated by injecting the ^{111}In-labeled nanoparticles in nude mice bearing human breast cancer tumor (MDA-MB-435) xenografts. Upon calculation of the pharmacokinetic parameters from the plasma and tumor data, the PEG-modified nanoparticles were found to have enhanced circulation times and increased tumor accumulation.

Keywords: Thiolated gelatin nanoparticles, PEG modification, biodistribution and pharmacokinetics, breast cancer xenograft.

2. INTRODUCTION

Non-viral vectors for gene delivery applications are becoming increasingly popular owing to several advantages, which include ease of production and administration, scale-up, and most importantly lack of immunogenicity and mutagenesis that are associated with viral vectors. Among the non-viral vectors, polymeric nanoparticles offer an attractive alternative for intracellular gene delivery because of their high surface to volume ratio and their ability to encapsulate DNA without a pre-condensing step [1]. In addition, these colloidal carriers are stable and can be prepared and modified with relative ease. Tissue- and cell-targeted polymeric nanocarriers can be obtained by attaching specific ligands such as antibodies or lectins in order to achieve site-specific delivery and the surface can be modified with hydrophilic polymers such as poly(ethylene glycol) (PEG) resulting in long circulating properties in the systemic circulation. Due to the small size these nanoparticles can be used to achieve tumor targeting by the enhanced permeability and retention (EPR) effect [2-4]

In order to enhance the intracellular delivery potential of plasmid DNA using non-viral vectors, we have developed thiolated gelatin nanoparticles that can release the payload in the presence of glutathione-rich (redox) environment. The thiolated gelatins with different degrees of thiolation were synthesized by reacting type B gelatin with varying amounts of 2-iminothiolane (namely the Gelatin, SHGel-20, SHGel-40 and SHGel-100). Cytotoxicity evaluations carried out by MTS assay showed that the thiolated gelatin SHGel-100 was slightly toxic when compared to the others, which had cell viability profile similar to that of the unmodified gelatin. The nanoparticles were prepared from gelatin and thiolated gelatin by desolvation using ethanol followed by crosslinking using 0.1 ml of 40 % (v/v) glyoxal for desired time interval [5].

The release profiles of fluorescein isothiocyanate (FITC)-dextran (Mol. wt. 70,000 da) encapsulated non-crosslinked and crosslinked nanoparticulate systems in phosphate buffered saline (PBS) containing glutathione (0-5mM GSH) were compared. The presence of GSH was found to enhance the release by about 40% in case of thiolated gelatin and 20% in case of gelatin nanoparticles. The predominant difference in the release characteristics can be attributed to the non-enzymatic reducing properties of GSH which cleaves off the disulfide bonds within the thiolated gelatin nanoparticles resulting in a rapid release of the payload [5].

Qualitative and quantitative analysis of transfection in NIH-3T3 murine fibroblast cells by the nanoparticles carrying plasmid DNA encoding for enhanced green fluorescent protein (EGFP-N1) was done by fluorescence microscopy and fluorescence–activated cell sorting (FACS). Qualitative results showed highly efficient expression of GFP in the crosslinked and non-crosslinked, and thiolated and non-thiolated nanoparticulate systems that remained stable for up to 96 hours. Quantitative results from FACS showed that the crosslinked, thiolated gelatin nanoparticles (SHGel-20) were effective in transfecting NIH-3T3 cells than other carrier systems examined. The high transfection efficiency associated with these particles could be attributed to the increased stability from crosslinking the particle and reduced cytotoxicity. The results of this study show that thiolated gelatin nanoparticles would serve as a biocompatible intracellular delivery system that can release the payload in highly reducing environment [5].

Despite numerous advantages of thiolated gelatin, it is still necessary to chemically modify the biopolymer so that it has longer circulation time that can lead to efficient expression at the target site. Long-circulating nanoparticles can be achieved upon surface modification of the conventional nanoparticles with hydrophilic polymers, such as PEG, that minimize the interactions with plasma proteins

(opsonins) and reduce the uptake by the reticuloendothilial system. A significant increase in circulation time would ultimately increase the probability of extravasation and retention of the colloidal carriers in areas of discontinuous endothelium.

So, in this study we have modified the thiolated gelatin nanoparticles by post-PEGylation technique, characterized them *in vitro* and evaluated their biodistribution and long circulating ability *in vivo* in a human breast cancer xenograft model.

3. EXPERIMENTAL METHODS

3.1 PEG-Modification of Gelatin and Thiolated Gelatin Nanoparticles

Thiolated gelatin SHGel-20 and gelatin were used for the preparation of nanoparticles by desolvation using ethanol. The nanoparticles were separated by centrifugation at a speed of 16,000 rpm for 30 min. The separated nanoparticles were re-dispersed in 10 ml of phosphate buffer and surface modified using 5 times molar excess of methoxypoly(ethylene glycol) succinimidyl glutarate (PEG-SG) of molecular weight 2,000 daltons. The nanoparticles were incubated with PEG-SG for varying time periods to achieve different degrees of PEGylation.

3.2 Determination of Degree of PEGylation

The degree of modification of thiolated gelatin nanoparticles by PEG was determined by 2,4,6 - trinitrobenzenesulfonic acid (TNBS) method wherein the number of free amino groups is estimated by a colorimetric reaction. The control gelatin and thiolated gelatin nanoparticles, the PEG conjugated gelatin and thiolated gelatin nanoparticles were dispersed in pH 8.5 alkaline borate buffer and allowed to react with trinitrobenzenesulfonic acid (TNBS) at room temperature. The reaction mixture is centrifuged and the absorbance of the supernatant solution is measured at 420 nm using Shimadzu UV160U spectrophotometer (Columbia, MD).

3.3 Characterization by Particle Size and Surface Charge

The controls and PEG-conjugated nanoparticles were characterized for size and surface charge. The mean particle size and surface charge measurement was carried out by ZetaPALS (Brookhaven Instruments Corporation). The measurement of particle size by this instrument is based on light scattering. The colloidal suspension of the nanoparticles is diluted with deionized distilled water and the particle size analysis is carried out at a scattering angle of 90° and a temperature of 25°C. Zeta potential or the surface charge on the particles with a thin film of liquid was measured at the default parameters of dielectric constant, refractive index and viscosity of water.

3.4 Surface Analysis by ESCA

The kinetic energy of the extracted electrons from the surface of the nanoparticles using a source of x-rays was measured which is an indicative of the element from which the electron came from. Only those electrons that escape from the surface of the sample without loss of energy contribute to the signal peak and were analyzed for carbon-1s (C_{1s}), oxygen-1s (O_{1s}), and nitrogen-1s (N_{1s}) envelopes.

3.5 Culture Conditions for MDA-MB-435

Estrogen-negative human breast adenocarcinoma cell line MDA-MB-435 (ATCC) derived from breast ductal carcinoma of a caucasian female was used for growing tumors in mice. These cells were grown as a monolayer in culture using RPMI supplemented with L-glutamine, Pen-strep and fetal bovine serum at 37°C and 5% CO_2. These cells are metastatic in origin and can be used to grow tumor models in mice.

3.6 Radiolabeling of the Nanoparticles

Nanoparticles of the unmodified and PEGylated gelatin derivatives were prepared by desolvation as described earlier. The free amino groups remaining after PEG conjugation were used to couple cyclic anhydride of DTPA. The nanoparticles conjugated with DTPA were then labeled with ^{111}In. To the nanoparticles suspended in HBS, 1M HEPES was added to prevent any change in pH. This was followed by the addition of sodium citrate and indium chloride. The mixture was incubated for 1h at room temperature and any free radioactivity was removed by centrifugation. The radiolabeled nanoparticles were re-suspended and centrifuged repeatedly, and the supernatant was measured for any traces of free radioactivity using a gamma counter. The specific radioactivity in microCi/mg of nanoparticles was assessed in order to estimate the amount of radiolabeled nanoparticles to be injected per animal.

3.7 Tumor Model Development

Six weeks old female Nu/Nu (athymic) mice, weighing approximately 20 grams, were the animal models chosen for the *in vivo* studies. For the purpose of biodistribution study, a total of 112 mice were used with 5 mice at every time point for four sets of experiments with a total of 7 time points per set. The development of the tumor model in mice was done according to the approved Northeastern University IACUC animal protocol. Tumors are implanted by subcutaneous injection of 2.0 million MDA-MB-435 cells in 200 µl of 0.9% saline in the breast pad of lightly-anesthetized Nu/Nu mice. The animals were sacrificed at predetermined time intervals and the radioactivity in different organs was measured using gamma counter. The data obtained was subjected to non-compartmental analysis using WinNonLin.

4. RESULTS AND DISCUSSION

4.1 Determination of Degree of PEGylation

The free amino groups on the surface of gelatin and thiolated gelatin nanoparticles were considered as 100%. There was an increase in PEGylation of the surface amino groups of the nanoparticles with increase in incubation time to up to 2 h. At the end of 2 h about 90% of the amino groups have been modified upon incubation with 5 Molar excess of PEG.

4.2 Characterization by Particle Size and Surface Charge

The mean particle size of the nanoparticles is shown in **Table 1.** For the controls gelatin (Gel) and thiolated gelatin (SHGel-20) nanoparticles the size was found to be in the range of 220-250 nm. However, owing to the surface modification both the PEG-modified nanoparticles were found to have larger particle size ranging from 290 – 350 nm. The controls gelatin and thiolated gelatin nanoparticles were found to have zeta potential values of -8.45 and – 9.36 mV respectively. In comparison, the PEG-modified gelatin and thiolated gelatin nanoparticles were found to have a surface charge of - 7.72 and -6.67 mV respectively.

Table 1: Summary of Characterization of Nanoparticles

Nanoparticle Type	Empty Nanoparticles	
	Size (nm)	**Zeta Potential (mV)**
Gelatin (Gel)	230 ± 11.5	- 8.45 ± 0.45
PEG- Modified Gelatin (PEG-Gel)	329 ± 17.7	- 7.72 ± 0.88
Thiolated Gelatin (SHGel-20)	244 ± 6.8	- 9.36 ± 0.64
PEG-Modified Thiolated Gelatin (PEG-SHGel-20)	302 ± 9.2	- 6.67 ± 0.77

4.3 Surface Analysis by ESCA

The results of ESCA show the peak intensities of the species –C-H- (hydrocarbon), -C-O- (ether), and –C=O (carbonyl) obtained from C_{1S} scan of the control and PEG-modified nanoparticles. The surface presence of PEG chains in the PEG-modified nanoparticles was confirmed by the increase in the relative peak intensities of ether linkage from the high resolution spectra. The peak intensities of ether linkage in case of PEG-modified gelatin were found to be 79.2% and PEG-modified thiolated gelatin nanoparticles resulted in 82.6%. In comparison, the gelatin and thiolated gelatin nanoparticles have shown ether peak intensities of 36% and 28.4% respectively.

4.4 Biodistribution of Indium Labeled Nanoparticles

The percent activity recovered was calculated and the distribution profile of the radiolabeled nanoparticles was plotted as a function of time. The results indicate longer circulation of the PEG-modified nanoparticles when compared to the unmodified nanaoparticles. PEG-modification has improved the circulation time of the nanoparticles resulting in enhanced extravasation and retention in the tumors with about 13% of the recovered dose of PEG-SHGel-20 and PEG-Gel nanoparticles being retained in the tumor at 12 h time point as opposed to SHGel-20 and Gel nanoparticles with 9% and 7% retention respectively. In case of gelatin nanoparticles almost 50% of the recovered dose was found to get accumulated in the liver as opposed to 20 – 30% of the recovered dose in case thiolated and PEG-modified nanoparticles. The animals injected with PEG-modified nanoparticles have shown a progressive increase in radioactivity in the kidney at later time points. Accumulation of the nanoparticles in lungs and heart was found to be negligible.

Upon calculation of the pharmacokinetic parameters from the plasma-time curves, the PEG-SHGel-20 nanoparticles were found to have a half-life of 15.34 h when compared to PEG-Gel with a half-life 10.69 h. The unmodified SHGel-20 and Gel nanoparticles had a half life of about 3 h. The kinetic parameters calculated from the tumor data show an increased half-life of the PEG-SHGel-20 nanoparticles to up to 37 h followed by PEG-Gel nanoparticles (34 h). The prolonged circulation of the modified nanoparticles has resulted in an enhanced ability to extravasate leaky vasculature of the tumors.

5. CONCLUSIONS

The results of this study show that the thiolated gelatin nanoparticles have sensitivity towards highly reducing environment wherein the disulfide bonds are reduced to trigger-release the payload. Upon modification with PEG the nanoparticles have longer circulation potential and hence can be used to passively target solid tumors *in vivo*.

6. ACKNOWLEDGEMETS

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