Pharmaceutical Research, Vol. 19, No. 7, July 2002 (© 2002) *Research Paper*

Long-Circulating Poly(Ethylene Glycol)-Modified Gelatin Nanoparticles for Intracellular Delivery

Goldie Kaul[1] and Mansoor Amiji[1,2]

Received Nov 26, 2001; accepted March 26, 2002

Purpose. The objective of this study was to develop and characterize long-circulating, biodegradable, and biocompatible nanoparticulate formulation as an intracellular delivery vehicle.

Methods. Poly(ethylene glycol) (PEG)-modified gelatin was synthesized by reacting Type-B gelatin with PEG-epoxide. The nanoparticles, prepared by pH and temperature controlled ethanol-water solvent displacement technique, were characterized for mean size, size distribution, and surface morphology. Electron spectroscopy for chemical analysis (ESCA) was used to confirm the surface presence of PEG chains. *In vitro* release of tetramethylrhodamine-labeled dextran (TMR-dextran, Mol. wt. 10,000 daltons) from the nanoparticle formulations was examined in PBS, with and without 0.2-mg/ml protease, at 37°C. Relative cytotoxicity profile of control and PEGylated gelatin was evaluated in BT-20 a human breast cancer cell line. The nanoparticles were incubated with BT-20 cells to determine uptake and cellular distribution using confocal microscopy.

Results. Gelatin and PEGylated gelatin nanoparticles were found to be spherical in shape with a smooth surface in a size range of 200–500 nm and a unimodal size distribution. ESCA results showed an increase in the ether carbon (-C-O-) peak in the PEGylated gelatin nanoparticles due to the presence of PEG chains. The presence of PEG chains decreased the percent release of TMR-dextran in the presence of proteolytic enzyme due to steric repulsion. Cytotoxicity assays indicated that both gelatin and PEGylated gelatin were completely non-toxic to the cells. A large fraction of the administered control gelatin and PEGylated gelatin nanoparticles were found to be concentrated in the perinuclear region of the BT-20 cells after 12 hours indicating possible vesicular transport through initial uptake by endocytosis and endosomal processing.

Conclusion. The results of this study show that PEGylation of gelatin may prove beneficial as long-circulating delivery system *in vivo*. Additionally, the nanoparticles could encapsulate hydrophilic macromolecules and are internalized by tumor cells.

KEY WORDS: intracellular delivery vehicle; PEGylated gelatin nanoparticles; rhodamine-labeled dextran (TMR-dextran); BT-20 cells; endocytosis.

INTRODUCTION

With advances in molecular and cell biology, there are increasing numbers of opportunities for cell-specific targeting and delivery of drugs (1–7). Safe and efficacious DNA delivery with non-viral vectors represents the most important intracellular delivery challenge of the new millennium. For DNA delivery, cationic lipid complexes (lipoplexes), and cationic polymer complexes (polyplexes) have been studied extensively for somatic gene therapy (8–10). These vector systems, however, suffer from poor efficiency of transfection, lack of selectivity, and in some cases, poor stability of the DNA after intracellular delivery (11,12). One strategy for improving the transfection efficiency with non-viral vectors is by increasing the DNA concentration and stability in the cell through encapsulation in a polymeric matrix (13–15). Hirosue *et al.* (16) formulated plasmid DNA-containing poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles with improved loading and enhanced stability in the presence of DNA degrading enzymes. Being a hydrophobic slow degrading polymer that results in highly acidic local microenvironment, PLGA is not a very effective polymer matrix for DNA encapsulation (17,18).

For the development of polymeric nanoparticulate carriers with improved encapsulation and efficient intracellular delivery of DNA, we have outlined the following design criteria. First, the matrix polymer must be biocompatible and preferably biodegradable. Second, the carrier system should efficiently encapsulate the DNA and protect it during transit in the systemic circulation. Third, the carrier should be able to reach specific tissues and cells in the body and avoid uptake by the mononuclear phagocytic system after systemic administration. This is achieved through the use of cell-specific ligands and attachment of poly(ethylene glycol) (PEG) chains on the nanoparticle surface. Fourth, the nanoparticulate system should be able to deliver the DNA inside the cell through nonspecific or receptor-mediated endocytosis and protect the DNA during cellular transit. Lastly, for industrial production, the system should be amenable to scale-up and manufacturing under the GMP guidelines.

Based on these criteria, in the present study, we have developed PEG-modified gelatin nanoparticles as long-circulating intracellular delivery systems for hydrophilic macromolecules like DNA. Gelatin is a proteinaceous biopolymer obtained by hydrolytic degradation of naturally occurring porcine or bovine collagen. Gelatin is a colorless, odorless, nonirritating, biocompatible, and biodegradable material. Gelatin has a long history of safe use in a wide range of medical and pharmaceutical applications, cosmetics, as well as in food products (19,20). It is a relatively inexpensive material that is available in sterile and pyrogen-free form. Type-A gelatin is obtained by acid treatment of collagen and has an isoelectric point (pI) between 7.0 and 9.0. Type-B gelatin, on the other hand, is obtained by alkaline hydrolysis of collagen and has a pI between 4.8 and 5.0. The large number of pendant functional groups in the gelatin structure aid in chemical cross-linking and derivatization. Truong-Le *et al.* (14) have described the use cross-linked gelatin nanospheres, formed by complex coacervation with DNA, for controlled gene delivery.

MATERIALS AND METHODS

Materials

Type-B gelatin (225 bloom strength) with 100–115 millimoles of free carboxylic acid per 100 g of protein, an isoelectric point of 4.7–5.2, and an average molecular weight 40,000–

[1] Department of Pharmaceutical Sciences, School of Pharmacy, Northeastern University, 110 Mugar Life Sciences Building, Boston, Massachusetts 02115

[2] To whom correspondence should be addressed (e-mail: m.amiji@neu.edu)

0724-8741/02/0700-1061/0 © 2002 Plenum Publishing Corporation

50,000 daltons was purchased from Sigma Chemical Company (St Louis, Missouri). Monomethoxy-poly(ethylene glycol) (PEG) with a molecular weight of 5,000 daltons was obtained from Fluka Chemika/Biochemika (Ronkonkoma, New York). Type-I protease enzyme from bovine pancreas with an activity of 6.9 units per mg, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), and polyethyleneimine (PEI, M_n = 10,000 Da) were all purchased from Sigma as well. Tetramethylrhodamine labeled-dextran (TMR-dextran) of molecular weight 10,000 daltons and a degree of substitution of 0.83 moles of rhodamine per mole of dextran was obtained from Molecular Probes (Eugene, Oregon). N,N-dimethylformamide, epichlorhydrin and reagent grade ethanol were obtained from Fisher Scientific (Milwaukee, Wisconsin). The BT-20 estrogen receptor negative human breast carcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, Maryland) and maintained in Eagle's minimum essential media (MEM) supplemented with 10 mM pyruvate, non-essential amino acids, L-glutamine, and 10% fetal bovine serum at 37°C and 5% CO_2 atmosphere. General-purpose serum free medium (UltraCulture®) was obtained from Bio-Whittaker, (Walkersville, Maryland). Lipofectin® reagent was obtained from Invitrogen (Carlsbad, California). All aqueous solutions were prepared exclusively in deionized distilled water (Nanopure II, Barnstead/Thermolyne, Dubuque, Iowa).

Synthesis and Purification of PEG-Epoxide

For synthesis of PEG-epoxide, 25 g (5 mM) of PEG was dissolved in 100 ml of dehydrated N,N-dimethylformamide containing 1% (w/v) of triethanolamine at 40°C for 4 h under constant stirring conditions. To this mixture, five times the molar excess (2.31 g) of epichlorhydrin was added and the reaction to convert the terminal hydroxyl group of PEG into epoxide continued overnight at room temperature under reflux conditions. The epoxide derivative was precipitated in cold diethyl ether. The PEG-epoxide was then washed thrice with cold diethyl ether and dried *in-vacuo*. Typical yield was about 88%.

Synthesis and Characterization of PEG-Modified Gelatin

To a known quantity of PEG-epoxide dissolved in 20 ml of pH 8.5 alkaline borate buffer, 2.0 g of gelatin was added and the reaction for grafting PEG-epoxide to the primary amine groups of basic amino acids was carried out for 14 h at 40°C. The mixture was precipitated in 3-fold excess of acetone to remove any unreacted PEG-epoxide, washed with acetone, and dialyzed extensively against deionized distilled water, using a membrane with a molecular weight cut-off of 12–14 KDa, for up to 24 h at room temperature, and lyophilized.

To determine the degree of gelatin modification by PEG-epoxide, control gelatin or PEGylated gelatin derivatives were dissolved in pH 8.5 alkaline borate buffer to prepare 1.0 mg/ml solution. Ten-ml of the solutions were mixed with 250 μl of 30 mM trinitrobenzenesulfonic acid (TNBS) solution in methanol. After 30 m of reaction at room temperature, the absorbance of solution at 420 nm was measured by Shimadzu UV160U spectrophotometer (Columbia, Maryland) and the percentage of amine groups of gelatin that were derivatized by PEG-epoxide was calculated (21).

Preparation and Characterization of Nanoparticles

Preparation of Nanoparticles

Nanoparticles of the unmodified gelatin and PEGylated gelatin derivative were prepared by the ethanol precipitation method under controlled conditions of temperature and pH. Two hundred mg of gelatin or PEGylated gelatin derivative was dissolved in 20 ml of deionized distilled water at 37°C until a clear solution was obtained. The pH of the resulting solution was adjusted to 7.00 with 0.2 M sodium hydroxide. The nanoparticles were formed by controlled precipitation of the aqueous gelatin solution with ethanol under continuous stirring. In the final mixture of 100 ml, the ethanol:water volume ratio was maintained at 65:35. The nanoparticle precipitate was centrifuged at 14,000 rpm for 90 m, washed twice with deionized distilled water, and lyophilized.

Particle Size Analysis

The resulting turbid colloidal system of gelatin or PEGylated gelatin nanoparticles was analyzed for mean particle size and size distribution by a Coulter counter. A sample of the diluted (1:4) nanoparticulate suspension in deionized distilled water was used for particle size analysis at a scattering angle of 90° and a temperature of 25°C using Beckman/Coulter N4 plus (Fullerton, CA, USA) instrument.

Scanning Electron Microscopy (SEM)

The nanoparticle suspension was centrifuged at 14,000 rpm for 90 min and the resulting pellet was lyophilized for SEM analysis. The gelatin and PEGylated gelatin nanoparticle samples were mounted on an aluminum sample mount and sputter coated with gold-palladium to minimize surface charging. The samples were then observed for surface morphology with an AMR-1000 (Amray Instruments, Bedford, Massachusetts) scanning electron microscope at an accelerating voltage of 10 kV.

Electron Spectroscopy for Chemical Analysis (ESCA)

Both control and PEGylated gelatin nanoparticles were analyzed by ESCA, a technique that measures the elemental composition and identifies chemical functional groups of 100 Angstroms-thick surface layer. These experiments were carried out at National ESCA and Surface Analysis Center for Biomedical Problems (NESAC/BIO) at the University of Washington (Seattle, Washington). The spectra were taken on a Surface Science Instrument X-probe spectrophotometer with a monochromatized Al X-ray source. A 5eV flood gun of was used to neutralize surface charge. Surface elemental composition was determined using the standard Scofield photoemission cross-sections (23). Identification of chemical functional groups was obtained from the high-resolution peak analysis of carbon-1s (C_{1s}), oxygen-1s (O_{1s}), and nitrogen-1s (N_{1s}) envelopes.

TMR-Dextran Loading

TMR-dextran was used as a model of hydrophilic macromolecular drugs. To an aqueous solution of gelatin or PEGylated gelatin adjusted to pH 7.0, TMR-dextran was added at a final concentration of 1.0% (w/w) and the loaded nanoparticles were prepared as described above. The TMR dextran-loaded nanoparticles were separated from the free TMR-dextran by centrifugation and washing steps. The loading efficiency of TMR-dextran in the nanoparticle formulations was determined by dissolving the nanoparticle sample in protease (0.2 mg/ml)-containing PBS at 37°C until a clear solution was observed (~30 m). The resulting solution was filtered through a 0.22 μm filter and the fluorescence intensity was measured at 560 nm excitation and 580 nm emission wavelengths, using a Perkin Elmer LS50B fluorescence spectrophotometer (Norwalk, Conneticut) interfaced with an FLWINLAB® software for data analysis and processing. The efficiency of TMR-dextran loading in the nanoparticle formulations were determined using a standard curve of TMR-dextran in protease-containing PBS.

In Vitro Release Studies

The release kinetics of TMR-dextran from gelatin and PEGylated nanoparticles was carried out in PBS, with and without 0.2-mg/ml protease, at 37°C. Ten-mg of TMR-dextran containing gelatin or PEGylated gelatin nanoparticles was placed with 1.5 ml of buffer solutions in an Eppendorf-type microcentrifuge tubes. Periodically, the tubes were removed from the water-bath and centrifuged at 14,000 rpm for 5 min. Subsequently, 500 μl of the supernatant was removed and diluted to 1.0 ml with either PBS or protease-containing PBS. The buffer was replaced each time to maintain constant volume and sink conditions. The fluorescence intensity of TMR-dextran released was measured using the parameters described under the loading studies and the cumulative amount released was calculated from appropriate calibration curves.

Cytotoxicity Assays

Immortalized BT-20 cells were grown in 96 well plates at an initial seeding density of 10,000 cells per well in 200 μl of supplemented Eagle's MEM. Cells were grown for 24 h, after which the growth medium was removed and replaced with 180 μl of serum-free medium. Appropriate concentrations of gelatin and PEGylated gelatin, along with serum alone, Lipofectin®, and PEI as negative and positive controls, respectively, were added in 20 μl aliquots. Samples were incubated at 37°C for 5 h and the metabolic activity of each well determined by the MTT assay (24). To each well was added 25 μl of a 5-mg/ml solution of MTT stock solution in sterile PBS buffer. The samples were incubated at 37°C for 24 h and 100 μl of extraction buffer (20% w/v SDS in DMF/water (1:1), pH = 4.7) was added to each well. Samples were incubated at 37°C for another 5.0 h and the absorbance was measured at 540 nm with a Labsystems Multiskan MCC/340 microplate reader. The percent viability of cells was expressed as the ratio of absorbance of the polymer-treated cells relative to those in serum alone multiplied by 100.

Cell Uptake and Distribution Studies

After initial passage in tissue culture flasks, BT-20 cells were grown to semi-confluence in supplemented Eagle's MEM in 6-well tissue culture plates on Corning's circular glass cover-slips at 37°C and 5% CO_2 atmosphere. A suspension of TMR-dextran containing gelatin or PEGylated nanoparticles was prepared at a concentration of 0.5 mg/ml in the culture medium. After filtration, the nanoparticle suspension was incubated with the cells at 37°C for a period of 12 h. The media was then removed and the plates were washed thrice with sterile PBS. After the final wash, the cells were fixed with 4% (v/v) paraformaldehyde in PBS for 1.0 h at room temperature and were washed four times with PBS. Individual cover-slips were then mounted cell side up on clean glass slides with fluorescence-free glycerol based mounting medium (Fluoromount-G®, Southern Biotech Associates, Birmingham, Alabama). Both differential interference contrast (DIC) and fluorescence images were acquired with a Zeiss Axioplan-2® confocal microscope (Thornwood, New York). The images were digitized and processed with Adobe Photoshop® software.

RESULTS AND DISCUSSION

Characterization of PEGylated Gelatin

The percentage of free amine groups of gelatin that were reacted by PEG-epoxide in the modified gelatin derivative was determined by TNBS assay as a function of the amount of PEG-epoxide added per gram of gelatin. With increasing amount of PEG-epoxide, there was an increase in the percent amine groups that were occupied by PEG as shown in Fig. 1. For instance, when 0.5 g of PEG-epoxide was added per gram of gelatin, percent PEGylation, as determined by TNBS assay was approximately around 30%. The 30% PEG-modified derivative was subsequently used for preparation of PEGylated gelatin nanoparticles.

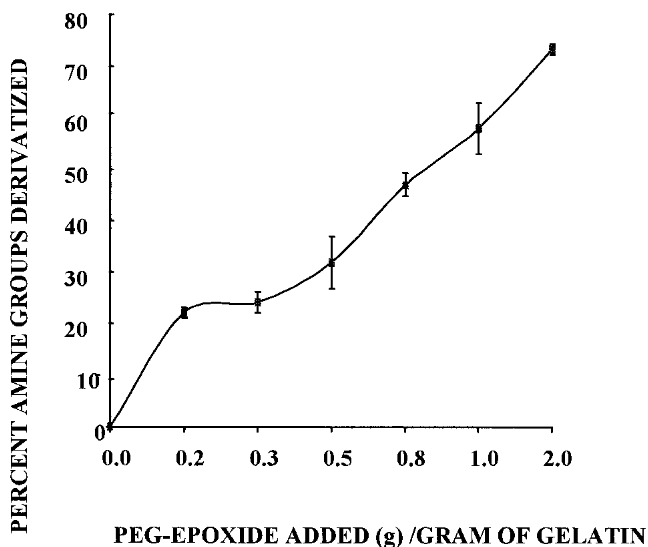

Fig. 1. Percentage of amine groups of gelatin (Type B) that were derivatized with poly(ethylene glycol) (PEG)-epoxide. The amount of PEG-epoxide added was per unit mass of gelatin.

Characterization of Nanoparticles

Particle Size Analysis

The mean nanoparticle size and size distribution, as measured by Coulter, are shown in Fig. 2. For both control and PEGylated gelatin nanoparticles, the mean particle size was around 300 nm and a range of 200–500 nm and with a narrow size distribution.

SEM Analysis

The SEM micrographs of the lyophilized control and PEGylated gelatin nanoparticle samples, shown in Fig. 3, verified that the nanoparticles were smooth and with spherical shape. The SEM micrographs also indicated that high-speed centrifugation and freeze-drying did not affect the nanoparticle morphology. Based on the studies by Jain and co-workers (22), most peripheral human tumor vessels have a permeability cut-off of less than 600 nm. The gelatin and PEGylated gelatin nanoparticles, therefore, should provide an effective means of DNA delivery to solid tumors after intravenous or intratumoral administration of the formulation in future *in vivo* studies.

ESCA Results

ESCA exploits the photoelectric effect to obtain information about the chemical composition and structure of the surface layer (23). When a photon source (e.g., X-rays) is directed at a sample, the photons interact with the electrons present in the sample material. If the photon has sufficient energy, it causes an electron from the surface atom to be emitted from its orbital. The measured kinetic energy of the electron is indicative of the element from which the electron came from and the chemical environment of that element. ESCA is a surface sensitive technique because only those electrons that leave the surface without energy loss will contribute to the peak signifying that element. Those electrons originating from far below the surface (>100 Angstroms) suffer energy loss through collisions and are unable to make it out of the surface, or they escape the surface with considerable energy loss (23).

Table I shows the high resolution -C-H- (hydrocarbon), -C-O- (ether) and –C=O- (carbonyl) peaks in the C_{1s} envelop of the control and the PEGylated gelatin at their characteristic binding energies of 285.0, 286.4 and 288.1 eV respectively. The surface presence of PEG chains in the PEGylated gelatin nanoparticle sample was confirmed by comparing the high-resolution ESCA spectra of the modified gelatin sample with the unmodified gelatin. The observed increase in the relative peak intensity of the ether peak in the PEGylated gelatin derivative (from 30% in the control sample to 38% in the PEGylated gelatin) is consistent with the presence of ethylene oxide residues of PEG on the surface of modified gelatin derivative.

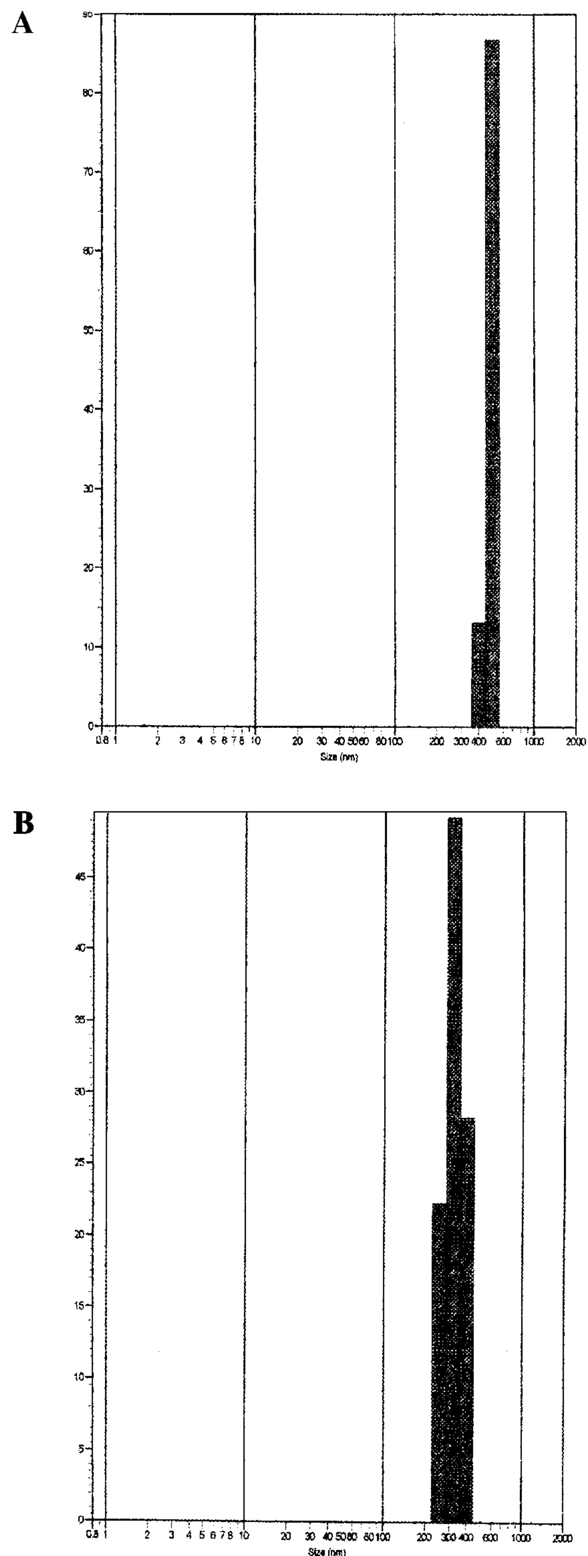

Fig. 2. Analysis of particle size and size distribution by Coulter counter. Gelatin (A) and poly(ethylene glycol)-modified gelatin (B) nanoparticles were analyzed using Beckman/Coulter N4 plus instrument at 25°C.

TMR-Dextran Loading

TMR-dextran was used as a model macromolecular compound since it allows for easier evaluation of loading and release by fluorescence spectrophotometry. Additionally, TMR-dextran containing nanoparticles can be easily visualized by fluorescence microscopy in cell trafficking studies. Using 1.0% (w/w) TMR-dextran concentration, we determined that the loading efficiency in gelatin and PEGylated gelatin nanoparticles was 51% and 48%, respectively. Based

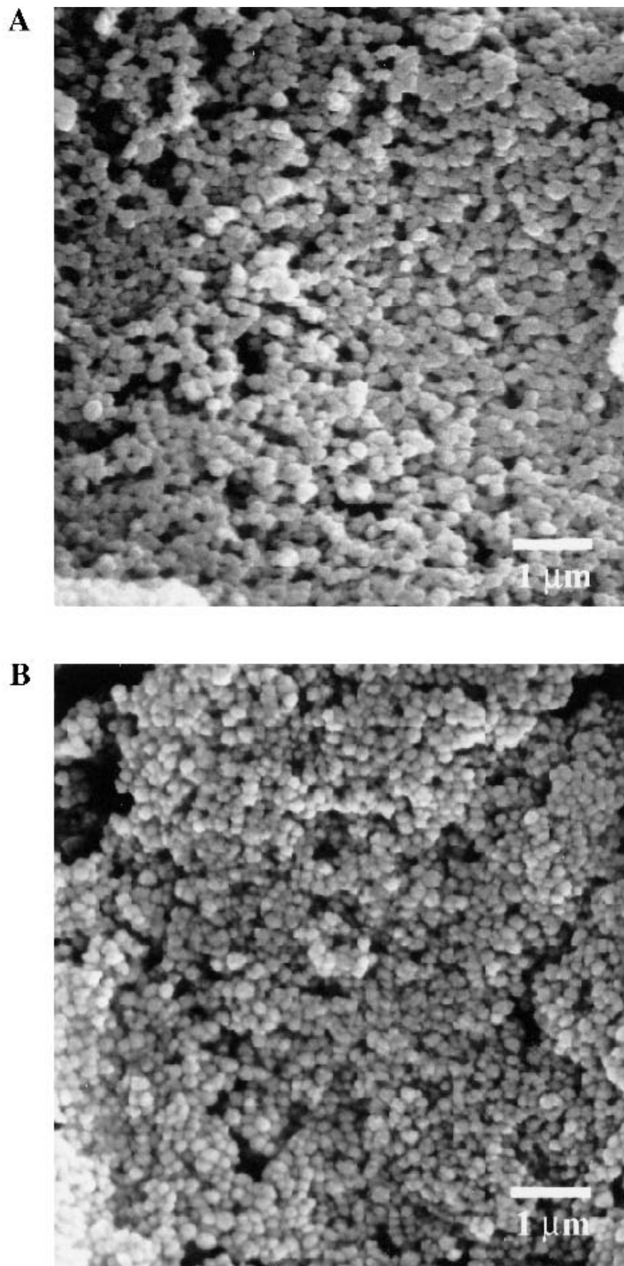

Fig. 3. Scanning electron micrographs of gelatin (A) and poly(ethylene glycol)-modified gelatin (B) nanoparticles. The original magnification was 13,500× and the scale bar represents a distance of 1 µm.

on these results, we found that the PEG-modification of gelatin does not affect the loading capacity for hydrophilic macromolecular compounds.

In Vitro Release Studies

The *in vitro* release of TMR-dextran was performed in PBS at 37°C, with and without 0.2-mg/ml protease. Protease was included in the release medium to evaluate the effect of the enzyme on the release of macromolecular compound, if any, from PEGylated gelatin nanoparticles. As shown in Fig. 4, the release of TMR-dextran from PEGylated gelatin nanoparticles was significantly greater than from the unmodified gelatin nanoparticles when the release medium did not contain protease (Fig. 4A). The greater fractional release of TMR-dextran from PEGylated gelatin nanoparticles in the absence of protease was due to the greater hydrophilicity of the matrix that allowed for higher swelling and subsequent release of a macromolecule. In contrast, when the medium

Table I. High Resolution C_{1s} Peak Analysis of ESCA on Surfaces of Gelatin and PEGylated Gelatin Nanoparticles[a]

	Relative peak area (%)		
Nanoparticle sample	–C–H– (285.0 eV)	–C–O– (286.4 eV)	–C=O– (288.1 eV)
Gelatin	45	30	25
PEGylated Gelatin	35	38	26

[a] Electron spectroscopy for chemical analysis (ESCA) was performed at National ESCA and Surface Analysis center for Biomedical Problems (NESAC/BIO), University of Washington, Seattle, Washington.

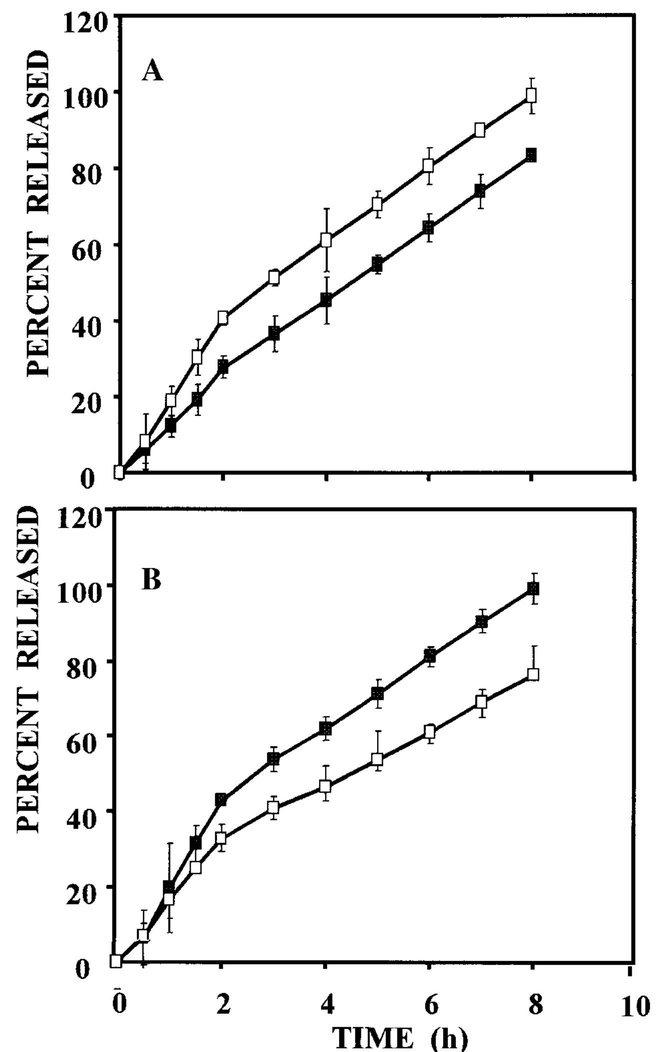

Fig. 4. Release kinetics of tetramethylrhodamine-labeled dextran (Mol. wt. 10,000 daltons) from gelatin (■) and poly(ethylene glycol)-modified gelatin (□) nanoparticles. The release studies were performed in the absence (A) and presence (B) of 0.2 mg/ml protease in phosphate-buffered saline (pH 7.4) at 37°C.

contained protease, the fractional release of TMR-dextran from PEGylated gelatin nanoparticles was decreased significantly (Fig. 4B). Predominant difference in the release characteristics between gelatin and PEGylated gelatin nanoparticles in protease-containing buffer was seen in earlier time points. At 6 h, for instance, the PEGylated sample released only about 60% as compared to approximately 80% release of the gelatin nanoparticles. In contrast to diffusional release in the absence of protease, the predominant mechanism of TMR-dextran release in the presence of protease is thought to be due to enzymatic degradation of the biopolymer. The decrease in the release of TMR-dextran from PEGylated gelatin nanoparticles was probably due to steric repulsion of the enzyme by surface accessible PEG chains, which resulted in decrease degradation of the matrix. As the nanoparticles continued to hydrate and the enzyme was able to access the active site, and the differences in the release behavior between gelatin and PEGylated gelatin nanoparticle became insignificant.

Cytotoxicity Assays

New polymeric vectors are often compared to standard DNA condensing agents and transfection vectors such as PEI and Lipofectin® for their transfection. PEI and Lipofectin®, however, are also associated with significant levels of cytotoxicity and thus high levels of cellular uptake and transfection are accompanied with considerable loss of cell viability. To determine the toxicity profile of the newly synthesized PEGylated gelatin, we conducted the standard MTT cytotoxicity assay with BT-20 cells (24).

As can be seen in Fig. 5, cells incubated with gelatin and PEGylated gelatin remained 100% viable relative to control (serum-free media) at concentrations as high as 200 μg/ml. In contrast, cells treated with Lipofectin® and PEI showed significant decrease in viability. For instance, at 50 μg/ml concentration, cells viability was 68% and 17% for Lipofectin®- and PEI-treated cells, respectively. It is encouraging to observe from the cytotoxicity profiles of gelatin and PEGylated gelatin that these polymers merit further studies as biocompatible and non-toxic vectors.

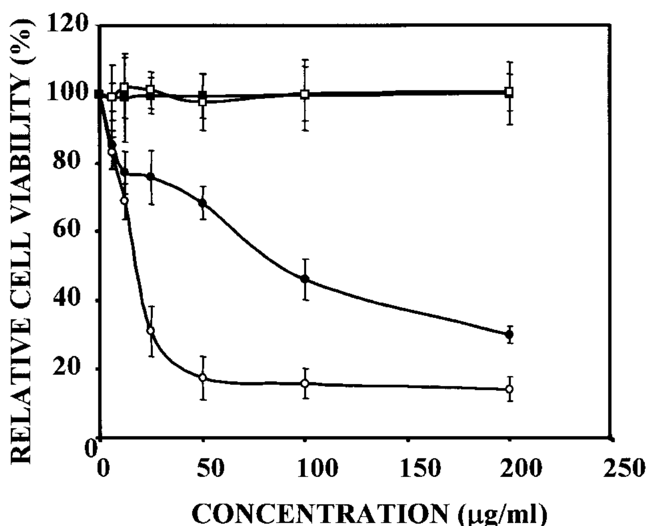

Fig. 5. Cytotoxicity profiles of gelatin (■), PEG modified gelatin (□), Lipofectin® (●) and polyethyleneimine (○). Percent viability of BT-20 cells was expressed as a function of polymer concentration relative to serum-free media.

Cell Uptake and Distribution Studies

The DIC and fluorescence images of nanoparticle uptake and distribution in BT-20 human breast cancer cells is shown in Fig. 6. After 12 h of incubation with the cells, the figures clearly show that both control gelatin and the PEGylated gelatin nanoparticles containing TMR-dextran were localized in the perinuclear region. Also interesting to note here that the cells remained viable during the course of this study and, as such, these nanoparticles do not confer any overt cytotoxicity. At initial time points, we observed the nanoparticles to present mainly on the cells surface with subsequent uptake though the vesicular transport system. Once the nanoparticles were endocytosed, they were able to escape the endosome and found primarily in the cytoplasm around the nuclear membrane. In the case of gelatin nanoparticles, the fluorescence confocal image (Fig. 6B) shows that some of the fluorophore was released and stained the nucleus. PEGylated gelatin nanoparticles, on the other hand, remained intact as the fluorescence image (Fig. 6D) clearly shows discrete particles around the nucleus. When TMR-dextran was added to the cells in solution and incubated for 12 h, the fluorescence was completely diffused (results not shown).

Although some investigators have found that PEG-modified drug carriers do not enter the cells probably due to steric repulsion (25,26), our results were different. We observed that a large fraction of the incubated nanoparticles were internalized by the cells and remained stable during vesicular transport. Based on the results of cell uptake studies, PEGylated gelatin nanoparticles show great promise as an intracellular delivery system for hydrophilic macromolecules, like DNA.

CONCLUSIONS

PEG-modified (PEGylated) gelatin derivative was synthesized for preparation of long-circulating nanoparticles with capacity for intracellular delivery of drugs and genes. The

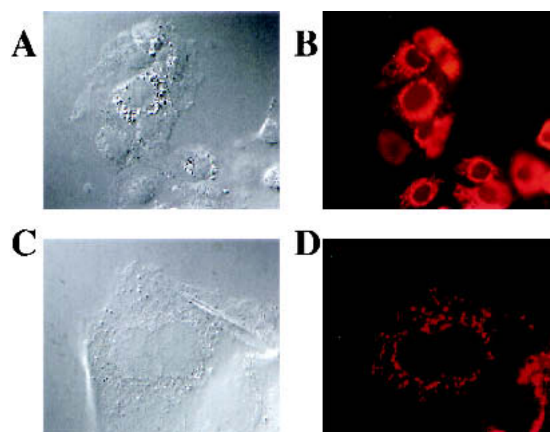

Figure 6. Differential interference contrast and fluorescence microscopic images of tetramethylrhodamine-labeled dextran (Mol. wt. 10,000 daltons) containing gelatin (A & B) and poly(ethylene glycol)-modified gelatin (C & D) nanoparticles in BT-20 cells. The cells were incubated with the nanoparticles for 12 h at 37°C.

nanoparticles in the size range of 200–500 nm were prepared and the presence of PEG chains on the surface was confirmed by ESCA. TMR-dextran, used as a model macromolecule, was loaded at around 50% in both control gelatin and PEGylated gelatin nanoparticles. During the release *in vitro* studies, we observed that the release of TMR-dextran was significantly decreased when protease was added to the medium. In the presence of protease, PEGylation afforded protection against enzymatic degradation, which subsequently affected the release of TMR-dextran. Gelatin and PEGylated gelatin were found to be completely non-toxic to cells even at 200 μg/ml concentration. Cell uptake studies of the control and PEGylated gelatin nanoparticles confirmed that they were internalized by endocytotic pathway and remained stable during the vesicular transport process. The results of this study are very encouraging for the development of an intracellular delivery system for drugs and genes that can offer long-circulating property, is efficiently internalized by cells and remains intact in the endosomes and lysosomes.

ACKNOWLEDGMENTS

This study was partially supported by the Northeastern University Research and Development Grant. ESCA was performed at the NESAC/BIO, University of Washington, Seattle, WA, which is supported by NIH grant RR-01296. We are also very grateful to Dr. Vladimir Torchilin and his postdoctoral associate Ram Rammohan for assistance with particle size analysis and cell culture facilities.

REFERENCES

1. A. V. Schally and A. Nagy. Cancer chemotherapy based on targeting of cytotoxic peptide conjugates to their receptors on tumors. *Eur. J. Endocrinol.* **141**:1–14 (1999).
2. G. A. Pietersz and I. F. C. McKenzie. Antibody conjugates for the treatment of cancer. *Immunol. Rev.* **129**:57–80 (1992).
3. D. J. Rodi and L. Makowski. Phage-display technology - finding a needle in a vast haystack. *Curr. Opin. Biotechnol.* **10**:87–93 (1999).
4. R. Pasqualini, E. Koivunen, and E. Ruoslahti. αv-Integrins as receptors for tumor targeting by circulating ligands. *Nat. Biotechnol.* **15**:542–546 (1997).
5. M. Hashida, M. Nishikawa, and Y. Takakura. Receptor-mediated cell specific delivery of drugs to the liver and kidney. In N. Ogata, S. W. Kim, J. Feijen, T. Okano (eds.), *Advanced Biomaterials in Biomedical Engineering and Drug Delivery Systems,* Springer-Verlag, Tokyo, Japan, 1996 pp. 86–90.
6. E. A. L. Biessen, H. F. Bakkeren, J. Kuiper, and T. J. C. Van Berkel. Ligand size is a major determinate of high-affinity binding of fucose- and galactose-exposing (lipo)proteins by the hepatic fucose receptor. *Biochem. J.* **299**:291–296 (1994).
7. D. V. Schaffer, N. A. Fidelman, N. Dan, and D. A. Lauffenburger. Vector unpackaging as a potential barrier for receptor-mediated polyplex gene delivery. *Biotech. Bioeng.* **67**:599–606 (2000).
8. P. L. Felgner and G. M. Ringold. Cationic liposome-mediated transfection. *Nature* **337**:387–389 (1989).
9. D. Scherman, M. Bessodes, B. Cameron, J. Herscovici, H. Hofland, B. Pitard, F. Soubrier, P. Wills, and J. Crouzet. Application of lipids and plasmid design for gene delivery to mammalian cells. *Curr. Opin. Biotech.* **9**:480–485 (1998).
10. M. C. Garnett. Gene-delivery systems using cationic polymers. *Crit. Rev. Ther. Drug Carrier Syst.* **16**:147–207 (1999).
11. C. Wang and L. Huang. pH-sensitive immunoliposomes mediate target-cell-specific delivery and controlled expression of a foreign gene in mouse. *Proc. Natl. Acad. Sci. USA* **84**:7851–7855 (1987).
12. F. Liu, J. Yang, L. Huang, and D. Liu. New cationic lipid formulations for gene transfer. *Pharm. Res.* **13**:1856–1860 (1996).
13. K. W. Leong. Biopolymer-DNA nanospheres. In *Nonviral Vectors for Gene Therapy*. L. Huang, M. C. Hung and E. Wagner (eds.). Academic Press, Inc., San Diego, California, 1999 pp 267–287.
14. V. L. Truong, J. T. August, and K. Leong. Gene delivery by DNA-gelatin nanospheres. *Human Gene Ther.* **9**:1709–1717 (1998).
15. V. L. Truong, S. M. Walsh, E. Schweibert, H. Q. Mao, J. T. Guggino, and K. W. Leong. Gene transfer by DNA-gelatin nanospheres. *Arc. Biochem. Biophys.* **361**:47–56 (1999).
16. S. Hirosue, B. Miller, R. Mulligan, and R. Langer. Plasmid DNA encapsulation and release from solvent diffusion nanospheres. *J. Control. Release* **1**:41–48 (2000).
17. H. Okada, Y. Doken, Y. Ogawa, and H. Toguchi. Preparation of three-month depot injectable microspheres of leuprorelin acetate using biodegradable polymers. *Pharm. Res.* **11**:1143–1147 (1994).
18. T. G. Park, W. Lu, and G. Crotts. Importance of in vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein encapsulated poly (DL-lactide-co-glycolide) microspheres. *J. Control. Release* **33**:211–222 (1995).
19. A. G. Ward and A. Courts. *The Science and Technology of Gelatin.* Academic Press, New York, 1977.
20. A. Veis. *The Macromolecular Chemistry of Gelatin.* Academic Press, New York, 1964
21. S. L. Snyder and P. Z. Sobcinski. An improved 2,4,6-trinitrobenzene sulfonic acid method for the determination of amines. *Anal. Biochem.* **64**:284–288 (1975).
22. R. K. Jain. Transport phenomena in tumors. *Adv. Chem. Eng.* **20**:129–200 (1994).
23. M. A. Sherwood. Data analysis in X-Ray photoelectron spectroscopy. In D. Briggs and M. P. Seah (eds.). *Practical Surface Analysis, Second Edition,* John Wiley and Sons, New York, 1990 pp. 555–586.
24. M. B. Hansen, S. E. Nielsen, and K. Berg. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods* **119**:203–210 (1989).
25. A. L. Klibanov, K. Maruyama, V. P. Torchilin, and L. Huang. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett.* **268**:235–237 (1990).
26. V. P. Torchilin, A. L. Klibanov, L. Huang, S. M. O'Donnell, N. Nossiff, and B. A. Khaw. Targeting accumulation of polyethylene glycol-coated (immuno)liposomes in infarcted rabbit myocardium. *FASEB J.* **6**:2716–2719 (1992).