Biotechnology Letters **24:** 2093–2098, 2002.

Intracellular drug delivery using poly(D,L-lactide-*co*-glycolide) nanoparticles derivatized with a peptide from a transcriptional activator protein of HIV-1

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Received 15 October 2002; Accepted 21 October 2002

Key words: biodegradable polymer, conjugates, nanoparticles, protein transduction domain (PTD), Tat peptide

Abstract

Biodegradable poly(D,L-lactide-*co*-glycolide) (PLGA) nanoparticles were derivatized with Tat_{49-57} peptide, which is the protein transduction domain from the transcriptional activator Tat protein of human immunodeficiency virus type-1 (HIV-1). The Tat_{49-57} peptide-modified PLGA nanoparticles, with a mean diameter of ca. 238 nm, was effectively adsorbed on to the membrane of HaCaT cells and delivered into the nuclei without cytotoxicity.

Introduction

Nanoscopic particulates have been extensively investigated for controlled drug delivery via intravenous, ocular, and oral administration routes (Müller *et al.* 2001). Depending on the desired route of administration, particle size and surface properties should be optimized to achieve targeted and extended drug delivery to the affected tissues. Surface modification of particulate systems using cell-adhesion ligands is being popularly studied to improve the cell-to-particle interactions (Cho *et al.* 1997). However, particle adsorption on to a cell surface cannot guarantee sufficient intracellular translocation across the cellular membrane to show therapeutic effects (Dawson & Halbert 2000).

Recently, protein transduction domains (PTDs) have received considerable attention due to their excellent membrane spanning properties. These peptides expedite lipid membrane-reorganizing processes, such as fusion and pore formation, involving temporary membrane destabilization and subsequent reorganization (Bayley 1999). Structural similarities between PTDs include a high content of arginine residues as well as the ability to adopt an alpha-helical conformation (Schwartz & Zhang 2000). Among the PTDs, Tat peptides from a transcriptional activator protein of human immunodeficiency virus type-1 (HIV-1) have been the most studied partly due to their high translocational activity. Vivès *et al.* (1997) established that a 9-mer basic amino acid-rich sequence (Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) was strictly required but was sufficient to be taken up effectively by cells and to accumulate in the nuclei. Interestingly, this cationic peptide has been reported to mediate the cellular uptake of relatively large molecules in the tissue culture. For example, a fused Tat peptide (amino acid 47 to 57) at the *N*-terminal end of β-galactosidase (120 kDa) enhanced the tissue uptake of the protein *ex vivo* and *in vivo* after intraperitoneal injection into mice (Schwarze *et al.* 1999). Furthermore, the Tat peptide can mediate the trans-cellular uptake of supramolecular nanostructures. Liposomes, having a diameter of ca. 200 nm, could be effectively delivered into cells by anchoring Tat peptide to the liposome surface (Torchilin *et al.* 2001).

We propose here the use of Tat_{49-57} peptide-derivatized poly(D,L-lactide-*co*-glycolide) (Tat-PLGA) nanoparticles as a potent intracellular drug carrier. PLGA was chosen because it has been extensively utilized for biodegradable drug delivery carriers because of its superior biocompatibility. Tat-PLGA nanoparti-

cles were prepared by a spontaneous phase inversion technique, and their cellular internalization into HaCat cells was examined by using confocal laser scanning microscopy.

Materials and methods

Peptide synthesis

Tat-peptide (GRKKRRQRRRGYKC-NH_2) was synthesized by solid phase peptide synthesis (SPPS) using 4-methylbenzhydrylamine·HCl resin with a ABI 433 synthesizer, following the Fmoc[N-(9-fluorenyl)methoxycarbonyl]/*tert*-butyl method. It was purified (>95%) by using HPLC, and its molecular mass (1,846) was determined by mass spectroscopy (Agilent 1100 series).

Preparation of Tat peptide-PLGA conjugates

The $Tat_{49\text{-}57}$ peptide was conjugated to poly(D,L-lactide-*co*-glycolide) (PLGA, lactide/glycolide ratio 50/50, RG503H, Boehringer Ingelheim, Germany) via a simple coupling reaction between maleimide-derivatized PLGA and thiol-terminated Tat-peptide, as described in Figure 1. Briefly, the carboxylic acid end group of PLGA was activated to the succinimidyl ester using *N*-hydroxysuccinimide (Aldrich) and 1,3-dicyclohexylcarbodiimide (Aldrich), and then, was converted to primary amine groups using an excess amount of hexamethylene diamine (Aldrich). Maleimide-terminated PLGA was then prepared by reacting *N*-succinimidyl 4-(4-maleimidophenyl)butyrate (Sigma) to the primary amine terminated PLGA. Finally, $Tat_{49\text{-}57}$ peptide carrying a sulfhydryl group was dissolved in 400 μl Imject maleimide conjugation buffer (Pierce, Rockford, IL) and poured into the maleimide-terminated PLGA solution in DMSO with vigorous stirring. The sulfhydryl group of cysteine at *N*-terminal end of $Tat_{49\text{-}57}$ peptides was attached to a maleimide group in PLGA, forming a stable thioether bond. The final product was purified by dialysis against excess deionized distilled water and freeze-drying. The reaction was confirmed by FT-IR spectroscopy (Nicolet Magma-IR 550, WI). Degree of Tat-PLGA conjugation was determined by a fluorescamine assay, which allowed the determination of the amount of lysine residues in $Tat_{49\text{-}57}$ peptide. The detailed procedure was described in a previous report (Nam & Park 1999).

Fluorescein-labeled PLGA was also prepared by conjugating fluorescein amine (isomer I, Sigma) to the NHS-activated carboxylic terminal group in PLGA.

Preparation of nanoparticles

Polymer nanoparticles were prepared by a phase inversion method: 100 mg of a 95/5 (w/w) mixture of $Tat_{49\text{-}57}$-conjugated PLGA (or PLGA) and fluorescein-labeled PLGA dissolved in 10 ml acetone was slowly added to 10 ml of PBS containing 0.5% w/v polyvinylalcohol (88% hydrolyzed, M_W 25 K, Polysciences, Inc.) as an emulsifier under vigorous stirring conditions. The size distribution and zeta potential of the prepared nanoparticles were evaluated by using a dynamic laser light scattering (DLS) technique (Zetasizer 3000HS, Malvern, UK). Transmission electron microscopy (TEM) analysis was performed on a JEOL 1010 electron microscope (Akishima, Japan) with negative staining using 0.5% uranyl acetate.

Cell study

HaCaT cells, obtained from Dr Norbert E. Fusenig (Heidelberg, Germany), were cultured in Dulbecco's modified eagle medium (DMEM, Gibco BRL, Gaithersburg, USA) supplemented with 1% (v/v) antibiotics and 10% (v/v) fetal bovine serum. The cells grown in 35 mm ΔT culture dishes (Bioptechs, Butler, USA) were incubated with 1.5–50 mg polymer nanoparticles ml^{-1} at 37 °C in a 5% (v/v) CO_2 humidified incubator. The medium was then removed, and the cells were washed three times with fresh medium. Intracellular translocation of polymer nanoparticles was examined using confocal laser scanning microscopy (CLSM, Radiance 2000/MP, Bio-rad). Cytotoxicity was also determined using an 3-4,5-dimethylthiazol-2-*yl*)-2,5-diphenyl tetrazolium bromide (Sigma) assay and a lactate dehydrogenase (CytoTox 96 kit, Promega, Madison, USA) release assay, following the literature protocols (Mosman 1983, Behl *et al.* 1994).

Results and discussion

Tat-PLGA conjugates were synthesized via a coupling reaction between a maleimide-terminated PLGA and sulfhydryl group-terminated Tat peptide, as described in Figure 1. The FT-IR spectrum of Tat-PLGA conjugates indicates that the Tat-peptide moiety was successfully conjugated to PLGA, where the stretching peak of amide bonds appears in the vicinity of

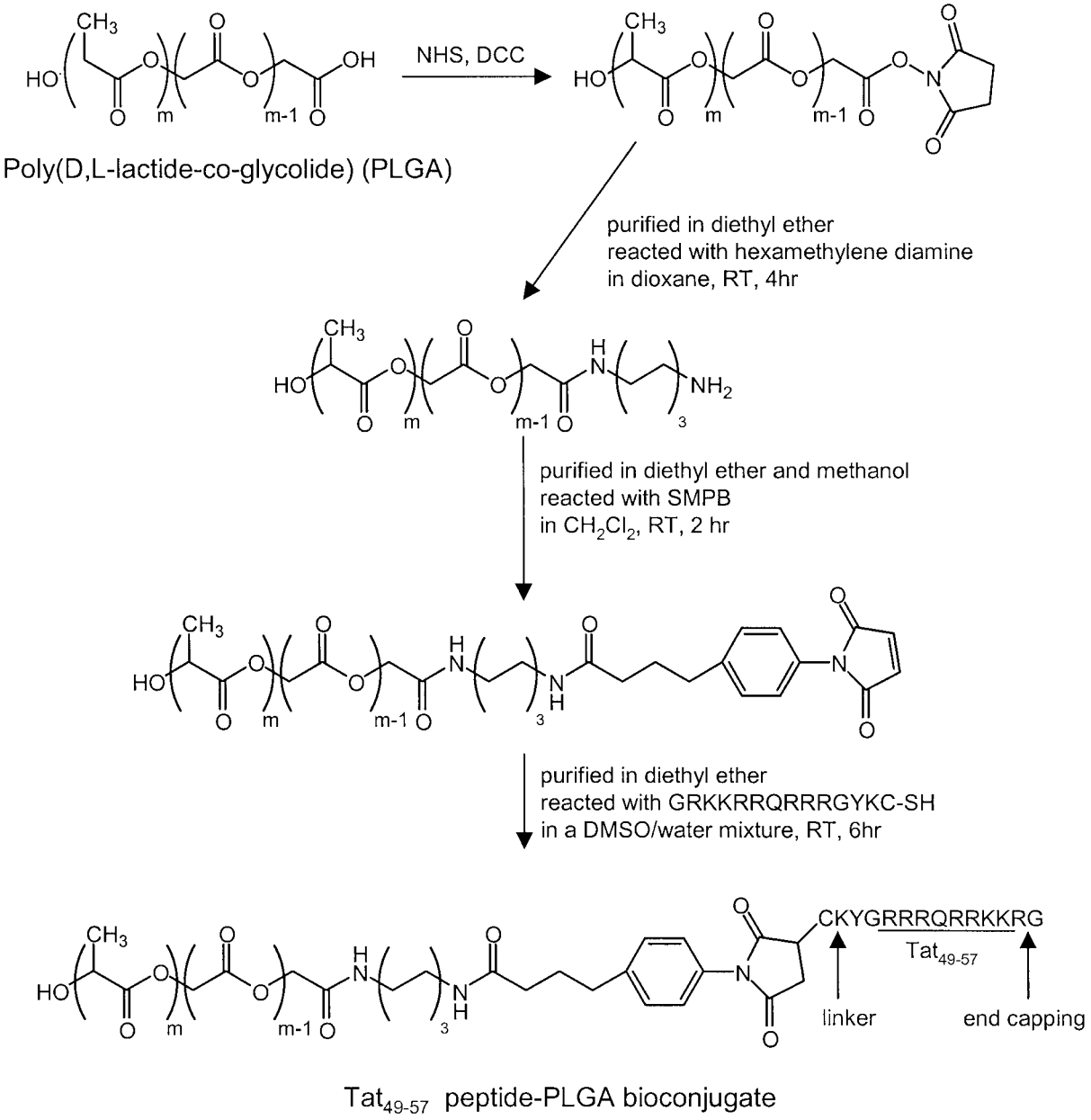

Fig. 1. Synthetic route of Tat-PLGA bioconjugates.

1656 cm^{-1} (Figure 1A). The conjugation degree was about 52%, as determined by a fluorescamine method.

Tat-PLGA nanoparticles were prepared by a spontaneous phase inversion method. Z-average diameter and ζ potential of the nanoparticles was 128 nm and -7.8 mV for the PLGA nanoparticles, and 238 nm and -0.9 mV for the Tat-PLGA nanoparticles, respectively (Figure 2B). The increase of the ζ potential is due likely to the contribution from cationic amino acid residues in Tat peptide. However, it is not clear why the Tat-PLGA nanoparticles have a particle size larger than that of the PLGA nanoparticles. One possible explanation is that the different chemical compositions of Tat-PLGA might have resulted in different internal morphology of the nanoparticles (Zhang & Eisenberg 1995), although the spherical shapes are the same as those of the unconjugated PLGA nanoparticles as observed in Figure 2C.

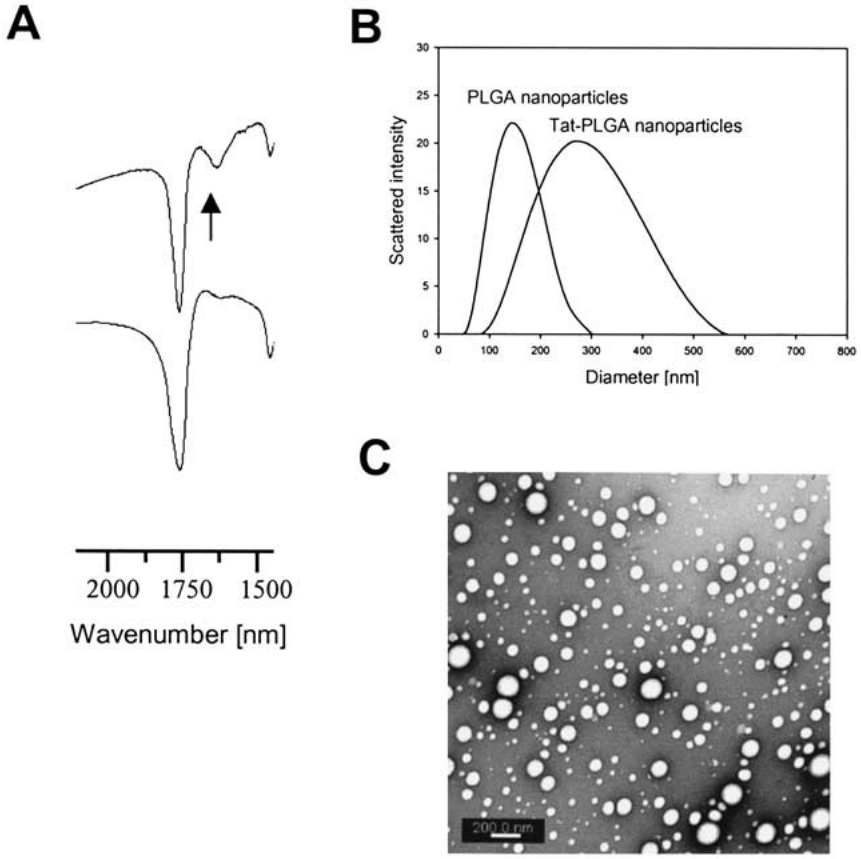

Fig. 2. (A) FT-IR spectra of PLGA and Tat-PLGA conjugates; (B) size distributions of nanoparticles; and (C) TEM image of Tat-PLGA nanoparticles (scale bar = 200 nm).

Toxicity of intracellular carriers is crucial. To deal with this concern, we utilized MTT and LDH assay protocols. Two-day cell incubation with nanoparticles did not show significant toxicity up to 50 μg ml^{-1} (data not shown). This indicates that we can exclude the effects of cell viability on the cellular uptake of particles if the concentration is less than 50 μg ml^{-1}.

Figures 3A and B show the fluorescence images of HaCat cells incubated with nanoparticles. Tat-PLGA nanoparticles (B) were much more effectively adsorbed on the cell membrane and were subsequently delivered into the cell cytoplasm, as compared with plain PLGA nanoparticles (A).

It should be noted that Tat peptide is known as a nuclear localization signal (NLS) for proteins. However, it has not been clear whether or not Tat peptide could also act as an NLS for synthetic polymer nanostructures. To address this issue, the cell nuclei were stained with the DNA-binding dye, propidium iodide (PI), as shown in Figures 3C and D. Subsequently, the locations of green fluorescence from nanoparticles were compared with those of red one from DNA-binding PI by composing two fluorescence images. The composite images (Figures 3E and F) clearly represented that Tat-PLGA nanoparticles accumulated in the cell nuclei, while plain PLGA nanoparticles were hardly observed. Thus, it is inferred that the NLS activity of Tat peptide is still alive on hard particulates.

Liposomes attached to Tat could accumulate in the cell nuclei (Torchilin *et al.* 2001). However, their structural alterations following the intracellular uptake was not considered. Membrane fusion and lipid dissociation/reorganization may happen when liposomes interact with cell membranes (Düzgüne & Nir 1999). This implies that the structural integrity of liposomes can be easily destroyed during the cellular uptake. Thus, we cannot exclude the possibility that the fluorescence found in the nuclei is just a sig-

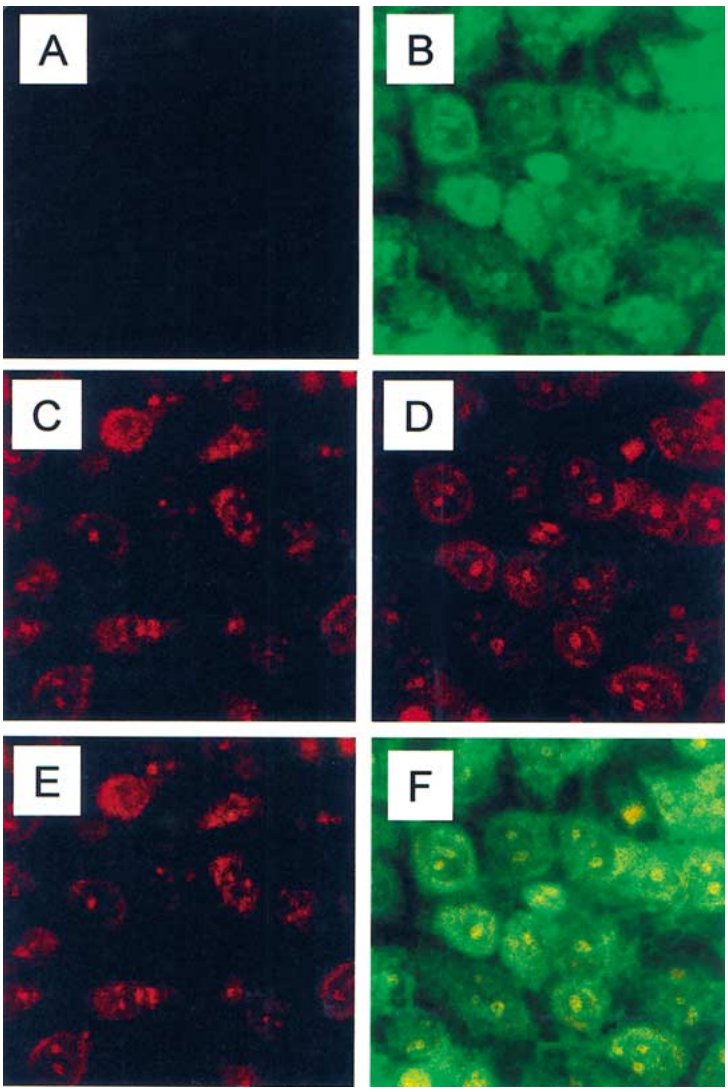

Fig. 3. Cofocal laser scanning microscopy images of HaCaT cells after incubation with PLGA (A, C, and E) and Tat-PLGA nanoparticles (B, D, and F) at 37 °C for 1 h: cells under FITC filter (A and B); cells under PI filter (C and D); composite of superimposed layeres from A and C (E); and composite of superimposed layers from B and D (F) (scale bar = 10 mm).

nal from the dissociated fluorescein-labeled Tat-lipid molecules. This issue remains to be examined further.

As for polymer nanoparticles, their structure is not easily destructed because of chain entanglement and/or high crystallinity (Allen *et al.* 1999). It is likely that the structural maintenance of nanoparticles during the cellular uptake would affect the drug delivery efficiency to the cell nuclei because of different release kinetics of encapsulated drugs (Lavasanifar 2002).

Conclusion

In conclusion, we propose a new nanoparticulate system based on Tat-PLGA bioconjugates for intracellular drug delivery. We demonstrated that Tat-PLGA nanoparticles have superior translocation ability into cells to conventional particles. Moreover, they were localized in cell nuclei presumably due to the NLS property of the $Tat_{49\text{-}57}$ peptide attached. These results imply that Tat-PLGA nanoparticles are particularly attractive for gene therapy, because nuclear targeting of gene vectors is a prerequisite for successful gene expression.

Acknowledgements

We gratefully acknowledge the Ministry of Health and Welfare in South Korea for financial support (the IMT-2000 program, Project No. 01-PJ11-PG9-01NT00-0050). We would like to thank Su Jung Kim (NICEM) for the TEM analysis.

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