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Cellular Uptake Behavior of Poly(D,L-lactide-co-glycolide) Nanoparticles Derivatized with HIV-1 Tat_{49-57} Peptide

(Abbreviated Title: Tat-PLGA Nanoparticles)

Ju Young Park, Yoon Sung Nam†, Junoh Kim, Sang-Hoon Han and Ih-Seop Chang

Nanotechnology Research Team, Amore Pacific R&D Center, 314-1, Bora-ri, Giheung-eup, Yongin-si, Gyeonggi-do, South Korea, 449-729

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ABSTRACT–This work aims at examining the cellular uptake behavior of poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles derivatized with a protein transduction domain (PTD) using HeLa cells. For this purpose, Tat_{49-57} peptide derived from transcriptional activation (Tat) protein of HIV type-1 was covalently conjugated to the terminal end of PLGA. Nanoparticles were then prepared with the Tat_{49-57}-PLGA conjugates by a spontaneous phase inversion method. The prepared particles had a mean diameter of *ca.* 84 nm, as measured by dynamic light scattering. The interaction of the Tat-PLGA nanoparticles with cells was examined by using confocal laser scanning microscopy. It was found that Tat-PLGA nanoparticles incubated with HeLa cells could efficiently translocate into cytoplasm, while plain PLGA nanoparticles showed negligible cellular uptake. In addition, even at 4°C or in the presence of sodium azide significant cellular internalization of Tat-PLGA nanoparticles was still observed. These results indicate that a non-endocytotic translocation mechanism might be involved in the cellular uptake of Tat-PLGA nanoparticles.

Key words–Intracellular delivery, Nanoparticles, Biodegradable polymers, Protein transduction domains (PTD), Tat peptide

Recently, "protein transduction domains" (PTD) or "cell penetrating peptides" (CPP) have been extensively studied as a strategy to improve the cellular uptake of proteins via non-endocytotic pathway.[1-6] Although the precise mechanism has not yet been proved, these peptides seem to temporarily destabilize and reorganize biological lipid membranes through direct interaction.[5] Representative PTDs are *Drosophila* Antennapedia homeotic transcription protein (Antp), herpes simplex virus structural protein VP22, and human immunodeficiency virus 1 (HIV-1) *trans*-activating transcriptional activator (Tat) protein. General structural similarities between PTDs are high contents of arginine residue and the ability to adopt an alpha helical conformation.[6] Among the PTDs, Tat protein from of HIV-1, an 86 amino acid polypeptide involved in the viral replication, has been extensively studied partly due to its high translocational activity. It was also demonstrated that a 9-mer sequence (Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) corresponding to 49-57 amino acids of Tat protein was strictly required but was sufficient to effectively translocate into the cytoplasm.[7]

Apart from intracellular translocation of Tat peptide itself, one interesting feature of this peptide is its ability to mediate cellular uptake of peptides,[8] pathogenic epitopes,[9] proteins,[4] and DNA.[10] Furthermore, Tat peptide has been known to mediate the transcellular delivery of nanostructured materials, such as liposomes and magnetic or polymeric nanoparticles.[11-13] Torchilin *et al.* (2001) reported that Tat peptide-attached liposomes having a diameter of *ca.* 200 nm could effectively translocate inside various cell lines.[11] They could be internalized into the cytoplasm even at a low temperature and in the presence of metabolic inhibitors, implying that Tat peptide mediated cellular uptake of liposomes seems to follow non-endocytotic pathway. Furthermore, fluorescein-dextran encapsulated within Tat modified liposomes could be delivered into the cells, although structural alterations of liposomes (membrane fusion and lipid dissociation/reorganization) following the intracellular uptake were not examined.[11] Tat_{48-57} peptide-derivatized super-paramagnetic iron oxide nanoparticles, known as Tat-CLIO (Tat peptide cross-linked iron oxide), have been investigated as an efficient vehicle of magnetic imaging agents *in vivo*.[12] In addition, it was shown that the surface modification of shell cross-linked (SCK) nanoparticles with Tat peptide enhances the cellular uptake of the nanoparticles *in vitro*.[13] All of these results indicate that Tat peptide can be utilized as a drug delivery enhancer if it is adequately introduced on the surface of polymer nanoparticles, which can be loaded

†본 논문에 관한 문의는 이 저자에게로
Tel : 031)280-5831 E-mail : ysnam@amorepacific.com

with therapeutic molecules.

In this study, 48-57 residues of Tat protein, Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg (GRKKRRQRRR), was chemically conjugated to the end of poly(D,L-lactide-co-glycolide) (PLGA). This sequence corresponds to the minimum PTD segment of Tat protein. Gly-Tyr-Lys-Cys (GYKC) segment was used as a spacer between PLGA and Tat_{48-57} peptide. PLGA was selected because it has a number of advantages over other polymers for drug delivery including biodegradability, biocompatibility, and the FDA approval for human uses.[14] This conjugate was processed into nanoparticles using a spontaneous phase inversion method. Tat-PLGA nanoparticles were incubated with HeLa cells and their cellular uptake was compared with that of plain PLGA nanoparticles using confocal laser scanning microscopy. Their cellular internalization was also observed at 4°C or in the presence of sodium azide.[7, 11]

Experimental

Materials

Poly(D,L-lactide-co-glycolide) (PLGA, lactide:glycolide ratio 50:50, RG502H, M_w 8,600) was supplied from Boehringer Ingelheim (Ingelheim, Germany). This polymer has an uncapped free carboxylic acid group in its terminal end. N-hydroxysuccinimide (NHS), 1,3-dicyclohexylcarbodiimide (DCC), and hexamethylene diamine were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Propidium iodide (PI), formaldehyde solution, and sodium azide were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and 4-(maleimidomethyl)cyclohexanecarboxylic acid N-succinimidyl ester (SMCC) from Fluka Chemie GmbH (Buchs, Switzerland). The peptide including Tat_{48-57} sequence and linker segment (GRKKRRQRRRGYKC-NH_2) was synthesized as previously reported.[15] All other chemicals were of analytical grade.

Conjugation of Tat_{48-57} peptide to PLGA

Tat_{48-57} peptide-PLGA conjugates were prepared via a simple coupling reaction between maleimide-terminated PLGA and thiol-terminated Tat_{48-57} peptide. Briefly, carboxylic acid end group of PLGA was activated to the succinimidyl ester using NHS and DCC, and then converted to primary amine groups by the reaction with an excess amount of hexamethylene diamine. Maleimide-terminated PLGA was then prepared by coupling SMCC to the PLGA-NH_2. Lastly, Tat_{49-57} peptide carrying a sulfhydryl group was dissolved in the Imject® maleimide conjugation buffer (Pierce, Rockford, IL, USA) and then vigorously mixed with the maleimide-terminated PLGA solution in DMSO. The sulfhydryl group of cysteine at N-terminal end of Tat_{49-57} peptide was attached to a maleimide group in PLGA, forming a stable thioether bond. The final product was purified by dialysis (Spectra/Por® membrane 4, M_W cutoff 12-14 kDa, Spectrum, The Netherlands) against excess deionized distilled water, freeze-dried, and stored at −20°C until use. As a control material, cysteine-capped PLGA (Cys-PLGA) was prepared by conjugating cysteine to maleimide-terminated PLGA, following the same procedure. Tat-PLGA conjugate was examined using FT-IR spectroscopy (Nicolet Magma-IR™ 550, WI, USA).

Preparation and Characterization of Nanoparticles

Tat-PLGA nanoparticles were prepared by a spontaneous phase inversion method. 50 mg of Tat-PLGA were dissolved in 10 ml DMSO and mixed with 50 ml deionized distilled water at 40°C under vigorous stirring. One weight percent of fluorescein-PLGA was blended with Tat-PLGA for fluorescence microscopic observation. The dispersion phase contained 0.25% (w/v) cetyltrimethylammonium chloride (CTAC) as an emulsifier, which was largely eliminated by dialysis (M_W cut-off 12 – 14 kDa) against excess water after the particle formation. Size distribution of the prepared nanoparticles was measured by dynamic light scattering with a vertically polarized He-Ne laser (Zetasizer 3000HS, Malvern, UK). Particle morphology was observed using transmission electron microscopy (TEM) on a JEOL 1010 electron microscope (Akishima, Japan). More detailed procedures were described in a previous report.[16]

Cell Study

HeLa (Human cervix adenocarcinoma) was pre-incubated in four well Lab-Tek II chamber slide (Nunc Inc, Naperville, IL) with Dulbeccos modified Eagles medium (DMEM) supplemented with 1% (v/v) antibiotics (streptomycin, 10,000 μg/ml; penicillin, 10,000 IU/ml) and 10% (v/v) FBS (Gibco BRL, Gaitthersburg, MD) at 37°C in a humidified atmosphere containing 5% CO_2. Cells grown to 75% confluency were incubated with 50 μg/ml Tat-PLGA or plain PLGA nanoparticles in serum free culture media for 1 hr. After incubation with the nanoparticles, the cells were washed several times with 10 mM phosphate buffered saline (PBS, pH 7.4, 138 mM NaCl) and then treated with 4% (w/v) formaldehyde in PBS for 10 min. Nucleic acids were then stained with 1 μg/ml PI in PBS for 20 min at room temperature. The cells were washed with excess PBS, and treated with ProLong Antifade solution (Molecular Probes, Eugene, OR, USA). Mounted slides were kept in the dark at 4°C until microscopic observation. The cells were

examined on a confocal laser scanning microscope (CLSM, model LSM 510, Zeiss, Jena, Germany).

Results and Discussion

Tat-PLGA conjugates were synthesized via a coupling reaction between the maleimide-terminated PLGA and the thiol-terminated Tat peptide, as shown in Figure 1. The carboxylic acid end of PLGA was first activated with NHS and DCC, and then conjugated to a primary amine group of hexamethylene diamine. SMCC was then conjugated to the amine-terminated PLGA, in order to prepare the maleimide group-terminated PLGA. Finally, Tat-PLGA conjugates were prepared through a stable thioether formation between the maleimide group of PLGA and the sulfhydryl group of Tat peptide. This conjugation reaction was confirmed by FT-IR analysis. The stretching peak of the amide bond appeared at 1,656 cm^{-1}, indicating the successful conjugation of Tat peptide to PLGA. The conjugation efficiency was estimated about 58%, as determined by a fluorescamine assay, which allowed the determination of the amount of lysine residues in Tat_{49-57} peptide.[17]

Polymer nanoparticles were prepared by a spontaneous phase inversion method. Without interfacial stabilizers, Tat-PLGA conjugates themselves could form nanoparticles having a diameter of *ca.* 400 nm, while PLGA was just immediately precipitated in water. This might be due to the amphiphilic

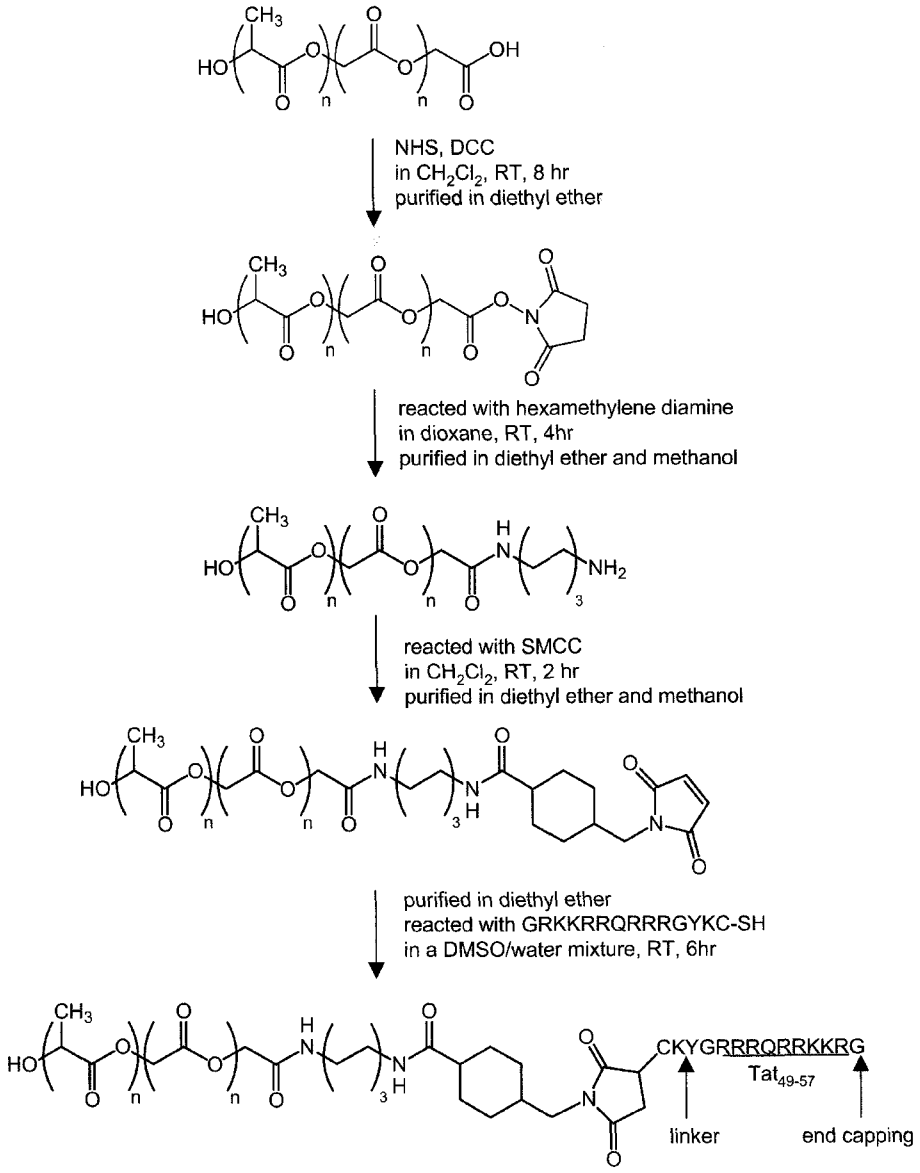

Figure 1–Synthetic scheme of Tat_{48-57} peptide-PLGA conjugate.

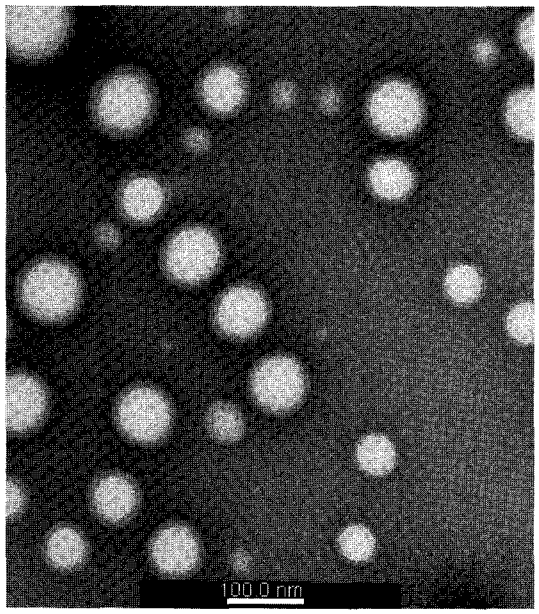

Figure 2–TEM image of the prepared Tat-PLGA nanoparticles.

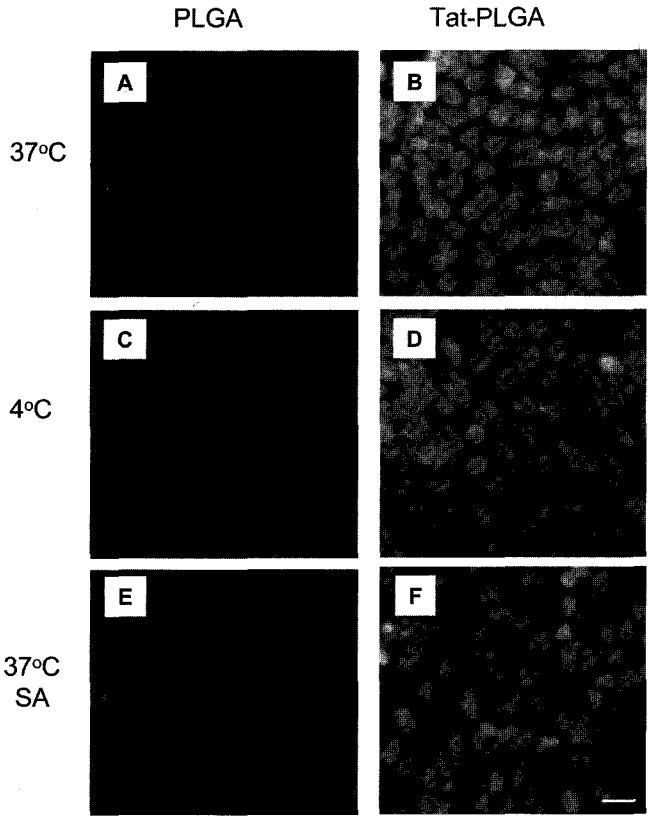

Figure 3–Confocal laser scanning microscopy images of HeLa cells after incubation with PLGA nanoparticles (A, C, and E) and Tat-PLGA nanoparticles (B, D, F) for 1 hr. The cells were incubated at 37°C (A and B), at 4°C (C and D), and at 37°C in the presence of 0.1% sodium azide (E and F) (scale bar=10 μm).

nature of Tat-PLGA conjugates, consisting of hydrophilic Tat peptide and hydrophobic PLGA blocks. However, 0.25% CTAC was used as an emulsifier during particle formation, in order to obtain both Tat-PLGA and PLGA nanoparticles having a similar particle size for cellular uptake study. Hydrodynamic mean diameters of the prepared nanoparticles, as determined by dynamic light scattering, were 88 nm for the plain PLGA nanoparticles and 84 nm for the Tat-PLGA nanoparticles, respectively. TEM image of Tat-PLGA nanoparticles showed a discrete spherical morphology of nanoparticles (Figure 2).

In order to determine the effect of Tat_{48-57} peptide upon the intracellular uptake of PLGA nanoparticles, cellular uptake of Tat-PLGA nanoparticles by HeLa cells was compared with that of plain PLGA nanoparticles. In agreement with our previous studies with HaCat cells,[15] confocal microscopy of Tat-PLGA conjugates after 1-hr incubation with HeLa cells at 37°C showed distinctly strong fluorescence, as shown in Figure 3B. Indeed, optical sectioning showed that the fluorescence was observed throughout the cell as well as in the plasma membrane, indicating that Tat-PLGA nanoparticles were translocated into the cytoplasm (data not shown). In contrast, the cells incubated with plain PLGA nanoparticles exhibited only faint fluorescence intensity in the cellular membranes (Figure 3A).

In general, cellular uptake of nanoparticles can be suppressed either by lowering temperature or by adding metabolic inhibitors because endocytosis, a major mechanism of internalization of particulates into cells, is an energy-dependent process. This is why many studies have been performed at low temperature or in the presence of a metabolic inhibitor, in order to examine the mechanism of intracellular translocations of molecules.[7,11,18,19] Interestingly, Tat-PLGA nanoparticles were efficiently internalized into cytoplasm even at 4°C, as shown in Figure 3D. The presence of a metabolic inhibitor (0.1% sodium azide) had also no significant influence on the intracellular delivery of Tat-PLGA nanoparticles into cytoplasm (Figure 3F). Although the exact mechanism could not be discussed here, it is conceivable that an energy-independent and non-endocytotic process might be involved in the intracellular translocation of Tat-PLGA nanoparticles across the plasma membrane into cells.

Tat peptide is known as a nuclear localization signal (NLS), which is involved in the active transport of exogenous proteins and macromolecules into the cell nucleus.[7,18] To address whether Tat-PLGA nanoparticles are localized in the cell nuclei, confocal microscopy was performed after staining the cell nuclei with a DNA-binding dye, propidium iodide (PI), as shown in Figure 4B. Subsequently, the locations of green flu-

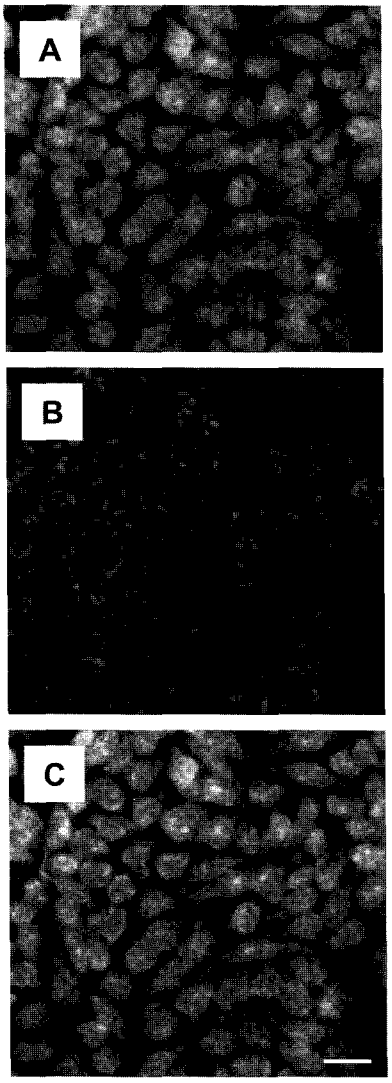

Figure 4–Confocal laser scanning microscopy images of HeLa cells after incubation with Tat-PLGA nanoparticles at 37°C for 1 hr and stained with PI: cells under FITC filter (A); cells under PI filter (B); composite of superimposed layers from A and B (C) (scale bar=10 μm).

orescence from nanoparticles were compared with those of red one from PI by overlapping two fluorescence images (Figure 4C). The composite image shows that faint fluorescence in the nuclear region was associated with areas of PI staining, indicating that considerable amount of Tat-PLGA nanoparticles were located in the nuclear area. Intranuclear translocation of large molecules is known as a selective process through nuclear pore complexes that involves energy dependent carrier proteins.[20,21] However, the size-exclusion limit for particulates that can enter the nucleus by this signal-mediated nuclear transportation was estimated *ca.* 25 nm, which is much smaller than the size of Tat-PLGA nanoparticles (~84 nm in diameter).[22,23] In addition, experimental conditions (a low temperature or a metabolic inhibitor) did not affect the nuclear localization of the nanoparticles, indicating an energy-independent process. This means that the translocation of Tat-PLGA nanoparticles into nuclear region might also be mediated by Tat peptide attached on the surface of the particles.

Conclusion

In conclusion, we have examined here the cellular uptake behavior of Tat-PLGA nanoparticles using HeLa cells. It was found that Tat-PLGA nanoparticles have superior translocation ability into cells to plain PLGA nanoparticles and this activity was not significantly suppressed even at 4°C or in the presence of a metabolic inhibitor. Furthermore, Tat-PLGA nanoparticles were found in cell nuclei possibly due to the NLS property of the Tat_{49-57} peptide attached. These results imply that Tat-PLGA nanoparticles are particularly attractive for intracellular drug delivery, where efficient translocation into cytoplasm and nucleus is a prerequisite for high drug efficacy.

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