Protocol

Synthesis of a non-viral vector for gene transfer via the high-affinity neurotensin receptor

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Abstract

We describe herein a method for synthesizing a non-viral gene vector that exploits the internalization properties of neurotensin (NT), as well as the procedures for a successful gene transfer to cells via the high-affinity NT receptor. The gene vector is NT cross-linked with poly-L-lysine via N-succinimidyl-6-[2-pyridyldithio]propionamido]hexanoate (LC–SPDP). The SPDP-derivatives containing either NT or poly-L-lysine are purified by gel filtration. The non-viral vector resulting from the reaction of NT–SPDP with HS–SPDP–poly-L-lysine is purified on Biogel A-1.5 m. This vector is complexed with plasmid DNA at a specific molar ratio to form the NT–polyplex, which ensures the delivery of the gene of interest to cells under conditions of receptor-mediated internalization. The NT–polyplex has shown ability to mediate transient gene expression in vitro [Brain Res. Mol. Brain Res. 69 (1999) 249] and in vivo [Soc. Neurosci. Abstr. 25 (1999) 67.7]. This approach holds great promise for research and therapy.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Uptake and transporters

Keywords: Neurotensin internalization; Receptor-mediated endocytosis; Gene transfer; Gene therapy; Transgenic animal

1. Type of research

Non-viral vectors for receptor-mediated gene transfer systems are synthesized by cross-linking poly-L-lysine with a ligand for cell-surface receptors that undergo endocytosis [20]. The plasmid DNA is electrostatically bound to the poly-L-lysine moiety of the vector leading to the formation of a complex that is known as polyplex [9]. When the ligand of the polyplex recognizes its cell-surface receptor, the polyplex is internalized via receptor-mediated endocytosis, cotransporting the foreign DNA (polyfection) [9]. Neurotensin (NT) is a suitable ligand for a vector capable of transferring genes to cells via the high-affinity NT receptor (NTRH) [14]. Once NT binds to NTRH, the ligand–receptor complex is endocytosed, and NT is later localized unaltered near the cell nucleus [5]. These evidences suggest that the transport of NT bypasses the lysosomal compartment, the rate-limiting barrier to receptor-mediated systems. Accordingly, NT as polyplex ligand will provide the escape of the cDNA from the endosome during its transport, thus assuring an effective gene transfer to NTRH-expressing cells [14]. In the brain of experimental animals, some putative targets for gene deliver via NTRH are neurons of the mesolimbic and nigrostriatal dopaminergic systems and of the basal forebrain cholinergic system [10]. Polyfection of these neuronal systems can be used to develop strategies for gene therapy of the central nervous system. The neuroblastoma N1E-115 cell line and the human colon cancer adenocarcinoma HT-29 cell line express NTRH [2,3], being thus useful systems for a rapid evaluation of the efficacy of the NT–polyplex.

We report herein the detailed method for the synthesis of the NT–SPDP–poly-L-lysine conjugate (the non-viral vector), guidance to select the optimal molar ratio of the NT–polyplex, and procedures for successful gene transfer to cells via NTRH.
2. Time required

Phase I. Conjugation of neurotensin to poly-l-lysine: 5 days.

- Formation of NT–SPDP and SH–SPDP–poly-l-lysine moieties: 8 h.
- Synthesis of NT–SPDP–poly-l-lysine conjugate: 36 h.
- Purification, concentration and dialysis of the NT–SPDP–poly-l-lysine conjugate: 48 h.
- Determination of poly-l-lysine content in the conjugate by spectrophotometry: 1 h.

Phase II. Determination of the optimal molar ratio of DNA: NT–SPDP–poly-l-lysine conjugate: 3 h.

- Formation of the NT–polypelex: 1 h.
- Agarose gel electrophoresis and DNA detection by UV transillumination: 2 h.

Phase III. Internalization assay: 3 h.

- Formation of NT–polypelex and fluorescent labeling of DNA with propidium iodine: 1 h.
- Cell incubation with propidium iodine-labeled NT–polypelex, fixation and mounting for confocal microscopy analysis: 2 h.

Phase IV. Expression assay: 51 h.

- Formation of NT–polypelex: 1 h.
- Cell incubation with NT–polypelex: 12 h.
- Additional incubation in serum-supplemented medium: 36 h.
- Cell fixation and mounting for confocal microscopy analysis: 2 h.

3. Materials

3.1. Special equipment

Econo-Pac 10DG column, Sephadex G-10 (22×1 cm) column and Biogel A-1.5 m (45×1.5 cm) column, electrophoresis power supply model 1000/500, horizontal minigel apparatus (Bio-Rad Laboratories; Richmond, CA). Spectrophotometer DU 650 (Beckman Instruments Inc.; Palo Alto, CA), vacuum concentrator Heto (ATR; Laure, MD), cell model 12 for ultrafiltration under nitrogen atmosphere and membrane 25, PM 10 (Amicon Corporation; Lexington, MA), Eagle Eye II (Stratagene; La Jolla, CA), vortex Genie 2 (Scientific Industries Inc.; Bohemia, NY), Sorval RMC14 microcentrifuge (Dupont; Newtown, CT). Biological safety cabinet and CO2 incubator (Nuaire Inc.; Plymouth, MN) and confocal imaging system equipped with a krypton–argon laser beam (Bio-Rad MRC-600; Watford, UK).

3.2. Chemicals and reagents

Neurotensin, poly-l-lysine hydrochloride (25 000 Da), dimethyl sulfoxide, EDTA disodium salt, ethidium bromide, propidium iodine, agarose, guanidine and molecular-weight markers (dextran blue, β-amylase, bovine serum albumin, horse cytochrome C and bromophenol blue) were purchased from Sigma Chemical Co. (St. Louis, MO). LC–SPDP and dithiothreitol (DTT) were from Pierce Chemical Co. (Rockford, IL). Dialysis membrane (10 000 Da cut off), pGreen Lantern™-1, sodium bicarbonate, Hepes, Dulbecco’s Modified Eagle Medium, fetal bovine serum and antibiotic–antimycotic solutions were obtained from GIBCO-BRL (Grand Island, NY). Vectashield was purchased from Vector Laboratories, Inc. (Burlingame, CA) and calcein AM was from Molecular Probes (Eugene, OR). Other chemicals were of analytical reagent grade quality obtained from usual commercial sources. Four-well culture plates Nunc lon (Cat. #176740) were purchased from Nunc, Inc. (Naperville, IL), 10×10 mm cover glass from Thomas Scientific (Swedesboro, NJ), and 0.22 μm filter units Millex-GV from Millipore (Bedford, MA).

Column PBS (17.42 mM Na2HPO4, 2.58 mM KH2PO4, 150 mM NaCl, 1.0 mM EDTA, 0.02% sodium azide, pH 7.2).

Cell PBS (8.1 mM Na2HPO4, 1.2 mM KH2PO4, 138 mM NaCl, 2.7 mM KCl, pH 7.4).

TAE buffer (0.04 M Tris–acetate and 0.001 M EDTA, pH 8.0).

6× Gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 15% Ficoll Type 400, Pharmacia, in water).

4. Detailed procedure

4.1. Phase I: Conjugation of neurotensin to poly-l-lysine

4.1.1. Theoretical considerations

Since free NH2 groups are involved in DNA binding, the extent of NT cross-linked to NH2 groups of poly-l-lysine should be minimal but sufficient to activate NTRH-mediated endocytosis. NT has four NH2 groups per molecule, while poly-l-lysine has 195, which can react with SPDP. The chemistry of cross-linking of NT with poly-l-lysine at 5/1 ratio is presented in Fig. 1. Based on the assumption that the reaction is 100% efficient, an average of five molecules of NT would cross-link with one molecule of poly-l-lysine.

4.1.2. Formation of SH–SPDP–poly-l-lysine moiety

- Dissolve 10 mg of poly-l-lysine (25 000 Da) in 970 μl
Fig. 1. Conjugation of neurotensin to poly-L-lysine using the cross-linker LC-SPDP. The reaction has three steps: (A) Conjugation of LC-SPDP to poly-L-lysine and reduction with DTT. (B) Conjugation of LC-SPDP to neurotensin. (C) Cross-linking of neurotensin-SPDP to SH-SPDP-poly-L-lysine. The broad arrows indicate the site of the reaction. $E_{435} = 8.08 \times 10^3$ M$^{-1}$cm$^{-1}$.

- Dissolve 1.7 mg of LC-SPDP (425.5 Da) in 30 μl of DMSO (the concentration of LC-SPDP is 133.2 mM). Transfer the complete volume to the tube containing poly-L-lysine at once, and mix vigorously to avoid precipitation. Final concentrations are 4 mM LC-SPDP and 0.4 mM poly-L-lysine.
- Stir the reaction mixture for 30 min at room temperature in the dark (SPDP is light sensitive).
- To purify SPDP-poly-L-lysine, apply the sample to an Econo-Pac 10 DG column previously equilibrated with
column PBS at room temperature, and collect 17 1-ml fractions.

• Transfer 100 µl of each fraction to 0.5-ml tubes. Add 200 µl of column PBS, mix well, and read absorbance simultaneously at 215 and 280 nm. Obtain the chromatogram by plotting absorbance versus eluent volume (Fig. 2).

• Pool the first peak (generally 5 ml) that elutes in fractions 3 to 7 (Fig. 2), and reduce the volume to 1 ml in a vacuum concentrator (Heto). The first peak contains the poly-t-lysine–SPDP moiety.

• When 1 ml is achieved, immediately add 0.5 ml of 156 mM DTT (12 mg DTT in 0.5 ml of column PBS) to reduce SPDP–poly-t-lysine to SH–SPDP–poly-t-lysine.

• Stir the mixture for 30 min at room temperature.

• After incubation, dilute 20 µl of the reaction mixture with 180 µl of column PBS (10× dilution), and read the absorbance at 343 nm. To calculate the reaction efficiency, use the equation:

\[
C = \left( \frac{\text{Abs}_{343\,\text{nm}}}{E_{343\,\text{nm}}} \right) \text{D.F.}
\]

Where \( C \) is the concentration of pyridine-2-thione, \( \text{Abs}_{343\,\text{nm}} \) is the absorbance of pyridine-2-thione at 343 nm, \( E_{343\,\text{nm}} \) is the molar extinction coefficient of pyridine-2-thione at 343 nm whose value is \( 8.08 \times 10^3 \,\text{cm}^{-1}\,\text{M}^{-1} \), and D.F. is the dilution factor. Given an \( \text{Abs}_{343\,\text{nm}} = 1.21 \):

\[
C = (1.21 \,\text{cm}^{-1}/8.08 \times 10^3 \,\text{cm}^{-1}\,\text{M}^{-1}) \times 10^{-3} \,\text{M}
\]

Considering 4 mM as the initial concentration of LC–SPDP, the direct source of pyridine-2-thione (see Fig. 1A), the reaction efficiency will thus be 1.497 mM/4 mM×100% = 37.4%.

• To purify the SH–SPDP–poly-t-lysine moiety, apply the sample to an Econo-Pac 10 DG column equilibrated with column PBS at room temperature, and collect 17 1-ml fractions.

• Transfer 100 µl of each fraction to 0.5-ml tubes. Add 200 µl of column PBS into each tube, mix well, and read absorbance simultaneously at 215, 280 and 343 nm. Obtain the chromatogram by plotting absorbance versus eluent volume (Fig. 3).

• Pool the first peak (generally 5 ml) that elutes in fractions 3 to 7 (Fig. 3), and reduce the volume to 1 ml in a vacuum concentrator (Heto). The first peak contains the SH–SPDP–poly-t-lysine moiety.

4.1.3. Formation of the NT–SPDP moiety

• In a parallel assay, dissolve 3.34 mg of NT (1673 Da) in 970 µl of column PBS. The NT concentration is 2.06 mM.

• Dissolve 1.7 mg of LC–SPDP (425.5 Da) in 30 µl of DMSO (the concentration of LC–SPDP is 133.2 mM). Transfer the complete volume to the tube containing neurotensin at once, and mix vigorously to avoid precipitation. Final concentrations are 4 mM LC–SPDP and 2 mM neurotensin.

• Stir the mixture of neurotensin and LC–SPDP for 30 min at room temperature in the dark.

• To purify the SPDP–neurotensin moiety, apply the sample to a Sephadex G-10 column equilibrated with column PBS at room temperature, and collect 22 0.5-ml fractions.

• Transfer 100 µl of each fraction to 0.5-ml tubes. Add 200 µl of column PBS into each tube, mix well, and read absorbance simultaneously at 215 and 280 nm. Obtain the chromatogram by plotting absorbance versus eluent volume (Fig. 4).

• Pool the first peak containing the NT–SPDP moiety.
the absorbance at 343 nm. Calculate the reaction efficiency according to the equation:

\[ C = \frac{(\text{Abs}_{343\text{ nm}})}{(E_{343\text{ nm}})} \times \text{D.F.} \]

Where \( C \) is the concentration of pyridine-2-thione, \( \text{Abs}_{343\text{ nm}} \) is the absorbance of pyridine-2-thione at 343 nm, \( E_{343\text{ nm}} \) is the molar extinction coefficient of pyridine-2-thione at 343 nm \( (8.08 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}) \), and D.F. is the dilution factor. Given an \( \text{Abs}_{343\text{ nm}} = 0.924 \)

\[ C = \frac{(0.924 \text{ cm}^{-1}) \times (8.08 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1})}{10} \]

\[ C = 1.14 \times 10^{-4} \text{ M} \]

Considering 4 mM as the initial concentration of LC-SPDP, the direct source of pyridine-2-thione (see Fig. 1A), the reaction efficiency will thus be 1.14 mM/4 mM \times 100\% = 28.5\%.

- To purify the NT-SPDP-poly-\( \ell \)-lysine conjugate, apply the sample to a Biogel A-1.5 m column equilibrated with 2 M guanidine in 10 mM Heps buffer, pH 7.4. Collect 100 1-ml fractions.
- Transfer 100 \( \mu \)l of each fraction to 0.5-ml tubes. Add 200 \( \mu \)l MilliQ water into each tube, mix well, and read absorbance simultaneously at 215, 280 and 343 nm. Obtain the purification chromatogram considering the three wavelengths (Fig. 5A).
- Pool the peak 1 (generally 21 ml) corresponding to fractions 40 to 60 (Fig. 5A), which contain NT-SPDP-poly-\( \ell \)-lysine, and reduce the volume to 1 ml under \( \text{N}_2 \) atmosphere using the ultrafiltration cell model 12 with a 25, PM 10 membrane (Amicon).
- Dialyze the NT-SPDP-poly-\( \ell \)-lysine conjugate against 1 l of cell PBS for at least 4 h. Repeat the

Fig. 4. Purification of neurotensin-SPDP moiety on Sephadex G10. The mobile phase is column PBS, pH 7.2. The collected fractions are equivalent to 0.5 ml of eluent. The numbers show the elution peaks.

that elutes in fractions 10 to 14 (5 to 7 ml, Fig. 4), and reduce the volume to 1 ml in a vacuum concentrator.

4.1.4. **Synthesis of NT-SPDP-poly-\( \ell \)-lysine conjugate (the gene vector)**

- Mix the NT-SPDP moiety with the SH-SPDP-poly-\( \ell \)-lysine moiety under vigorous stirring to avoid precipitation.
- Stir the reaction mixture for 36 h at room temperature in the dark.
- After incubation, dilute 20 \( \mu \)l of the reaction mixture with 180 \( \mu \)l of column PBS (10 \times dilution) and read

Fig. 5. Purification of neurotensin-SPDP-poly-\( \ell \)-lysine conjugate (the non-viral vector) on Biogel A-1.5 m (Panel A). The mobile phase is 2 M guanidine in 10 mM Heps buffer, pH 7.4. The collected fractions are equivalent to 1 ml of eluent. The numbers show the elution peaks. Panel B is the calibration curve obtained with five molecular-weight markers: Dextran Blue (MW 2000 KDa), \( \beta \)-Amylase (MW 200 000 Da), Bovine Serum Albumin (MW 66 000 Da), Horse Cytochrome C (MW 12 000 Da) and Bromophenol Blue (MW 692 Da). \( V_e = \) elution volume; \( V_0 = \) the void volume corresponding to Blue Dextran elution volume.
dialysis procedure four times using freshly-prepared cell PBS.

- Sterilize the NT–SPDP–poly-l-lysine conjugate using a 0.22-µm filter (Millex-GV), and distribute it in 0.1-ml aliquots. Store the aliquots at −70°C for up to 1 year.

4.1.5. Determining the molecular-weight of the NT–SPDP–poly-l-lysine conjugate

- Calibrate the Biogel A-1.5 m column with five molecular-weight markers (Fig. 5B). The mobile phase is 2 M guanidine in 10 mM Hepes buffer, pH 7.4.
- Dissolve all the molecular-weight markers in 1 ml of mobile phase as follows: Blue dextran MW= 2,000,000 (Peak #1), 0.5 mg. β-Amylase MW= 200,000 (Peak #2), 1.0 mg. Bovine serum albumin MW=66,000 (Peak #3), 1.0 mg. Horse cytochrome C MW=12,000 (Peak #4), 0.5 mg. Bromophenol blue MW=692 (Peak #5), 0.1 mg.
- Apply the 1-ml standard mixture to a Biogel A-1.5 m column equilibrated with the mobile phase. Collect 100 1-ml fractions and read absorbances at 280 nm.
- Obtain the calibration chromatogram by plotting absorbance versus eluent volume, and the calibration curve by plotting the logarithms of the known molecular-weights of protein standards versus the respective $V_e/V_o$ values (Fig. 5B). $V_e$ is the elution volume and $V_o$ is the void volume corresponding to blue dextran elution volume.
- Extrapolate the ratio $V_e/V_o$ of the NT–SPDP–poly-L-lysine conjugate (Fig. 5A, peak 1) in the calibration curve to calculate its mean molecular-weight.

4.1.6. Determination of NT–SPDP–poly-l-lysine conjugate concentration

- Obtain the standard curve of poly-L-lysine (25,000 Da) by plotting absorbance at 215 nm versus concentration (from 0.1 to 1.0 mg/ml cell PBS).
- Aliquot 15, 20, and 30 µl of the NT–SPDP–poly-L-lysine conjugate into separate 0.5-ml tubes, and add cell PBS to bring the volume to 300 µl (dilutions 1:20, 1:15 and 1:10, respectively). Read the absorbance of the diluted samples at 215 nm, and extrapolate the values in the standard curve to calculate the concentration of poly-L-lysine. Correct by the dilution factor to obtain the concentration of poly-L-lysine in the conjugate (mg/ml).
- To obtain the molar concentration of poly-L-lysine in the conjugate, divide the concentration value in mg/ml by the mean molecular-weight value of the conjugate.

4.2. Phase II. Determination of the optimal molar ratio of DNA: NT–SPDP–poly-l-lysine

4.2.1. Retardation microassay

- Prepare nine 2-µl dilutions of the NT–SPDP–poly-L-lysine conjugate with serum-free culture medium corresponding to the following molar concentrations: 486, 540, 594, 648, 702, 756, 810, 864 and 918 nM. Mark the tubes from #2 to #10 according to the increasing concentrations.
- In 10 separate 500-µl tubes, dispense 4 µl of 9 nM plasmid DNA. Mark the tubes from #1 to #10.
- Add 2 µl of serum-free culture medium to tube #1, and 2 µl of each dilution of the NT–SPDP–poly-L-lysine conjugate to the corresponding tube containing the plasmid DNA (pGreen Lantern™-1). The final concentration of DNA is 6 nM and that of the NT–SPDP–poly-L-lysine conjugate is 0, 162, 180, 198, 216, 234, 252, 270, 288 and 306 nM, respectively. The molar ratio of DNA versus NT–SPDP–poly-L-lysine conjugate will thus be 1:0, 1:27, 1:30, 1:33, 1:36, 1:39, 1:42, 1:45, 1:48 and 1:51, respectively (Fig. 6).
• Stir the tubes for 30 min at room temperature to allow formation of the NT–polyplex.
• After incubation, add 1.2 μl of 6× loading buffer to each tube, vortex, and gather dispersed drops by centrifugation in a microcentrifuge (3000 rpm for 10 s).
• Transfer the complete volume of each ratio to the respective well of 0.8% agarose gel. Electrophorese the samples in a horizontal minigel apparatus using TAE buffer (80 V, 2 h). Stain with ethidium bromide (0.5 μg/ml), and examine the gel with a UV transillumination apparatus (Eagle Eye II) to analyze DNA migration. Fig. 6 shows that the optimal molar ratio is in the range 1:36 to 1:42.

4.2.2. Preparing 400 μl of NT–polyplex at molar ratio 1:36 DNA: NT–SPDP–poly-l-lysine for internalization and expression assays

• Dilute 13 μl of 6.7 μM NT–SPDP–poly-l-lysine conjugate with 107 μl of serum-free medium. NT–SPDP–poly-l-lysine concentration is 726 nM.
• Dilute 0.8 μl of 3 μM pGreen Lantern™-1 with 280 μl of serum-free medium. DNA concentration is 8.5 nM.
• To form the NT–polyplex, add drop by drop the complete 120-μl volume of NT–polyplex to 280-μl volume of DNA solution shaking manually after each addition. Finally, shake the mixture for at least 30 min at room temperature (final concentrations of DNA and NT–SPDP–poly-l-lysine conjugate are 6 and 217 nM, respectively; molar ratio 1:36).
• Electrophorese a 6-μl aliquot of the NT–polyplex and a sample of a similar concentration of plasmid DNA in 0.8% agarose gel (TAE buffer, 80 V, 2 h). Stain with etihdium bromide (0.5 μg/ml), examine the gel with a UV transillumination apparatus (Eagle Eye II), and compare the DNA migration with that obtained in the retention microassay (Fig. 6).
• Use the NT–polyplex for expression assays in cell cultures as described in Section 4.4.
• For internalization assays, label the plasmid DNA of the NT–polyplex with a fluorescent dye 10 min before its use. For this purpose, add 4 μl of 1 mM propidium iodine dissolved in serum-free culture medium to 400 μl of NT–polyplex (the final concentration of propidium iodine is 10 μM). Mix well for 10 min in the dark (propidium iodine is light sensitive), and add it to the cell cultures as described in Section 4.3.

4.3. Phase III. Internalization of propidium iodine-labeled NT–polyplex in NTRH-bearing cells

• Place a sterile 10×10 mm cover glass into each well of 4-well plates (Nunclon).
• Add 300 μl of 0.2% collagen (common gelatin prepared in MilliQ water) into each well. After 5 min, remove the collagen solution and let wells dry in a tissue culture biological cabinet.
• Seed NTRH-bearing cells at 70% confluence on cover glasses placed into multiwell culture plates. The culture medium is supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution.
• After a 20-h incubation, remove the culture medium and add 400 μl of 5 μM of calcein AM, a fluorescent marker of cell viability. Calcein must be dissolved in culture medium and be used at once. The removal and addition of medium must be performed carefully to prevent cells from detaching.
• After a 20-min incubation, remove the calcein solution and immediately add the 400-μl volume of propidium iodine-labeled NT–polyplex. Incubate the cells for different times to study the temporal course of internalization.
• Upon completion of incubations, remove the culture medium, wash three times with cell PBS, and fix cells with either 4% paraformaldehyde at room temperature for 30 min or methanol (absolute grade) at −20°C for 5 min.
• Remove the cover glasses from the multiwell culture plates and mount them on a microscope slide using Vectashield.
• Scan the fixed cells in a confocal imaging system equipped with a krypton–argon laser beam (Bio-Rad MRC-600, Watford, UK). Detect the fluorescence of propidium iodine and calcein present inside the cells with a 60× oil-immersion objective at excitation/emission wavelengths of 568/585 nm (red channel) and 488/522 nm (green channel).

4.4. Phase IV. Transient expression of pGreen Lantern™-1 polyfected in NTRH-bearing cells

• Seed NTRH-bearing cells at 40% confluence on cover glasses placed into culture plates as described in Section 4.3.
• After a 24-h incubation, remove culture medium and immediately add 400 μl of the NT–polyplex prepared in serum-free medium. The removal and addition of medium must be performed carefully to prevent cells from detaching.
• After 2 h, add 44 μl of fetal bovine serum to the NT–polyplex solution to yield a final concentration of 10%.
• After a 12-h incubation, remove the NT–polyplex solution, wash once with cell PBS, and immediately add medium supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution.
• Forty-eight hours after the addition of the NT–polyplex, carefully wash cells three times with cell PBS, and fix them with either 4% paraformaldehyde at room temperature for 30 min or methanol (absolute grade) at −20°C for 5 min.
• After fixation, wash cells three times with cell PBS, and add 200 μl of 1 μM propidium iodine dissolved in cell PBS. After 2 min, aspirate the propidium iodine and wash three times with cell PBS.
• Remove the cover glasses from the multwell culture plate and mount them on a microscope slide using Vectashield.
• Observe the fluorescence inside the cells with a confocal microscope system at excitation/emission wavelengths of 488/522 nm (green channel) for the green fluorescent protein, and 568/585 nm (red channel) for propidium iodine.

5. Results

5.1. Synthesis of NT–SPDP–poly-L-lysine (the non-viral vector)

The purification of SPDP–poly-L-lysine on Econo-Pac 10 DG column shows two peaks at both 215 and 280 nm (Fig. 2). High molecular-weight compounds (>6000 Da) elute in the first peak, while low molecular-weight compounds elute in the second peak (<6000 Da). Accordingly, the SPDP–poly-L-lysine moiety elutes in the first peak (fractions 3–7). The presence of absorbance at 280 nm in the first peak is evident of SPDP conjugation to poly-L-lysine, since the latter molecule does not absorb at that wavelength. Low molecular-weight compounds such as free SPDP and N-hydroxysuccinimide released from the reaction elute in the second peak (fractions 7–14).

Fractions 3–7, containing the SPDP–poly-L-lysine moiety, were concentrated and incubated with DTT to expose the highly reactive sulfhydryl group of SPDP. The conjugation efficiency was 37.4% as estimated by the concentration of pyridine-2-thione released in the reaction. The chromatogram of purification on Biogel A-1.5 m shows three peaks at 215 nm and only one peak at 343 nm, the latter corresponding to pyridine-2-thione (Fig. 5A). According to the calibration curve, the first peak (30 000 Da, mean MW) at 215 nm corresponds to NT–SPDP–poly-L-lysine conjugate, and the second one to free NT–SPDP (Fig. 5A). The fractions 40–50 containing compounds from 30 000 to 120 000 Da were pooled, concentrated in the Amicon cell under N₂ atmosphere, and tested in internalization and expression assays. The concentration of this conjugate based on the poly-L-lysine moiety was 0.5 mg/ml corresponding to 6.7 μM (75 000 Da, mean MW).

5.2. Determination of the optimal molar ratio DNA: NT–SPDP–poly-L-lysine conjugate

Cross-linking of NT with poly-L-lysine by means of SPDP resulted in conjugates capable of binding DNA as shown by the retention microassay (Fig. 6). Electrophoresis of NT–polyplexes formed at different molar ratios (DNA versus NT–SPDP–poly-L-lysine conjugate) showed a different migration pattern in 0.8% agarose gel (Fig. 6). DNA migration was gradually retarded in the respective wells up to the point of not entering the gel when increasing the NT–SPDP–poly-L-lysine conjugate concentration. The optimal molar ratio of DNA: NT–SPDP–poly-L-lysine conjugate was in the range from 1:36 to 1:42 as confirmed by the expression assays.

5.3. NT–polyfection of N1E-115 cells

N1E-115 cells stained with calcine (Fig. 7A) and exposed to propidium iodine-labeled NT–polyplex for 30 min show red marks inside the cells (Fig. 7B). On the contrary, calcine-stained cells (Fig. 7C) incubated with propidium iodine-labeled NT–polyplex for 30 min in the presence of 0.45 M sucrose show propidium iodine marks on the cell perimeter (Fig. 7D). These results demonstrate the mediation of endocytosis in plasmid DNA uptake by NTRH-expressing cells.

In agreement with the internalization results, Fig. 8 shows the expression of the gene encoding the green fluorescent protein in N1E-115 cells (Panel A). The
**Fig. 7.** Internalization of NT–polyplex by receptor-mediated endocytosis. Panels A and C show the fluorescence of calcein observed at Ex/Em wavelengths of 488/522 nm (green channel). Panels B and D show the fluorescence of propidium iodine-labeled NT–polyplex at Ex/Em wavelengths of 568/585 nm (red channel). Internalization of NT–polyplex in N1E-115 cells is shown in Panels A and B. Endocytosis blockade with 0.45 M sucrose in N1E-115 cells is shown in Panels C and D. All microphotographs are horizontal sections through cell nuclei obtained by a confocal imaging system. In all cases, the scale bars correspond to 20 μm.

Counterstaining with propidium iodine after cell fixation reveals the cell population (Panel B) in the same field where the expression was observed, showing a NT–polyfection efficiency of 6.5±1.5%.

**6. Discussion**

**6.1. Troubleshooting**

The synthesis of NT–polyplex is a simple, low-cost and reproducible procedure. However, the NT–polyplex must be formed at an optimal molar ratio between DNA and NT–SPDP–poly-lysine to obtain successful and consistent results in internalization and expression assays. Only the NT–polyplex formed at the optimal molar ratio keeps the internalization properties of NT, being able to endocytose and transport cDNA towards the cell nucleus [14]. Our results have shown that the retardation microassay is a fundamental, practical and rapid procedure to determine the optimal molar ratio [13,14]. Following the method for NT–SPDP–poly-lysine synthesis and the procedure to form the NT–polyplex at optimal molar ratio, both issues described herein, we have reported successful polyfection of reporter genes in vitro [14] and in vivo [1].

A wide range of molecular-weight conjugates is obtained when bifunctional cross-linkers (SPDP), poly-lysine (a polydisperse compound) and polyvalent ligands (–NH₂>2) are used [13,15]. This heterogeneity is due to the lack of control over the location of the cross-linking. It
has been reported that high molecular-weight conjugates (>500 000 Da) resulting from the cross-linking of some molecules of poly-l-lysine through ligand bridges are inefficient for gene transfer [13]. On the contrary, low molecular-weight conjugates (30 000–100 000 Da) have provided successful results in vitro [11,13–15] and in vivo [1]. Monovalent and low molecular-weight ligands at adequate proportion of conjugation to poly-l-lysine are factors that have improved gene transfer [11,13,15]. Based on these considerations, NT was selected as the targeting molecule because of its low molecular-weight (1600 Da), and the reaction was designed to conjugate about five molecules of NT per molecule of poly-l-lysine. The mean molecular-weight of NT–SPDP–poly-l-lysine conjugate calculated from the standard curve (30 000) was consistent with the theoretical molecular-weight (37 000). However, the molecular-weight of the conjugate varied in a range from 20 000 to 100 000 Da. This dispersion is typical of poly-l-lysine itself and of the SPDP derivatives [13,14].

A critical step in the process of conjugation with SPDP is to avoid partial precipitation of the reagents at the beginning of the reaction. This fact can change the conjugation ratio of neurotensin to poly-l-lysine, resulting in unpredictable mixtures of conjugates. A quick mixing and a strong shaking usually avoid precipitation. Another factor that reduces the efficiency of conjugation is the incomplete elimination of DTT from the SH–SPDP–poly-l-lysine solution. DTT, present in the final step of the conjugation, will compete with SH–SPDP–poly-l-lysine for the reduction of the NT–SPDP moiety, thus decreasing the conjugation efficiency. After chromatography, a 4-h dialysis of the sample containing SH–SPDP–poly-l-lysine against column PBS will provide complete elimination of DTT.

Since NT–SPDP–poly-l-lysine conjugates lack aromatic groups that absorb at 280 nm, monitoring at 215 nm is an alternative to detect the conjugate elution. At this wavelength, however, guanidine interferes with absorbance readings of the NT–SPDP–poly-l-lysine conjugate. Besides, the high concentration (2 M) of guanidine in the mobile phase saturates the detection capacity of some spectrophotometers. A 1/3 dilution of the samples with MilliQ water usually helps determine the elution pattern of the conjugate.

SPDP and its derivatives are sensitive to temperature, moisture and light [4,8]. However, NT–SPDP–poly-l-lysine conjugates stored at −70°C in 100-μl aliquots have proved effective and reliable for gene transfer up to 1 year. Thus, it is best to thaw one aliquot once and to store the remaining solution in 10-μl aliquots at −70°C only for one use. Freezing and thawing denaturalize the non-viral vector, thus diminishing the activity of the NT–polyplex.

Readings at 343 nm after both the DTT reduction and the conjugation reaction of NT–SPDP to SH–SPDP–poly-l-lysine are important steps to confirm that the reaction took place.

The molar ratio between DNA and the vector can be theoretically figured out taking into account the complete positive charges of NT–SPDP–poly-l-lysine and negative charges of DNA [9,12,18]. However, this theoretical
procedure requires experimental confirmation. The retention microassay is a practical and rapid procedure to determine the molar ratio [1,13,14]. Before each internalization and expression assay, it is recommended to confirm the optimal molar ratio of the NT–polyplex that will be used, since an improper molar ratio will yield unsuccessful results. NT–polyplex formed at molar ratios between 1:36 and 1:42 proved the most efficient for polyfection [1,14]. At ratios over 1:45, the resulting complexes were insoluble and unable to enter the agarose gel (Fig. 6), and consequently they failed for polyfection.

External factors that influence the gene transfer effectiveness of the NT–polyplex in vitro concern the intrinsic properties of cell lines. For instance, N1E-115 cells should be kept in the log phase of growth for at least 20 days in order to express functional NTRHs [7].

6.2. Comparison with alternative approaches

The limiting factor for in vivo receptor-mediated gene transfer seems to be the degradation of the expression vectors in the lysosomal compartment. In order to avoid the lysosomal degradation, diverse approaches have been successfully used together with receptor-mediated gene transfer systems. Examples are hepatectomy-induced liver regeneration after the injection of the asialoglycoprotein–polyplex [21], whole adenovirus to induce disruption of DNA-containing endosomes [6,19], and chloroquine to neutralize the acidic pH of lysosomes [16]. Experimental evidence has suggested that NT avoids the lysosomal compartment during its transport by endosomes [5,14], and this NT characteristic can account for the ability of the NT–polyplex for gene transfer in vitro [14] and in vivo [1].

In adult rat brain, we have shown that NT–polyfection of substantia nigra pars compacta cells resulted in transgene expression in dopaminergic neurons for up to 15 days [1]. The use of tissue-specific promoters in the plasmid might prolong the expression of polyfected genes. Specific promoters for dopaminergic cells such as the dopamine transporter promoter [17] can be used to drive long-term expression of transgenes in NT–polyfected neurons.

NT–polyplex offers a safe and low-cost strategy to develop protocols for treatment of neurological diseases in experimental animals.

7. Essential literature references

Original paper: Refs. [11–14,18,21].

8. Quick procedure

(i) Synthesis of the NT–SPDP–poly-L-lysine conjugate.


(iii) Internalization assay.

(iv) Expression assay.

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References


