Poly(l-lysine) nanostructured particles for gene delivery and hormone stimulation

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Abstract

In this work, we designed replica particles based on poly (l-lysine) (PLL) polymers crosslinked via a homobifunctional linker to support coadsorption of a plasmid DNA and a peptide hormone for concurrent transfection and induction of a cellular function. PLL replica particles (PLL RP) were prepared by infiltrating polymer into mesoporous silica (MS) particles, crosslinking the adsorbed chains by using a homobifunctional crosslinker and finally removing the template particles. Moreover, we verified their cytoxicity. Furthermore, based on this PLL RP gene delivery system, we simultaneously evaluated the melanin stimulation and gene expression in these cells by fluorescence microscopy. To further understand the bi-functionality, we labeled the SPI7pTPL and PAG-α-MSH with YOYO-1 and Rhodamine, respectively, to follow its intracellular pathway by confocal microscopy. Our data suggests that the PLL RP is a promising vector for gene therapy and hormone stimulation.

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of a plasmid DNA and a peptide hormone for concurrent transfection and induction of a cellular function. We outline the preparation of the PLL replica particles (PLLrp), verify their cytotoxicity, and demonstrate their successful use as colloidal carriers for the concurrent gene and drug delivery.

To investigate the gene expression and hormone simulation, we chose SPT7pTL, a vector expressing the human SPT7 nuclear transcription factor, and alpha-melanocyte-stimulating hormone (a-MSH) as a reporter hormone. B16-F1 cells were used because these cells respond specifically to a-MSH. a-MSH is a potent stimulator of melanogenesis in mammalian melanocytes and in melanoma cells which binds to a specific receptor on the cell surface, melanocortin-1 receptor (MC1-F), and induces activation of tyrosinase, the key enzyme for melanin formation, through stimulation of adenylyl cyclase and protein kinase A [13]. a-MSH is of interest as a model for signal transduction effects from cyclic AMP up to the final product, in this case the quantifiable pigment melanin. It is already known that a-MSH can be covalently coupled to molecules using well-established procedures [14]. From an earlier study [14], it became clear that a-MSH coupled to PLL can be incorporated into multilayer films and that certain biological properties of such a-MSH derivatives remain active in this formulation. Recently, we have also shown that it is possible to use poly-(l-glutamic acid) (PGA) as a carrier [15]. Furthermore, based on this PLLrp gene delivery system, we simultaneously evaluated the melanin stimulation and gene expression in these cells by fluorescence microscopy. To further understand the bi-functional nature, we labeled the SPT7pTL and PGA-MSH with YOYO-1 and Rhodamine, respectively, to follow its intracellular pathway by confocal microscopy.

2. Materials and methods

2.1. Materials

Poly-(l-lysine) hydrobromide (PLL, Mw = 30 kDa) and poly-(l-glutamic acid (PGA, 54 kDa) were purchased from Sigma. The SPT7pTL plasmid (5.3 kb) was grown in E. coli and purified by a Gigen kit (Qiagen, US). The purity and integrity of the plasmid were assessed by absorption spectrometry (A260/A280 ratio) and electrophoresis on a 1% agarose gel. The DNA concentration was determined by UV absorbance at 260 nm using a Cary 400 spectrophotometer.

2.2. PLL replica particle (PLLrp) preparation and -potentials characterization

PLL (or FTTC-PLL) was infiltrated into porous silica particles at pH 8. After removing the excess PLL, the polypeptide was crosslinked within the silica particles using DMPF (Sigma D6388), a homobifunctional crosslinker, which uses amine groups but does not remove the charges of the amine. The silica template particle was removed by aqueous HF buffered with ammonium fluoride to pH 5. The resulting PLLrp (or FTTC-PLLrp) replica particles were washed with PBS until pH of the supernatant became equal to that of the fresh buffer [16,17].

For all the experiments, the particle distribution was measured by High Performance Particles Sizer (Malvern) instrument. The -potentials were measured on a Zetasizer 2000 (Malvern) instrument.

2.3. a-MSH synthesis

The SPT7 was used as the primary antibody and a-MSH coupled to PLLrp as the secondary antibody. After overnight incubation at room temperature, the cells were washed with PBS-Tx and incubated with a fluorescein-conjugated secondary antibody diluted at 1/500 in PBS-Tx for 1 h at room temperature. Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch) was used as a secondary antibody. The cells were washed with PBS-Tx, rinsed with PBS, and counterstained with the Hoechst 33258 DNA dye (5 mg ml-1 bisbenzimidaze; Sigma) for 20 s. The cells were covered with mounting medium and analyzed by fluorescence microscopy.

2.4. Fluorescently labeled polyelectrolytes

Rhodamine B (Invitrogen, Cergy Pontoise, France) was coupled to PGA-MSH as previously described [15].

2.5. Fluorescence imaging

The particles were imaged on an Olympus IX 71 inverted fluorescence microscope using a FITC filter.

2.6. Transmission electron microscopy

Transmission electron microscopy (TEM) was used to examine the particle morphologies. The samples (2 ml) were placed on Formvar-coated copper grids and allowed to air-dry.

2.7. Flow cytometry

Flow cytometry was performed on a Becton Dickson FACs caliber flow cytometry using excitation wavelength of 488 nm and 405 nm for YOYO-1 and FITC, respectively.

2.8. PLLrp/(a-MSH + SPT7pTL) formation

First, PGA-a-MSH + SPT7pTL was prepared by mixing PGA-a-MSH with various amounts of SPT7pTL in equal volumes of phosphate buffered saline (PBS). Next, PLLrp/(a-MSH + SPT7pTL) was formed by adding the PGA-a-MSH + SPT7pTL to various amounts of PLLrp in equal volumes at room temperature by rotating for 15 min.

2.10. Cell viability

Cell viability was evaluated by quantification of the cellular content in proteins. After treatment, the cells were washed three times in PBS and lysed by the addition of 100 ml of a cell lysis reagent (Promega). The cellular proteins were measured using the bicinchoninic acid assay (Interchim, Montluçon, France) following an incubation time of 30 min at 60 °C. The absorbance was recorded at 562 nm [20].

2.11. Melanin assay

The melanin content was quantified after 1, 2, and 3 days of incubation of cells with different chemicals. The cells were seeded in 24-well plates at 10,000 cells/well [19]. Optical densities (OD) of the supernatant were measured at 405 nm using an Elisa reader. Before each measurement the cell number was evaluated using a Neubauer counting chamber. OD was then normalized for each measurement to allow comparison between different culture conditions. The melanin content was evaluated according to a standard curve of melanin concentration. All experiments were performed at least three times, each yielding at least triplicate values.

2.12. Transfection protocols

For transfection, B16-F1 cells were incubated in complete medium with PLLrp/(a-MSH + SPT7pTL, PGA-a-MSH + SPT7pTL, PLLrp/SPT7pTL and free SPT7pTL. For transfection, B16-F1 cells were incubated with PLLrp/(a-MSH + SPT7pTL, PGA-a-MSH + SPT7pTL, PLLrp/SPT7pTL and free SPT7pTL. The amount of PLLrp/SPT7pTL used was 0.5 mg and 0.1 mg for these two cases (the number of PLLrp to cells were 500 and 100), respectively. After incubation, the transfection medium was removed and rinsed by PBS [21].

2.13. Immunofluorescence

B16-F1 cells were fixed with 2% paraformaldehyde in PBS for 4 min at room temperature and incubated twice for 10 min with PBS containing 0.1 Triton X-100 (PBS-Tx) [22]. After a PBS wash, the cells were incubated overnight at room temperature with primary antibody diluted at 1/1000 in PBS. Mouse polyclonal anti-SPT7 was used as the primary antibody. After overnight incubation at room temperature, the cells were washed with PBS-Tx and incubated with a fluorescein-conjugated secondary antibody diluted at 1/500 in PBS-Tx for 1 h at room temperature. Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch) was used as a secondary antibody. The cells were washed with PBS-Tx, rinsed with PBS, and counterstained with the Hoechst 33258 DNA dye (5 mg ml-1 bisbenzimidaze; Sigma) for 20 s. The cells were covered with mounting medium and analyzed by fluorescence microscopy.

2.14. Confocal laser scanning microscopy (CLSM)

A Bio-Rad MRC 1024 ES confocal microscope, with a Nikon Eclipse TE 300 inverted microscope, was used for the optical sectioning of cells. An argon/krypton mixed gas laser was used to illuminate a Nikon 60×1.2 NA water immersion objective. Emitted light was detected with two photomultipliers through selected band pass filters. Confocal sections were taken every 0.2 μm. Digital image recording was performed using the LaserSharp 2.3 software (Bio-Rad).
Excitation of YOYO-1 was achieved using the 488 nm excitation line, with the resulting fluorescent wavelengths observed using a 506–538 nm band pass filter (green). Rhodamine fluorescence was detected after excitation at 543 nm, and emission long pass filter 585 nm (red). All the experiments were performed in aqueous conditions [23].

3. Results and discussions

3.1. Characterization of PLL replica particles

The preparation of PLLRP involved polymer infiltration into mesoporous silica (MS) particles, crosslinking of the adsorbed chains using a homobifunctional crosslinker and finally removal of the template particles [6]. This linker reacts with the substrate amine groups to form an amidine linkage which retains the positive charge (Scheme 1). The morphology of PLLRP was analyzed by TEM (Fig. 1A) and visualized by fluorescence microscopy (Fig. 1B). The diameter of the PLLRP analyzed by TEM was ~1.5 μm, which is somewhat smaller than that derived from fluorescence microscopy. This size difference is attributed to the TEM measurements being conducted under vacuum. Using fluorescently labeled polymers and flow cytometry as a means to quantify the fluorescence of the particles, we established that ~10^7 PLLRP particles contain 10 μg of the polypeptide, which effectively and near quantitatively adsorbs around 1 μg of nucleic acid and/or poly(l-glutamic acid) from their

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**Scheme 1.** Schematic illustration of the preparation of the poly(l-lysine) replica particles (PLLRP) and cargo loading. PLL was infiltrated into mesoporous silica particles, crosslinked using a homobifunctional crosslinker and the template particles removed.

**Fig. 1.** TEM image of PLLRP obtained by using MS porous silica template particles (A). Fluorescence image of PLLRP. PLLRP were fluorescently labeled with FITC (B).
solutions in phosphate buffered saline (1 mL) (Fig. 2). To characterize more the particles, we measured the zeta-potential of the loaded PLLRP particles before and after adding the PGA derivative. We have shown that the PLLRP particle has a zeta-potential of 21.5 mV. After PGA derivative loading, the zeta-potential of the particles decreased with the PGA derivative content increasing in the incubation solution. For instance, the PGA-loaded PLLRP particles have a zeta-potential of 16.3 mV when incubated with 0.1 mg mL⁻¹ and -20.2 mV when incubated with 1 mg mL⁻¹ of PGA suggesting the success loading of the negatively charged cargoes on the positively charged PLLRP particle.

3.2. Hormone stimulation and cell viability

The melanin secretion in the melanocytic cells was first monitored by the addition of various concentrations of free PGA-α-MSH after incubation for 1, 2 and 3 days (Fig. 3). The results showed that the PGA-α-MSH did not induce melanin production by cells after 1 day of incubation. However, after 2 days the melanin secretion significantly increased with increasing concentration of free PGA-α-MSH. Moreover, the melanin content reached a plateau when the concentration increased up to 0.02 μg mL⁻¹. After 3 days, the tendency of the melanin content was similar as that of 2 days.

Furthermore, the quantity of the melanin secretion for 3 days was slightly higher than that of 2 days.

The cell viability was also examined. For all cases, a high level of cell viability (≥90%) was found (Fig. 4). Since after 3 days incubation with free PGA-α-MSH the cells could secrete a relatively high amount of melanin and do not significantly decrease the cell viability, a 3-day incubation period was taken as a suitable time all of the following experiments.

In a second step, the melanin secretion in cells was measured after incubation with the PLLRP/PGA-α-MSH after 3 days. PLLRP/PGA-α-MSH were prepared by mixing PGA-α-MSH with PLLRP (500, 100, 10 and 1 PLLRP per cell). The melanin content in the cells, which was measured by the addition of PGA-α-MSH at a concentration of 0.001 μg mL⁻¹, lower than the plateau concentration, was at the same level as the untreated cells (Fig. 5). This suggests that the melanin secretion in B16-F1 needs a certain amount of PGA-α-MSH to stimulate the melanogenesis. In the following experiments we...
chose a free PGA-α-MSH concentration of 0.02 μg mL⁻¹, which corresponds to a plateau concentration, where the melanin secretion of B16-F1 is stable.

In each case, the final concentration of PGA-α-MSH was 0.02 μg mL⁻¹, and for each sample, with the exception of a particle to cell ratio of 1:1, the PGA-α-MSH content was below PLLRP saturation with adsorbed polypeptide (Fig. 6). That is, for a 500-, 100- and 10-fold excess of particles over the cells, the introduced PGA-α-MSH was adsorbed onto the surface of PLLRP. From this figure, we found that the melanin secretion increased with increasing amount of PLLRP, namely the PLLRP/cell ratios. Accordingly, this suggests that PLLRP not trouble the activity of PGA-α-MSH to stimulate the cellular melanin secretion. Furthermore, the cell viabilities in all these cases were high (≥ 90%) (Fig. 7). Therefore, we conclude that PLLRP not only improves the melanin secretion but also does not contribute to the cytotoxicity.

3.3. Bi-functionality of PLLRP

Since our aim was to evaluate the bi-functionality of PLLRP as a gene delivery vector, we investigated the efficiency to deliver SPT7pTL into B16-F1 melanoma cells and melanin stimulation.
Fig. 9. Expression of SPT7 in B16-F1 cells for 3 days. The expression of SPT7 (red) was detected by using a mouse monoclonal anti-SPT7 as primary antibody and Cy3-conjugated goat anti-mouse as a secondary antibody. Nuclei were visualized by Hoechst 33258 staining (blue). The number of PLLRP to cells was 500:1 (A) and 100:1 (B). Scale bars = 20 μm.
Accordingly, in a next step, the melanin production was monitored after a 3-day incubation period with PLLRP/(PGA-\(\alpha\)-MSH + SPT7pTL) and PGA-\(\alpha\)-MSH + SPT7pTL, respectively (Fig. 8). This study was performed at PLLRP concentrations of 500 and 100 per cell. The melanin production of PLLRP/(PGA-\(\alpha\)-MSH + SPT7pTL) was not impaired and at the same level as that of PGA-\(\alpha\)-MSH + SPT7pTL, approximately 20-fold higher than the controls. Furthermore, there were no significant differences in terms of melanin content for the various PLLRP concentrations, which is in line with our previous study (Fig. 6).

The transfection ability of PLLRP was next evaluated by expressing the human SPT7 nuclear transcription factor and the nuclei were visualized by Hoechst staining (Fig. 9). The gene expression was determined after 3-day incubation with PLLRP/(PGA-\(\alpha\)-MSH + SPT7pTL), PLLRP/SPT7pTL and free SPT7pTL, respectively. The number ratio of PLLRP to cells was 500. Images are representative of more than 90% of the observed cells. Scale bar = 20 \(\mu\)m.

### 4. Conclusions

Our data suggests that the PLLRP is a promising vector for gene therapy and hormone stimulation. PLLRP could lead to efficient gene delivery and hormone stimulation followed by melanin production, simultaneously. The pathway of the DNA molecules was also visualized by confocal microscopy, and we found that SPT7pTL could enter into the nucleus. The entry of hormone into the cytoplasm and nuclei of cells induces melanin secretion.

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### Appendix

Figure with essential colour discrimination. Fig. 4 in this article is difficult to interpret in black and white. The full colour image can be found in the online version, at doi:10.1016/j.biomaterials.2009.11.032.

### References


