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Nanoparticle-mediated conversion of primary human astrocytes into neurons and oligodendrocytes†

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Abstract

Central nervous system (CNS) diseases and injuries are accompanied by reactive gliosis and scarring involving the activation and proliferation of astrocytes to form hypertrophic and dense structures, which present a significant barrier to neural regeneration. Engineering astrocytes to functional neurons or oligodendrocytes may constitute a novel therapeutic strategy for CNS diseases and injuries. Such direct cellular programming has been successfully demonstrated using viral vectors *via* the transduction of transcriptional factors, such as Sox2, which could program resident astrocytes into neurons in the adult brain and spinal cord, albeit the efficiency was low. Here we report a non-viral nanoparticle-based transfection method to deliver Sox2 or Olig2 into primary human astrocytes and demonstrate the effective conversion of the astrocytes into neurons and oligodendrocyte progenitors following the transgene expression of Sox2 and Olig2, respectively. This approach is highly translatable for engineering astrocytes to repair injured CNS tissues.

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Author contributions

X. L. contributed to the design, execution, and analysis of the described experiments and preparation of the manuscript. K. K. contributed to the polymer synthesis and nanoparticle transfection condition screening and preparation of the manuscript. Y.-H. C. and H. L. contributed to the immunocytochemistry and qRT-PCR experiments. C. G. Z. contributed to the nanoparticle characterization data and manuscript review. J. G. contributed to the discussion and analysis of transfection studies. H.-Q. M. conceived the project, contributed to the design, and analysis of the study and preparation of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Introduction

Central nervous system (CNS) diseases and injuries occur with reactive gliosis and scarring. Gliosis is a common pathological process that involves the activation of astrocytes to proliferate and become hypertrophic and dense structures, which are initially beneficial, restricting the spread of damage, but ultimately are deleterious, acting as both a physical and chemical barrier to neuronal regeneration.^{1,2} Engineering these astrocytes into functional cells, such as neurons or oligodendrocytes, might be a potential strategy for cellular regeneration in CNS injuries and diseases.³

Recently, resident astrocytes have been directly or indirectly converted into functional neurons in the adult brain and spinal cord through a viral vector-mediated transduction of transcription factors, such as Sox2,^{4–6} Neurogenin 2,⁷ achaete–scute complex homolog-like 1 (Ascl1),⁸ and Neuro D1.⁹ Specifically, Niu *et al.* have demonstrated that the ectopic expression of Sox2 was sufficient to reprogram resident astrocytes to induce doublecortin (DCX)-positive neuroblasts.^{4–6} When supplied with neurotrophic factors, such as valproic acid (VPA), these neuroblasts could pass through a proliferative state and generate mature neurons.⁴ In addition, in the developing CNS, basic helix–loop–helix factors Olig1 and Olig2 are expressed in myelinating oligodendrocytes and their progenitors; and Olig2 is necessary for the specification of oligodendrocytes.¹⁰ Overexpression of Olig2 in neural stem cells (NSCs) by a viral vector has been shown to induce the maturation of oligodendrocytes and expression of major myelin-specific proteins and enhanced remyelination activity *in vivo*.^{11–13}

So far the success in cell reprogramming has mostly been achieved by viral vectors.³ From the perspective of clinical translation, non-viral transfection methods are preferred due to the risks of viral vectors regaining reproductive capability or tumor formation through insertional mutagenesis.^{14–16} Numerous biomaterials have been studied as potential non-viral gene delivery vectors that are comparatively easier to manufacture and scale-up than viral vectors, although they are less efficient in mediating transgene expression.^{17–20} One particularly interesting class of polymeric gene carriers are poly-(β -amino ester)s (PBAEs) due to their biodegradability *via* hydrolytically degradable ester linkages in the backbone, low cytotoxicity, and structural versatility.^{21–23} PBAEs can effectively condense plasmid DNA into nanoparticles with a high level of transfection activities in several stem cell types.^{24–30} Here we report an approach to convert primary human astrocytes into neurons or pre-mature oligodendrocytes *in vitro*, using PBAE-based nanoparticles to introduce Sox2 and Olig2 into astrocytes, respectively.

Results and discussion

Highly effective PBAE-based nanoparticle-mediated transfection of astrocytes

We selected five PBAE polymers (446, 447, 456, 536, 537, and 546) as vectors for the transfection of primary human astrocytes. Previous investigation on elucidating the structure–activity relationship of PBAE carriers for non-viral gene delivery has shown that PBAE characteristics such as pH-buffering, end-group structure,³¹ molecular weight,²⁷ and hydrophobicity³² significantly influence their gene delivery efficiency. While the

PBAE/DNA complexes are primarily taken up by caveolae-mediated endocytosis,³³ a higher buffering capacity, amine-terminated end-groups, intermediate molecular weights, balanced hydrophobicity/hydrophilicity, and polar terminal groups (*e.g.* E6 and E7)^{31,34} have all been shown to favor the transfection activity.³¹ Therefore, we have picked these five top-performing PBAE gene carriers based on our previous work for further screening to identify the optimal transfection conditions that yield a high level of transgene expression and low cytotoxicity using GFP as a reporter gene.

Screens used GFP plasmid DNA doses of 1 and 2 $\mu\text{g cm}^{-2}$ and a selected range of PBAE/plasmid DNA ratios of 30, 60, and 90 w/w in order to identify top polymers from this group. Using the monomers shown in Scheme 1, we were able to examine the role of the polymer structure on transfection efficiency by making small chemical changes incrementally (Fig. 1A). Polymers 446 and 447 differ only in the polymer endcap with 446 containing a secondary amine and hydroxyl, and 447 containing two tertiary amines. Although the backbone and therefore the majority of the polymer is the same, the small molecule endcap change resulted in increasing transfection from $1.7 \pm 0.5\%$ using Polymer 446 at 30 w/w and 2 $\mu\text{g cm}^{-2}$ DNA, to $64.3 \pm 1.6\%$ using Polymer 447 under the same conditions. Similarly, 456 which differs from 446 only in the polymer side chain being one hydrocarbon longer, achieved $45.7 \pm 2.0\%$ transfection at 60 w/w and 1 $\mu\text{g cm}^{-2}$ DNA *versus* $2.8 \pm 0.3\%$ using 446 under the same conditions.

Choosing the optimal PBAE formulation required considering both the transfection efficiency and cell compatibility, as some formulations achieved a high transfection efficiency but at the expense of low cell viability (Polymer 537 at 60 w/w and 1 $\mu\text{g cm}^{-2}$ DNA with $72.7 \pm 6.0\%$ transfection but $25.8 \pm 1.4\%$ viability) while others were ineffective but generally nontoxic (Polymer 456, 30 w/w and 1 $\mu\text{g cm}^{-2}$ DNA with $7.3 \pm 2.3\%$ transfection but no measureable toxicity). Among all these polymers, Polymer 536 showed relatively high transfection efficacies and low cytotoxicities compared with other PBAEs. PBAE 536 at 1 $\mu\text{g cm}^{-2}$ DNA and the polymer/DNA ratios of 30, 60, and 90 w/w, expressed transfection efficiencies of $13.8 \pm 2.1\%$, $55.9 \pm 2.7\%$, and $50.3 \pm 1.1\%$, respectively, with cell viabilities of $100.7 \pm 1.1\%$, $74.7 \pm 2.4\%$, and $52.8 \pm 4.8\%$ relative to non-transfected cells (Fig. 1B). We chose Polymer 536 at 60 w/w and 1 $\mu\text{g cm}^{-2}$ DNA for all further experiments. This is particularly interesting in the hard-to-transfect primary human astrocytes, as the commercially available transfection reagent Lipofectamine™ 2000 was unable to achieve a transfection higher than $6.4 \pm 2.2\%$ in any formulation tested. We have in a previous work often found the PBAE-based transfection to exceed that of Lipofectamine™ 2000 in varying cell types, including glioblastoma,^{24,25} mesenchymal stem cells,²⁶ and liver cancer.³⁵

Current studies on direct cellular reprogramming have primarily used viral vectors to introduce transdifferentiating factors,³ as non-viral vectors have traditionally been less effective.^{17–20} We have been able to demonstrate the safe and effective transfection of primary human astrocytes using Polymer 536, highlighting the potential for the clinical application of this approach.

Enhanced long-term viability of astrocytes after transfection by spheroid culture

Through the optimized transfection conditions identified above, we successfully delivered transcriptional factors Sox2 and Olig2 into astrocytes with the efficacies of $43.2 \pm 5.0\%$ and $52.9 \pm 1.7\%$, respectively. Immunostaining confirmed the expression of ectopic Sox2 and Olig2 in the nuclei of transfected astrocytes (Fig. 2).

Recently Corti *et al.* have shown human NSC culture conditions including a neuronal medium supplemented with EGF and FGF2 could increase the frequency of NSC colony emergence from astrocytes transduced with Sox2, Oct4, or Nanog.³⁶ In this study, we have applied NSC culture conditions to our transfected astrocytes to enhance their conversion efficiency. GFP, Sox2–GFP, and Olig2–GFP transfected cells showed viabilities of $73.4 \pm 2.9\%$, $83.6 \pm 2.2\%$, and $88.1 \pm 6.0\%$, respectively, relative to non-transfected cells at 1 day post transfection (Fig. 3); however, after a 2-week culture under NSC conditions, these transfected cells exhibited drastically decreased viabilities of $37.4 \pm 1.8\%$, $40.4 \pm 0.9\%$, and $41.2 \pm 2.5\%$, respectively (Fig. 3B).

In order to enhance the long-term survival of transfected primary astrocytes under NSC culture conditions, we applied a three-dimensional (3D) spheroid culture for these transfected cells.³⁷ Following our previously reported protocol,³⁸ the transfected cells were able to form spheroids in an agarose hydrogel microwell, but many cells died during the spheroid formation (Fig. S1†). In addition, we have directly transfected the pre-formed astrocyte spheroids. A high cell viability could be maintained after transfection; however, it was difficult to achieve transfection higher than 20% in any formulation tested (Fig. S2†).

Here we developed a novel protocol to fabricate astrocyte spheroids simultaneously with nanoparticle-mediated transfection. Single astrocytes (2×10^6 cells per mL) were mixed with prepared nanoparticles, and then seeded into the agarose hydrogel microwell.³⁹ Along with the spheroid formation, cells were transfected by the encapsulated nanoparticles in the hydrogel microwell. To obtain optimal transfection conditions for astrocyte spheroids, we chose Polymer 536 at the polymer/DNA ratio of 60 w/w, and adjusted the DNA doses (1, 3, 4.5, 6, and 9 μg DNA per 10^6 cells). Shown in Fig. 4, with the high-dose DNA (9 μg per 10^6 cells), cells failed to form a spheroid in the hydrogel microwell. The large amounts of loaded DNA were grossly toxic to the cells, which may interrupt the cell–cell adhesion and impair the spheroid formation. As for the DNA doses of 4.5 or 6 μg per 10^6 cells, the majority of cells could aggregate together in the microwell but with certain isolated cells around the formed spheroid. When the DNA dose was lower than 3 μg per 10^6 cells, almost all seeded cells were able to aggregate together to form a single spheroid. In consideration of the transfection efficiency, among all these DNA doses, 3 μg DNA per 10^6 cells has obtained the highest efficiency of $66.9 \pm 2.6\%$ at day 2, which decreased to $26.5 \pm 2.3\%$ at day 7, and then $14.8 \pm 0.6\%$ at day 14, while not showing a statistically significant decrease in cell viabilities (Fig. 4B and S3,† day 2: $94.8 \pm 1.9\%$; day 7: $102.3 \pm 9.0\%$, and day 14: $103.7 \pm 2.8\%$).

Considering the high transfection efficiency and low cytotoxicity of Polymer 536 at the DNA dose of 3 μg per 10^6 cells, we selected this condition to deliver Sox2 and Olig2 into astrocyte spheroids, respectively. The expression of transfected Sox2–GFP and Olig2–GFP

in astrocyte spheroids lasted about 14 days (Fig. 5A). The 3D spheroid culture drastically enhanced cell survival following transfection under NSC culture conditions. On day 14, GFP, Sox2–GFP, and Olig2–GFP transfected cells exhibited viabilities of $116.7 \pm 5.2\%$, $100.9 \pm 4.7\%$, and $100.2 \pm 3.7\%$, respectively, relative to non-transfected cells at day 1 (Fig. 5B).

We further compared the optimized 2D and 3D transfection conditions in terms of the transfection efficiency and cell viability (Table 1). Interestingly, the nanoparticle formulation which yielded the optimal transfection of cells cultured in 2D was drastically different from that used for 3D. Cells cultured in 3D spheroids required roughly 7-fold less DNA and polymer than those cultured in 2D, while achieving a significantly higher transfection (efficiency at day 2: $66.9 \pm 2.6\%$ vs. $55.9 \pm 2.7\%$). More importantly, enhanced survival with long-lasting gene expression was shown under 3D conditions (viability at day 14: $116.7 \pm 5.2\%$ vs. $37.4 \pm 1.8\%$; efficiency at day 14: $14.8 \pm 0.6\%$ as opposed to non-detected). This improved cell survival and extended transgene expression may be attributed to the spheroid culture as a result of the improved cell–cell anchorage, paracrine signaling, and trophic support from the neighboring cells. Furthermore, the overall size of spheroids was controlled at around 200 μm to ensure sufficient mass transport within the core of the spheroids.

Conversion of astrocytes into neuronal lineage by nanoparticle-mediated Sox2 expression

We next examined the effect of nanoparticle-mediated Sox2 expression on the conversion efficiency of primary human astrocytes. Here we introduced two control groups: one was non-transfected cells as a negative control and the other was the GFP-transfected group as a transfection control. On day 2 post transfection, in the Sox2-transfected group, there was a decreased GFAP expression compared to control groups (Fig. 6A). At the same time, the expression of neuroblast-specific proteins, nestin and Tuj1, was increased in the Sox2-transfected group. Specifically, none of the GFP-positive cells was Tuj1 positive in the GFP-transfected group. However, in the Sox2-transfected group, a few cells co-expressed transfected Sox2–GFP and Tuj1, which may imply that these Tuj1+ cells come from the conversion of astrocytes (Fig. 6A and S4†). Tuj1 expression in the Sox2-transfected group further increased along with the decrease of GFAP expression. Several bipolar shaped-neurons (Tuj1+) appeared in the Sox2-transfected group on day 4 post transfection (Fig. 6B and S5†). All of these results suggest that the nanoparticle-mediated delivery of Sox2 is capable of converting astrocytes into Tuj1+ neurons.

We also characterized the phenotypes of Sox2-transfected cells in a 3D spheroid culture through qRT-PCR and immunostaining. As shown in Fig. 7A, the nestin gene gradually increased with a significantly higher level at day 14 in the Sox2-transfected group compared to that in control groups ($n = 3$, $P < 0.05$). The Tuj1 gene was significantly enriched in Sox2-transfected cells compared with controls ($n = 3$, $P < 0.05$). Interestingly, the GFAP gene was downregulated by transfection, but upregulated with higher levels in the Sox2-transfected cells compared to that in controls at both days 7 and 14 ($n = 3$, $P < 0.05$). Immunostaining analysis confirmed that Sox2-transfected cells expressed higher levels of nestin and Tuj1 compared to controls on day 4 post transfection (Fig. 7B). Especially, the nestin expression

in Sox2-transfected cells further increased on day 10 post transfection (Fig. 7C). To further enhance the neuronal differentiation of Sox2-transfected spheroids, we re-seeded these spheroids on a tissue culture plate and cultured them in a growth factor-free NSC medium for another 7 days (Fig. 8A). At day 21, many cells had migrated out of these attached spheroids (Fig. 8B). A significantly larger number of Tuj1+ cells appeared around the Sox2-transfected cell spheroid compared to controls (Fig. 8B and C, Sox2: $45.0 \pm 8.1\%$, GFP: $11.8 \pm 2.8\%$, and Non: $6.4 \pm 1.0\%$; $n = 5$, $P < 0.05$). Similarly, a larger amount of Tuj1+ processes spread out of the Sox2-transfected cell spheroid. Furthermore, a larger number of MAP2+ neurons have been identified around the Sox2-transfected cell spheroids compared to controls (Fig. 8B and C, Sox2: $64.6 \pm 9.2\%$, GFP: $16.0 \pm 7.9\%$, and Non: $11.4 \pm 6.2\%$; $n = 5$, $P < 0.05$). In addition to neuronal markers, fewer GFAP+ cells appeared in the Sox2-transfected group when compared to those in the non-transfected group (Fig. 8B and C, Sox2: $28.0 \pm 9.9\%$, GFP: $37.5 \pm 13.9\%$, and Non: $61.5 \pm 14.5\%$; $n = 5$, $P < 0.05$). We have successfully developed a novel protocol to generate neurons from nanoparticle-mediated Sox2-transfected astrocytes.

Conversion of astrocytes into premature oligodendrocytes by nanoparticle-mediated Olig2 expression

In parallel, we tested the effects of nanoparticle-mediated Olig2 expression on the conversion efficiency of primary human astrocytes. Non-transfected cells were used as a negative control. As shown in Fig. 9A, two astrocyte-specific genes, GFAP and S100B, were downregulated following Olig2 transfection in astrocytes. The nestin expression did not show a significant difference compared to that in non-transfected cells during the time examined. In contrast, one oligodendrocyte progenitor-specific marker, PDGFR α , expressed significantly higher levels in Olig2-transfected cells at both 12 hours and 2 days compared to the control, whereas another marker, CNP, showed higher levels at both days 2 and 7. Subsequent immunocytochemistry confirmed that a few cells co-expressed Olig2–GFP with oligodendrocyte lineage-specific markers A2B5, Olig1, and O4, respectively (Fig. 9B), but no neuronal markers including nestin, Tuj1, or MAP2 (Fig. S6†).

We also investigated the cell phenotypes in Olig2-transfected astrocyte spheroids. As shown in Fig. 10, both PDGFR α and CNP in Olig2-transfected spheroids displayed higher levels compared to the control at both days 1 and 7 (Fig. 10A, $n = 3$, $P < 0.05$). By day 14 post transfection, the levels of these highly upregulated genes were decreased. In addition, Olig1 exhibited a significantly higher level in Olig2-transfected cells on both days 7 and 14. Immunostaining analysis confirmed that a large number of cells expressed PDGFR α and O4 in the Olig2-transfected group on day 10 post transfection (Fig. 10B). All these results have implied that transcription activation through the nanoparticle-mediated expression of Olig2 in astrocytes was effective in converting them into premature oligodendrocytes. Our further study will focus on examining the maturation of these converted oligodendrocytes in a long-term culture.

Conclusion

Here we identified top PBAE carriers for transfecting primary human astrocytes with a good gene expression efficiency and low cytotoxicity. We have successfully developed a protocol to generate neurons through the conversion of nanoparticle-mediated Sox2-transfected astrocyte spheroids. We also demonstrated that the nanoparticle-mediated expression of Olig2 in astrocytes was effective in converting them into oligodendrocyte progenitors. Future work will examine the ability of locally delivered PBAE/DNA nanoparticles *via* intracerebral injection into the scar tissue to engineer astrocytes around the lesion site after traumatic brain injury. This study provides the first step in developing a potentially translatable therapeutic approach to regenerate damaged brain tissues.

Materials and methods

Materials

Homo sapiens SRY (sex determining region Y)-box 2 (Sox2, CMV Promoter, RG200757) and oligodendrocyte transcription factor 2 (Olig2, CMV Promoter, RG208209) as transfection-ready DNAs were obtained from OriGene Technologies (Rockville, MD). pEGFP-N1 DNA was obtained from Elim Biopharmaceuticals (Hayward, CA). Recombinant human fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) were purchased from Peprotech (Rocky Hill, NJ). Poly-L-lysine was obtained from ScienCell Research Laboratories (Carlsbad, CA). 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI) was purchased from Molecular Probes (Eugene, OR). Primers and primary antibodies used in this study are shown in Tables S1 and S2,[†] respectively. Fluorophore-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Monomers used for synthesizing polymers (Scheme 1) were obtained as follows: 1,4-butanediol diacrylate (B4; Alfa Aesar, Ward Hill, MA), 1,5-pentanediol diacrylate (B5; Monomer-Polymer and Dajac Labs, Trevose, PA), 3-amino-1-propanol (S3; Alfa Aesar), 4-amino-1-butanol (S4; Alfa Aesar), 5-amino-1-pentanol (S5; Alfa Aesar), 2-(3-aminopropylamino) ethanol (E6; Sigma Aldrich, St Louis, MO), and 1-(3-aminopropyl)-4-methylpiperazine (E7; Alfa Aesar). All other chemical reagents were obtained from Sigma Aldrich (St. Louis, MO).

Polymer synthesis

Poly(β -amino ester)s (PBAEs) were synthesized in a two-step reaction as described previously using small commercially-available molecules (Scheme 1).⁴⁰ Briefly, for each polymer, one backbone monomer (labeled as B4 or B5) was mixed with a side-chain monomer (S3, S4, or S5), either neat or as a 500 mg mL⁻¹ solution in dimethyl sulfoxide (DMSO), at a 1.1 : 1 or 1.2 : 1 ratio of B : S. The mixture was stirred at 90 °C for 24 h to yield acrylate-terminated base polymers by Michael addition. Base polymers were end-capped with a small molecule (E6 or E7) by dissolving end-capping molecules in DMSO (167 mg mL⁻¹) and adding 480 μ L end-capping solutions to 320 μ L of a 0.5 M base polymer in DMSO, then shaking the mixture at room temperature overnight. Final polymers were

stored with a desiccant at 4 °C as 100 mg mL⁻¹ solutions in DMSO. Polymers used for optimization experiments were stored in small aliquots at -20 °C to minimize the freeze-thaw of samples. For the remainder of this report, PBAEs are named according to their component monomers in the format “BSE” along with the B : S synthesis ratio used. For example, B5 polymerized with S3 at a 1.1 : 1 ratio and end-capped with E6 is called “536, 1.1 : 1”.

The molecular weight (M_w) results obtained with gel permeation chromatography (GPC) and the degree of polymerization (DP) of all the PBAE polymers used on the screen are described in Table S3.† For complete additional information on the GPC and 1H-NMR analysis of PBAE polymers, see Sunshine *et al.* and Mangraviti *et al.*^{32,41} We have measured the average sizes of nanoparticles by a NanoSight NS500 (Malvern Instruments, UK), which were 170.8 ± 40.5 nm, 136.1 ± 41.2 nm, and 134.5 ± 33 nm for nanoparticles prepared with GFP plasmid, Olig2 plasmid, and Sox2 plasmid, respectively. Their corresponding zeta potential measured using the Malvern Zetasizer Nano ZS (Malvern Instruments, UK) were 13.2 ± 0.7 mV, 13.6 ± 0.9 mV, and 13.2 ± 0.3 mV, respectively.

Cell culture

Primary human astrocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA). Astrocytes were isolated from the cerebral cortex of human fetal brain tissue. In a conventional culture, poly-L-lysine (2 µg cm⁻²) was used to coat tissue culture plastic-ware at least one day before cell seeding. Cells were cultured in an Astrocyte Medium (Cat. #1801, Carlsbad, CA) and used before passage 3 in this study.

For the conversion of astrocytes, cells were cultured in a neural stem cell (NSC) medium [Neurobasal medium with B-27, N-2, 1× non-essential amino acids, 2 mM glutamine, 0.1% w/w bovine serum albumin, and growth factors of FGF-2 (20 ng mL⁻¹) and EGF (20 ng mL⁻¹)]. For neuronal differentiation, cells were cultured in the growth factor-free NSC medium.

Cell transfection

Nanoparticle compositions, including PBAE structures (446, 447, 456, 536, 537, and 546), polymer/plasmid DNA ratios (30, 60, and 90 w/w), and DNA doses (1 and 2 µg cm⁻²) were screened for their transfection efficiencies and cytotoxicities through eGFP plasmid DNA. The N/P ratios of different PBAE polymers at the polymer/DNA ratios of 30, 60, and 90 w/w are shown in Table S4.†

Briefly, before transfections, human astrocytes were seeded into flat-bottom, tissue culture treated 96-well plates (1.5 × 10⁴ cells per well, 100 µL medium per well). Cells were allowed to adhere overnight at 37 °C and 5% CO₂. Plasmid DNA coding eGFP was diluted in 25 mM sodium acetate buffer (NaAc, pH = 5). PBAEs were diluted into the NaAc buffer and then mixed with the DNA solution at various PBAE/DNA mass ratios (w/w). After allowing 10 min for self-assembly, nanoparticles were added directly to the cells in a 1 : 5 ratio (v/v) of particles to media. The final dosage was 300 and 600 ng of DNA added per well (1 and 2 µg cm⁻²). Each group contained 4 replicates. After 2 h of incubation at 37 °C, the media and particles were removed and replaced with fresh culture medium.

In consideration of spheroid transfection, a single cell suspension (2×10^6 cells per mL) was mixed with PBAE 536 nanoparticles at the polymer/DNA of 60 w/w and the DNA doses of 1, 3, 4.5, 6, and 9 μg per 10^6 cells, and then seeded into agarose hydrogel microwells to form spheroids as we reported before.³⁸ After 2 h, the cells with nanoparticles settled down to the bottom of the microwells. We gently used 1 mL PBS to remove the un-internalized nanoparticles in microwells as the 2D cell transfection. Then we added 1 mL medium to the microwells for the long-term culture.

Transfection efficiency

The GFP expression was measured by flow cytometry (Accuri C6, BD Biosciences, San Jose, CA) with a Hypercyt high-throughput robotic sampler (Intellicyt, Albuquerque, NM). Cells were transferred to round-bottom 96-well plates. Transfection data were analyzed with FlowJo7 software.

Cell viability

The cytotoxicity of PBAE/DNA nanoparticles was determined by WST-1 dye reduction assay at 24 h after cell transfection. The medium in each well was replaced with fresh medium containing 10% v/v of WST-1 reagent (Roche, Mannheim, Germany). The cells were incubated for 2 h at 37 °C. The absorbance of the supernatant at 450 nm (using 600 nm as a reference wavelength) was measured on a microplate reader (Infinite M200, TECAN, Männedorf, Switzerland).

AlamarBlue assay (Invitrogen, Carlsbad, CA) was conducted to characterize the cell viability in a long-term culture according to the manufacture's protocol. The metabolic reduction of alamarBlue was monitored by absorbance measurements at 570 nm and 600 nm using the microplate reader above.

Immunocytochemistry

The conversion of astrocytes after transfection was evaluated by immunocytochemistry. The cells were fixed in 4% w/w paraformaldehyde for 10 min. As for cell spheroids, after fixation, they were embedded in a tissue freezing medium and 10 μm thick sections were cut on a Leica CM3050 cryostat and mounted on microscope slides. The cells were stained with primary antibodies shown in Table S2,[†] including Sox2, Olig2, glial fibrillary acidic protein (GFAP), nestin, neuron-specific class III beta-tubulin (Tuj1), microtubule-associated protein 2 (MAP2), Olig1, platelet-derived growth factor receptor alpha (PDGFR α), A2B5, and O4. The cells were further stained with fluorophore conjugated secondary antibodies. The nuclei were counterstained with DAPI. Then cells were imaged using a Zeiss LSM 510 Meta Confocal Microscope (Thornwood, NY). At least 5 random fields per sample were analyzed.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was applied to investigate the gene expression of astrocytes after transfection with nanoparticles containing plasmids encoding GFP, Sox2–GFP, or Olig2–GFP. Non-transfected cells were used as a negative control. RNA was isolated by RNeasy@RT (R4533, Sigma). The High Capacity cDNA Reverse Transcription Kit (4374966, Life Technology) was used for the quantitative conversion of RNA to single-

stranded cDNA. Primers used for PCR are shown in Table S1.† The expression levels of the genes of interest were normalized to the expression of GAPDH.

Statistical analysis

Data are shown as mean $\pm$ standard deviation (SD). Statistical analyses were performed using one-way ANOVA (analysis of variance) followed by Tukey's post-test and the paired *t*-test where appropriate. A *P* value of <0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Identification of nanoparticle-mediated transfection conditions with high transfection efficacies and low cytotoxicities. (A) Screening used DNA doses of 1 and 2 $\mu\text{g cm}^{-2}$ and an abbreviated range of PBAE/plasmid DNA ratios (30, 60, and 90 w/w) in order to identify top polymers among 446, 447, 456, 536, 537, and 546 based on their transfection efficacies and cytotoxicities. (B) Expression of transfected GFP at day 2 of the tested PBAE 536 at the DNA dose of 1 $\mu\text{g cm}^{-2}$ and the polymer/DNA ratios of 30, 60, and 90 w/w, respectively. Scale bar = 100 μm .

Fig. 2.

Nanoparticle-mediated expression of Sox2 and Olig2 on primary human astrocytes. Immunostaining of astrocytes 2 days post transfection with PBAE 536 nanoparticles containing plasmids encoding GFP, Sox2–GFP, or Olig2–GFP, at the polymer/DNA ratio of 60 w/w and the DNA dose of $1 \mu\text{g cm}^{-2}$. The expression efficacies for GFP, Sox2–GFP, and Olig2–GFP were $55.9 \pm 2.7\%$, $43.2 \pm 5.0\%$, and $52.9 \pm 1.7\%$, respectively. Immunostaining confirmed the transfected Sox2 (red) or Olig2 (red) expressed in astrocytes, respectively. Cell nuclei were stained with blue by DAPI. Scale bar = 200 μm .

Fig. 3.

Long-term gene expression and viability of transfected cells under a 2D culture. (A) GFP, Sox2-GFP, or Olig2-GFP expressing in primary human astrocytes after transfection with PBAE 536 at the polymer/DNA ratio of 60 w/w and the DNA dose of $1 \mu\text{g cm}^{-2}$ at days 2, 5, and 10. Scale bar = 200 μm . (B) Viabilities of GFP, Sox2-GFP, or Olig2-GFP transfected cells were inspected by the alamarBlue kit on days 1, 4, 7, and 14 ($n = 4$). Non-transfected cells were used as a negative control.

Fig. 4. Optimization of nanoparticle-mediated transfection conditions in a spheroid culture. (A) GFP expression in astrocyte spheroids transfected with PBAE 536 at the polymer/DNA ratio of 60 w/w with varied DNA doses of 1, 3, 4.5, 6, and 9 µg per million cells. Scale bar = 200 µm. (B) Transfection efficacies and viabilities of astrocyte spheroids after transfection at days 2, 7, and 14.

Fig. 5. Long-term gene expression and viability of transfected cell spheroids. (A) GFP, Sox2-GFP, or Olig2-GFP expressing in astrocyte spheroids after transfection with PBAE 536 at the polymer/DNA ratio of 60 w/w and the DNA dose of 3 μg per million cells at days 2, 4, 7, and 14. Scale bar = 200 μm. (B) Viabilities of GFP, Sox2-GFP, or Olig2-GFP transfected astrocytes were inspected by the alamarBlue kit on days 1, 4, 7, and 14 ($n = 4$). Non-transfected cells were used as a negative control.

Fig. 6.

Conversion of astrocytes into neurons by nanoparticle-mediated Sox2 expression under a 2D culture. Immunostaining of astrocytes at days 2 (A) and 4 (B) post transfection with PBAE 536 nanoparticles containing plasmid encoding GFP or Sox2-GFP. Non-transfected cells were used as a negative control. Cells were stained with GFAP (red), nestin (red), and Tuj1 (red), respectively. Cell nuclei were stained with blue by DAPI. Scale bar = 200 μm.

Fig. 7.

Conversion of astrocyte spheroids by nanoparticle-mediated Sox2 expression. (A) Inspection of nestin, Tuj1, and GFAP gene expression in astrocyte spheroids after transfection with PBAE 536 nanoparticles containing plasmids encoding GFP or Sox2-GFP at days 1, 7, and 14 ($n = 3$, $*P < 0.05$). Non-transfected cells were used as a negative control. (B and C) Immunostaining of transfected astrocyte spheroids at days 4 (B) and 10 (C) post transfection. Cells were stained with nestin (red), Tuj1 (red), and GFAP (red), respectively. Cell nuclei were stained with blue by DAPI. Scale bar = 50 μ m.

Fig. 8. Neurons converted from Sox2-transfected astrocyte spheroids. (A) Schematic of the neuronal differentiation protocol for Sox2-transfected astrocyte spheroids. (B) Immunostaining of cell spheroids at day 21. Cells were stained with Tuj1 (red), MAP2 (red), and GFAP (red), respectively. Cell nuclei were stained with blue by DAPI. Scale bar = 200 μ m. (C) Quantity analysis of transfected cells at day 21 post transfection ($n = 5$, $*P < 0.05$).

Fig. 9.

Conversion of astrocytes into pre-mature oligodendrocytes by nanoparticle-mediated Olig2 expression under a 2D culture. (A) Inspection of the gene expression of astrocytes after transfection with PBAE 536 nanoparticles containing plasmid encoding Olig2-GFP. Non-transfected cells were used as a negative control. (B) Immunostaining of Olig2-GFP transfected astrocytes at day 4 post transfection. Cells were stained with GFAP (red), A2B5 (red), Olig1 (red), and O4 (red). Cell nuclei were stained with blue by DAPI. Scale bar = 200 μ m.

Fig. 10. Pre-mature oligodendrocytes converted from Olig2-transfected astrocyte spheroids. (A) Inspection of PDGFR α , CNP, and Olig1 gene expression of Olig2-transfected astrocyte spheroids at days 1, 7, and 14. Non-transfected cells were used as a negative control. (B) Immunostaining of astrocyte spheroids at day 10 post transfection. Cells were stained with PDGFR α (red) and O4 (red), respectively. Cell nuclei were stained with blue by DAPI. Scale bar = 50 μ m.

Polymer Synthesis Reaction

Monomers

Representative Polymer B5-S3-E6 (536)

Scheme 1.

Monomers and the reaction scheme used to synthesize the PBAE library. One backbone monomer (B, red) was polymerized with one side chain monomer (S, blue). The diacrylate B-S base polymer was then terminated with one end-capping monomer (E, green).

Table 1

Comparison of optimized 2D and 3D transfection conditions in terms of cell viability and transfection efficiency

		2D	3D
Nanoparticle	DNA dose (μg per 10^6 cells)	20	3
	PBAE 536 (μg)	1200	180
Cell viability (%)	Day 1	73.4 ± 2.9	95.1 ± 5.0
	Day 14	37.4 ± 1.8	116.7 ± 5.2
Transfection efficacy (%)	Day 2	55.9 ± 2.7	66.9 ± 2.6
	Day 14	ND	14.8 ± 0.6

ND: Not detected.