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Efficient gene transfection of suspension cells by highly branched poly(β-amino ester)

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ABSTRACT

Suspension cells play a crucial role in many biological processes. However, compared to adherent cells, it is particularly challenging to introduce exogenous genes into suspension cells to regulate their biological functions with non-viral gene vectors, mainly due to the low cellular uptake and endosomal escape of polyplexes. Herein, to improve the interactions of polyplexes with cellular membranes, we design and synthesize highly branched poly(β-amino ester) (HPAE) *via* an "**A2** + **B4** + **C2**" Michael addition strategy. Results show that branching significantly increases DNA condensation of HPAE, cellular uptake and endosomal escape of HPAE/DNA polyplexes. In mast cells (MCs), HPAE exhibits up to 80-fold higher gene transfection efficiency compared to the corresponding linear poly(β-amino ester) (LPAE) and the leading commercial gene transfection reagents PEI25k, jetPEI, and Lipofectamine 3000, without causing obvious cytotoxicity. Our study establishes a reliable non-viral platform for efficient gene transfection of suspension cells.

Gene transfection, *i.e.*, the introduction of exogenous nucleic acids into target cells to regulate the expression of specific proteins [1], has shown promise for CAR T cell-based therapies, treatment of cancer and genetic disorders, *etc.* [2–5]. Suspension cells including T cells, mast cells (MCs), human leukemia monocyte (THP-1), and ascites tumor cells (S180) *etc.* are the crucial effector cells in immunotherapy, allergic reactions, immune responses, and tumor treatment [6,7]. MCs, as the central effector cells in allergic reactions and other immune responses, play an important role in combating pathogens and regulating the inflammatory response of the immune system, and are therefore crucial to the first line of the body's immune defense [8]. MCs contain a large number of preformed and pre-activated immunomodulatory mediators. Once activated, they are degranulated and are involved in the regulation of physiological or pathological responses of the body, such as increased vascular permeability, tissue edema, and infiltration of inflammatory cells [9,10]. It is conceivable that targeted and highly efficient gene modulation of MCs would significantly improve their biological functions and thus the treatment of MC-related diseases [11–13].

However, safe and efficient intracellular delivery of exogenous genes in suspension cells has long been a challenge [5,14]. Compared with adherent cells, polyplexes forumulated by a gene vector and DNA usually have a lower interaction with the cell membranes of suspension cells, so that cellular uptake and endosomal escape of polyplexes are impaired, resulting in lower transfection efficiency [15,16]. The most commonly used gene transfection methods or transfection technologies for suspension cells are adenovirus vectors, liposomes, and electroporation [17]. However, their transfection efficiency is usually disappointing. For example, the transfection efficiency in MCs is only 16% even when virus-based vectors [12] or liposomes [11] are used. Moreover, polymer-based vectors [18–20] such as polyethylene imine (PEI), poly-L-lysine (PLL), poly[2-(dimethylamino)ethyl methacry-

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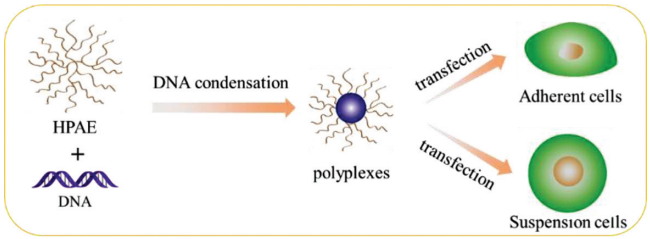

Scheme 1. HPAE condenses DNA to form polyplexes and transfect suspension cells.

late] (PDMAEMA), poly(amidoamine) (PAMAM), and poly(β-amino ester)s (PAEs) have been widely used for transfection of various types of adherent cells, but few of them have been used for transfection of suspension cells. Therefore, it is imperative to develop a safe and efficient platform for transfection of suspension cells.

Due to the wide availability of monomers, ease of synthesis, tailored chemical composition and structure, biodegradability in physiological environment, and high gene packaging capacity, poly(β-amino ester)s (PAEs) have been widely developed for gene delivery [21–25]. In 2005, our group proposed an "**A2** + **B3** + **C2**" Michael addition strategy to synthesize highly branched poly(β-amino ester)s (HPAEs) and further unleash the gene transfection potential of PAEs [12,26,27]. Optimized HPAEs confer up to 8521-fold higher gene transfection potency and low cytotoxicity in various types of adherent cells (*e.g.*, fibroblasts, keratinocytes, cancer cells, stem cells, and astrocytes) compared with the corresponding linear poly(β-amino ester)s (LPAEs). *In vivo,* HPAEs can effectively transfect *COL7A1* gene to restore the expression of recombinant type VII collagen (**C7**) in a recessive dystrophic epidermolysis bullosa (RDEB) graft mouse model for up to 10 weeks [22,28]. Despite the high transfection potency of HPAEs, to our knowledge, HAPEs have not yet been reported to enable gene delivery into suspension cells.

Herein, we report the development of HPAE for gene transfection of suspension cells *via* an "**A2** + **B4** + **C2**" Michael addition strategy (Scheme 1). We hypothesize that this strategy could improve the gene transfection efficiency of suspension cells for two reasons. First, the "**A2** + **B4** + **C2**" strategy using a **B4**-type branching monomer would generate HPAE with a higher branching degree and more terminal groups compared with our previous "**A2** + **B3** + **C2**" strategy using a **B3**-type branching monomer, which would facilitate DNA condensation. Second, the higher number of terminal groups of HPAE would facilitate the interaction of polyplexes with cellular membranes and promote their cellular uptake and endosomal escape, ultimately overcoming the major obstacles in the gene transfection of suspension cells. To this end, 1,4-butanediamine (**B4**) was chosen as a branching monomer to copolymerize with 4-amino-1-butanol (**A2**) and 1,4-butanediol diacrylate (**C2**) to generate an acrylate-terminated base polymer, which was then end-capped with 1-(3-aminopropyl)-4-methylpiperazine (**E7**). Studies showed that HPAE indeed have a strong DNA condensation ability, HPAE/DNA polyplexes exhibit high cellular uptake and endosomal escape ability. HPAE mediates up to 80-fold higher gene expression in laboratory of allergic diseases 2 (LAD2) human mast cells compared to the corresponding LPAE, even to the leading commercial transfection reagents PEI25k, jetPEI and Lipo3000. This study demonstrates for the first time that HPAE can efficiently introduce exogenous genes into suspension cells, providing valuable insights for the development of polymer-based gene delivery systems for gene transfection of suspended cells.

Previously, HPAEs were synthesized by the simple yet controllable one-pot "**A2** + **B3** + **C2**" Michael addition strategy using a **B3**-type branching monomer. To further increase the branching degree and the number of terminal groups, and thus the interactions of the polyplexes with cell membranes, an "**A2** + **B4** + **C2**" strategy was developed using **B4** as the branching monomer (Fig. 1). The stoichiometric ratio of acrylate to amine was set at 1.2:1.0 to initially generate an acrylate terminated HPAE base polymer (Table S1 in Supporting information). GPC was used to monitor the MW and $Đ$ of the base polymer throughout the polymerization process. As shown in Fig. 2a and Table S3 (Supporting information), MW and $Đ$ of the polymer reached 3.7 kg/mol and 1.9, respectively, after 2 h of polymerization. As polymerization progressed, both MW and $Đ$ of the polymer increased rapidly. After 4 h of polymerization, MW and $Đ$ increased to 7.1 kg/mol and 2.6, respectively, due

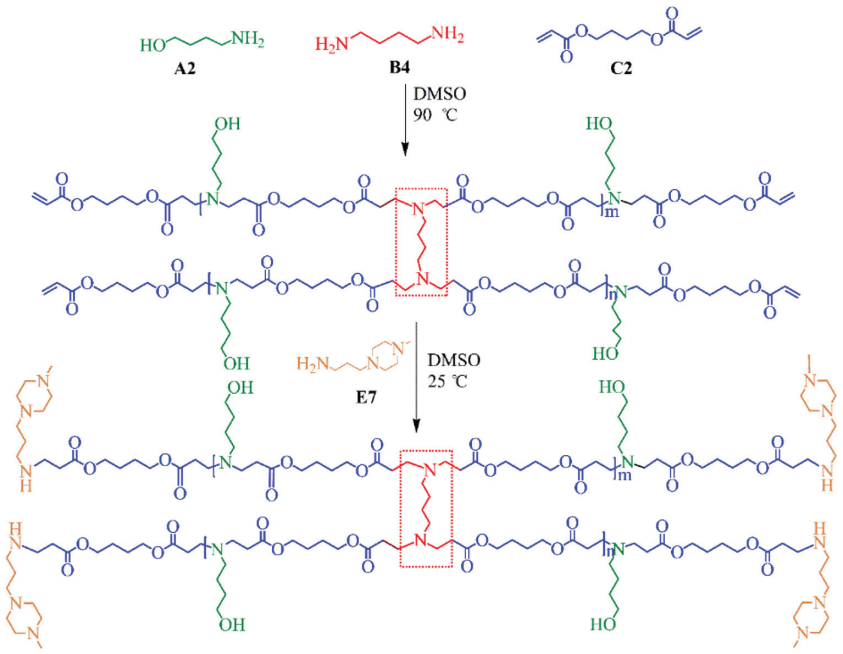

Fig. 1. HPAE was synthesized *via* an "**A2** + **B4** + **C2**" Michael addition strategy.

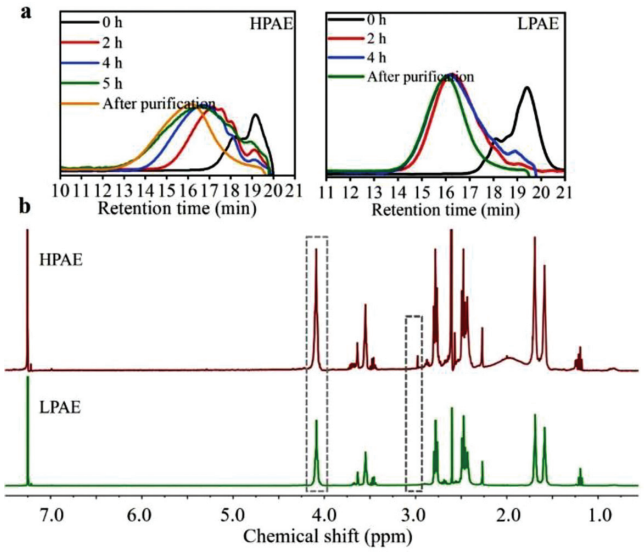

Fig. 2. (a) GPC traces of HPAE and LPAE; (b) ^{1}H NMR spectra of HPAE and LPAE.

to the combination of oligomers, a common feature of step-growth polymerization [18]. However, no gelation occurred even though a total monomer concentration of 500 mg/mL was used. After 5 h of polymerization, when MW reached 10.8 kg/mol, the reaction was stopped and end-capped with a second amine **E7**. After purification with diethyl ether, HPAE with a MW of 14.8 kg/mol and $Đ$ of 3.1 was obtained. For the synthesis of the corresponding LPAE (Table S2 in Supporting information), the monomers were polymerized directly without solvent to increase the polymerization rate. In general, the MW of the LPAE base polymer increased faster than that of the HPAE. After 2 h, MW and $Đ$ are 6.5 kg/mol and 2.0, respectively (Table S4 in Supporting information). The reaction was stopped after 4 h and end-capped with **E7**. The purified LPAE has a MW of 9.3 kg/mol and $Đ$ of 1.7. ^{1}H NMR confirmed the successful copolymerization of the monomers, purity and chemical composition of the polymers (Fig. 2b, Figs. S1 and S2 in Supporting information).

Effective condensation of DNA into nano-sized polyplexes with positive zeta potential is a prerequisite for high-performance gene transfection. Compared to the corresponding LPAE, the higher branching degree and multiple terminal groups of HPAE would facilitate DNA condensation, which was initially determined using PicoGreen assays. As shown in Fig. 3a, the DNA binding affinity of HPAE increased from 58% to 65% when the w/w increased from 30:1 to 60:1. In contrast, the DNA binding affinity of LPAE, although it also increased with w/w, was 20% to 30% weaker than that of HPAE. Even at the highest w/w of 90:1, the DNA binding affinity of LPAE was still weaker than that of HPAE at the lowest w/w of 30:1. Interestingly, although HPAE and LPAE have different DNA binding affinities, both can effectively retard DNA without observing DNA shift bands in the agarose gels (Fig. 3b). Consistent with DNA binding affinity, HPAE can condense DNA into polyplexes much smaller than LPAE as measured by dynamic light scattering (DLS) (Fig. 3c). All HPAE/DNA polyplexes have a diameter of $<$160 nm. In particular, at the w/w of 30:1 and 60:1, the diameters of the HPAE/DNA polyplexes are even smaller than 100 nm. In contrast, all the LPAE/DNA polyplexes, although having a diameter $<$300 nm, are much larger than HPAE/DNA polyplexes. Accordingly, HPAE/DNA have a relatively higher zeta potential than LPAE/DNA (Fig. 3d). The relatively smaller size and higher zeta potential of HPAE/DNA polyplexes may be attributed to the stronger DNA affinity of HPAE. The morphology of the polyplexes was further observed using TEM. As shown in Fig. 3e, the HPAE/DNA polyplexes exhibited a homogeneous and compact toroidal or spherical morphology. In contrast, the LPAE/DNA polyplexes had a loose shape and a diameter of approximately 100 nm. The small size, positive zeta potential and compact morphology would favor cellular uptake of HPAE/DNA polyplexes.

Cellular uptake and endosomal escape of polyplexes are the major obstacles to be overcome in gene delivery. To evaluate the efficiency of cellular uptake of the different polyplexes, DNA labeled with AF647 was used to prepare the polyplexes. As shown in Fig. 4a, after 4 h of transfection with HPAE/DNA polyplexes in the presence of serum, strong red fluorescence was observed in the cells. Notably, most of the red fluorescence was found around the nucleus, indicating the high uptake efficiency of the HPAE/DNA polyplexes into the cells. In contrast, much weaker red fluorescence was observed after incubation with LPAE/DNA for the same time, although it accumulated near the nucleus. These results suggest that the HPAE/DNA polyplexes have a higher efficiency of cellular uptake than the LPAE/DNA, possibly due to the stronger interaction of the polyplexes with cell membrane resulting from the multiple terminal groups of the HPAE. More importantly, the HPAE/DNA polyplexes also have a higher endosomal escape ability. As shown in Fig. 4b, the majority of HPAE/DNA polyplexes successfully escaped from endosomes (stained with Lyso-Tracker Green fluorescence dye) after 6 h of incubation, as shown by the de-localization of the red fluorescence and the green fluorescence, both of which have distinctly different plot profiles in the representative cell (marked by the white line). In contrast, most of the LPAE/DNA polyplexes were still trapped in the endosomes, as shown by the colocalization of the two fluorescence. Meanwhile, the two fluorescence in therepresentative cell shows almost the same plot profiles, indicating the relatively low endosomal escape ability of the polyplexes. All these results demonstrate that HPAE synthesized *via* the "**A2** + **B4** + **C2**" strategy indeed promote cellular uptake and endosomal escape of polyplexes, which may be attributed that the multi-terminal groups increase the interaction of polyplexes with cellular membranes.

The efficacy of transfection of HPAE was first investigated in adherent HeLa and HepG2 cells. The commercial gene transfection reagent jetPEI was used as a positive control. GFP was used as a marker gene and transfection was performed in the presence of serum. As shown in Fig. S3 (Supporting information), 24 h after transfection with HPAE, a high percentage of GFP-positive cells was observed in HeLa cells, particularly at the w/w of 30:1 and 60:1. In comparison, very weak green fluorescence was observed in the cells after transfection with LPAE. 48 h later, a higher percentage of GFP-positive HeLa cells was observed and the intensity of green fluorescence is much stronger than after 24 h. In particular, the gene transfection efficiency of HPAE is even comparable to that of jetPEI at the w/w of 30:1 (Fig. 5a). To further confirm the high gene transfection efficiency of HPAE, the Gluc activity of HeLa cells was quantified after transfection. It can be seen that at the w/w of 30:1, the Gluc activity of cells after transfection with HPAE was 2.0 to 3.6-fold higher than that of LPAE (Fig. 5b). Although the Gluc activity of the cells after transfection with jetPEI was similar to that of HPAE, the viability of the cells was only about 50%. In contrast, even at the highest w/w of 90:1, HPAE can still maintain $>$80% of cell viability (Fig. 5c). We speculate that this is due to the biodegradability of HPAE. Similar trends in gene transfection efficiency were observed in HepG2 cells. As shown in Figs. S4 and S5 (Supporting information), HPAE mediates the highest gene transfection at the w/w of 60:1, which is reflected in both GFP expression and Gluc activity. However, LPAE shows only moderate gene transfection efficiency at the highest w/w of 50:1. Gluc activity is only about 33% compared with that mediated by HPAE. Again, although jetPEI has the highest gene transfection efficiency, cell vi-

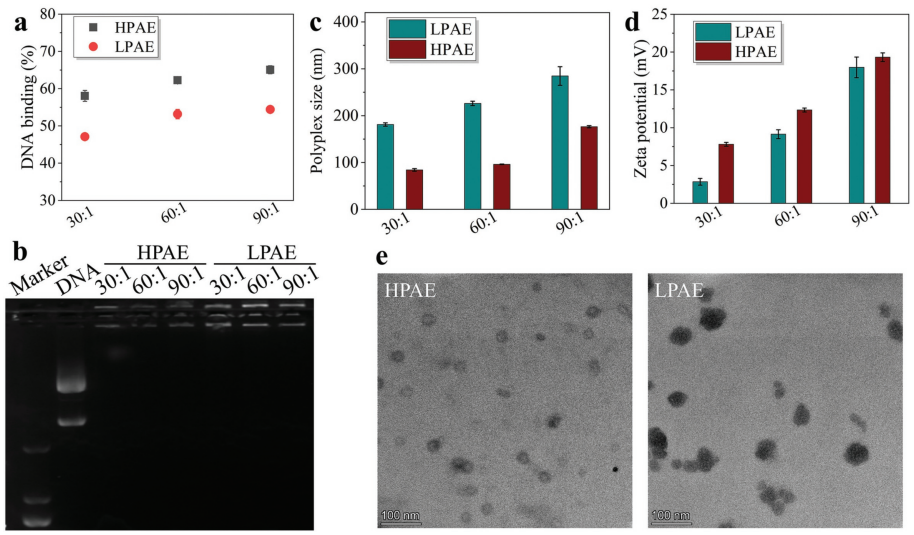

Fig. 3. Physicochemical properties of HPAE/DNA and LPAE/DNA polyplexes. (a) DNA binding affinity measured by PicoGreen assays; (b) DNA condensation ability measured by agarose gel electrophoresis; Size (c) and zeta potential (d) of polyplexes; (e) TEM images of HPAE/DNA and LPAE/DNA polyplexes at a w/w of 60:1. The scale bars are 100 nm.

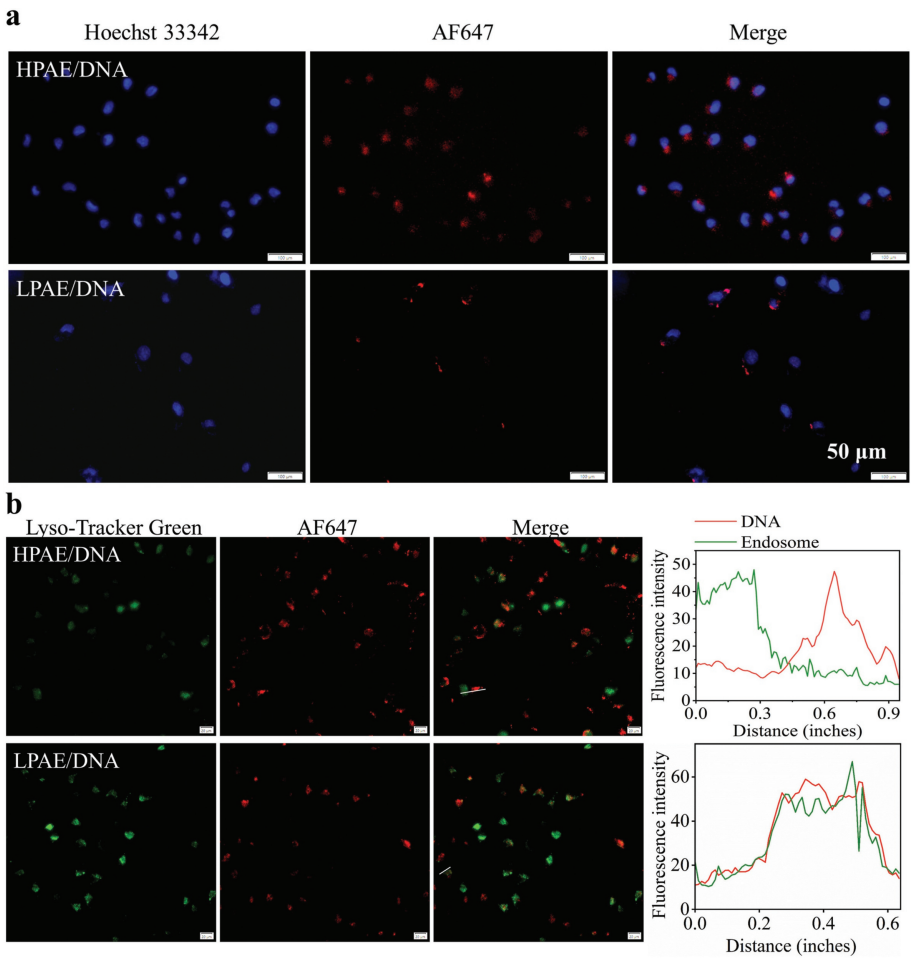

Fig. 4. HPAE/DNA polyplexes show high cellular uptake and endosomal escape ability. (a) Fluorescence images of HeLa cells after 4 h of incubation with polyplexes. The nucleus was stained with Hoechst 33342 (blue), and DNA was labeled with AF647 (red). (b) Fluorescence images of HeLa cells after 6 h of incubation with polyplexes. DNA was labeled with AF647 (red) and endosomes were stained with Lyso-Tracker Green (green). The scale bars represent 50 μm.

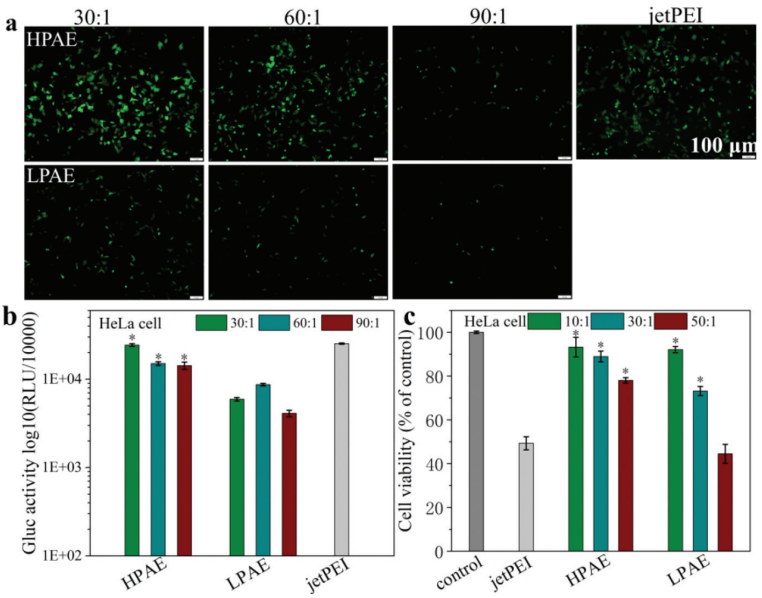

Fig. 5. Evaluation of gene transfection efficiency of HPAE in HeLa cells. (a) Representative fluorescence images of cells 48 h after transfection with different polyplexes, the scale bars are 100 μm; (b) Gluc activity of cells after transfection with different polyplexes. Data marked with an asterisk (*) are statistically significantly higher than those of the LPAE group (w/w = 30:1); (c) Viability of cells after transfection with different polyplexes. Data marked with an asterisk (*) are statistically significantly higher than those of the jetPEI group.

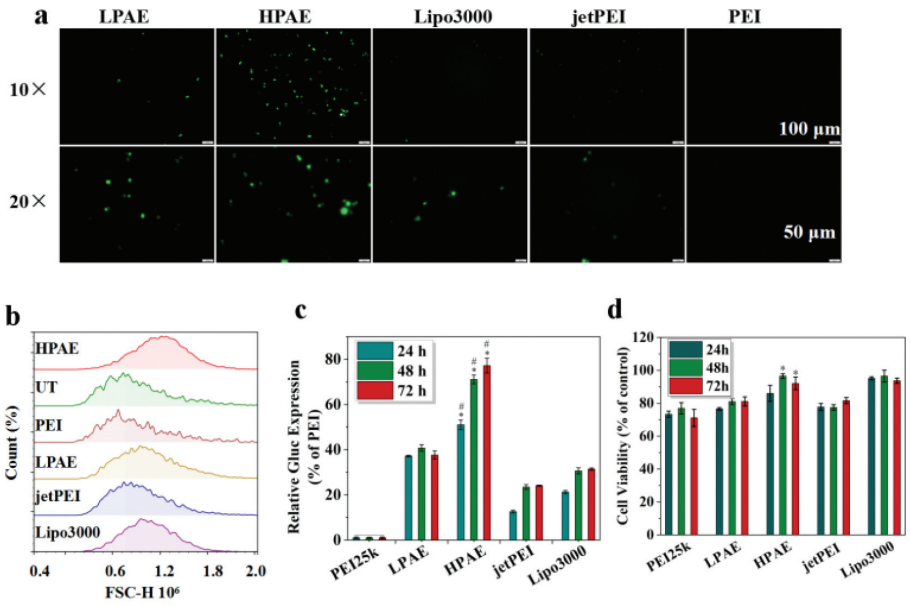

Fig. 6. HPAEs show high transfection efficiency in suspension LAD2 cells. (a) Representative GFP images of LAD2 cells 48 h after transfection, the scale bars are 100 μm (10×) and 50 μm (20×); (b) Representative histogram distributions of untreated (UT) LAD2 cells and cells transfected with HPAE, LPAE, and commercial gene transfection reagents; (c) Gluc activity of LAD2 cells after transfection. Data marked with an asterisk (*) are statistically significantly higher than those of the PEI25k group, and data marked with a number sign (#) are statistically significantly higher than those of the LPAE group at the w/w of 60:1; (d) Cells viability after transfection. Data marked with an asterisk (*) are statistically significantly higher than those of the PEI25k group.

ability is much lower than HPAE and LPAE (Fig. S6 in Supporting information). Taken together, these results demonstrate that HPAE have high gene transfection efficiency without causing obvious cytotoxicity in adherent cells, especially at a relatively low w/w, which could be an important reference for transfection of suspension cells.

MCs differentiate from hematopoietic stem cells (HSCs) along the myeloid pathway and play a key role in the allergic response by triggering a complex biochemical pathway [29,30]. As one of the major mast cell lines, LAD2 routinely degranulates in response to immune stimulation and has a high-affinity receptor for immunoglobulin E (IgE). LAD2 was used as a type of model suspension cells to evaluate the gene transfection of HPAE. Since the polyplexes have a lower interaction with the suspension cells, which would result in lower cellular uptake efficiency of the polyplexes. Therefore, the formulated polyplexes were directly mixed with the LAD2 cells and the cells were then seeded in the cell culture plate, which is obviously different from transfection of adherent

cells. As shown in Fig. S7 (Supporting information), GFP-positive cells were clearly observed 24 h after transfection with LPAE and HPAE at the w/w of 60:1. In comparison, there are almost no GFP-positive LAD2 cells after transfection with the leading commercial gene transfection reagents Lipofectamine 3000 (Lipo3000), jetPEI, and PEI25k. At 48 h after transfection, more GFP-positive LAD2 cells were transfected with HPAE and the intensity of green fluorescence is much stronger (Fig. 6a). However, very few GFP-positive cells were transfected with the commercial gene transfection reagents except for the transfection mediated by jetPEI. At 72 h after transfection, the number of GFP-positive cells transfected with HPAE and LPAE began to decrease. Interestingly, GFP-positive LAD2 cells transfected with Lipo3000 were observed (Fig. S8 in Supporting information). The gene transfection efficiency was further quantified by flow cytometry. As shown in Fig. 6b, the histograms of LAD2 cells shifted significantly to the right after transfection with HPAE compared with all other groups, indicating that there were more GFP-positive cells and the mean fluorescence intensity was stronger after transfection with HPAE. Correspondingly, the relative Gluc activity of LAD2 cells after transfection with HPAE was 50 to 80-fold higher than that with PEI25k, 3 to 4-fold higher than that with jetPEI, and 2 to 3-fold higher than that with Lipo3000 (Fig. 6c). At the same time, HPAE can maintain cell viability at over 85% (Fig. 6d). Although transfection in adherent HeLa and HepG2 cells has shown that jetPEI is highly efficient, it can only mediate very low transfection in suspension LAD2 cells. In sharp contrast, HPAE exhibits highest gene transfection efficiency in LAD2 cells compared to all leading commercial gene transfection reagents, highlighting its high potency in suspension cell transfection.

In summary, a new type of HAPE was synthesized *via* the "**A2** + **B4** + **C2**" Michael addition strategy and used in gene transfection of suspension cells. The branched structure enhances the DNA condensation ability of HPAE and leads to the formation of nano-sized HPAE/DNA polyplexes with positive zeta potential. HPAE/DNA polyplexes exhibit enhanced cellular uptake and endo/lysosomal escape ability. In adherent cells, HPAE mediates a high level of gene transfection. Most importantly, HPAE exhibits up to 80-fold higher gene transfection efficiency in LAD2 suspension cells compared to the leading commercial gene transfection reagents PEI25k, jetPEI, and Lipo3000, while maintaining cell viability above 85%. Considering the critical role of suspension cells in biological activities and the long-standing challenge to transfect suspension cells, HPAE would open a new avenue to enhance the biological functions of suspension cells by gene transfection.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.108066.

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