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Genipin-Cross-Linked Thermophilic Histone-Polyethylenimine as a Hybrid Gene Carrier

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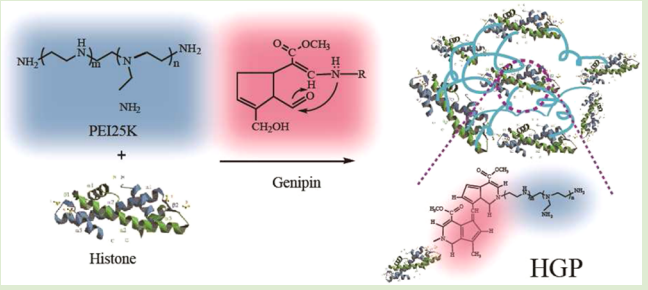
Supporting Information

ABSTRACT: A hybrid gene carrier, HGP, has been successfully constructed through the genipin-mediated cross-linking of thermophilic histone and PEI25K. The thermophilic histone gene GK2215 was cloned from *Geobacillus kastophilus* HTA426 and overexpressed in *Escherichia coli* BL21. The thermophilic histone was systematically characterized and then cross-linked with PEI25K by genipin to obtain HGP. Notably, HGP exhibited superior transfection efficiency due to the synergistic effects between these two components: PEI25K mainly contributed to the condensation and transfer of pDNA, while thermophilic histone could enhance the endosomal escape and further nuclear location to achieve high gene expression. Meanwhile, HGP showed much lower cytotoxicity and hemolytic activity than PEI25K due to the introduction of nontoxic thermophilic histone. In addition, a strong intrinsic red fluorescence could be obviously observed in HGP. In conclusion, the protein−polymer hybrid carrier could potentially be used as a theranostic delivery system for achieving both efficient gene therapy and in vivo imaging.



In recent years, gene therapy has emerged as a promising strategy for treating cancers, acquired and inherited diseases.[1,2] To achieve a successful gene therapy, it is a key issue to construct efficient gene carriers with high transfection efficiency and low cytotoxicity. Generally, there are two types of gene carriers: viral and nonviral. For viral gene carriers, the disadvantages have dramatically influenced their clinical applications, including limited nucleic acid cargo size, immune response, and mutagenesis.[3,4] Thus, nonviral carriers for gene delivery are significantly needed,[5] especially those with large gene loading capacity, high transfection efficiency, good biocompatibility, and low production cost. In nonviral gene carriers, polycations, such as chitosan, poly(L-lysine) and polyethylenimine (PEI) have been widely used for gene transfection due to large DNA loading capacity and no specific immune response.[6,7] However, their gene transfection efficiency is still limited owing to the existence of extra- and intracellular barriers, and relatively high cytotoxicity also restricted their potential application.

Histones as natural DNA-binding proteins have been considered as a potential type of vehicle for gene delivery due to their unique advantages.[8−14] For the composition of amino acids, histones generally consisted of a high content of positively charged residues lysine and arginine, and exhibited high isoelectric point (>9.0) and thus could form stable nanocomplex with plasmid DNA via electrostatic interaction. Meanwhile, nuclear localization signals have been identified at the N-terminal of histones, which would facilitate the efficient gene delivery and nuclear uptake. In addition, histone-mediated transmembrane was performed in a direct translocation manner (not by a typical endocytosis), which would facilitate the endosomal escape of carrier/DNA nanocomplexes.[15,16] More importantly, synergistic effects have been observed between histones and polymeric gene carriers, such as PEI and its derivative poly(L-glutamic acid)-*g*-PEI.[17−19] However, most of these histones were obtained from calf thymus or chick erythrocyte, and immune response was difficult to be avoided due to their intrinsic characteristic of high molecular weight. Compared with histones of these originations, thermophilic histones from thermophiles are potential to be efficient gene carriers with superior characteristics:[20−24] (1) they possess the basic fold of eukaryotic histones, and thus hold favorable DNA binding and package ability; (2) their molecular weights are much lower (<10 kDa), which will be favorable for decreasing the immunogenicity and cytotoxicity; and (3) they exhibit high stability against thermal, organic solvents, and denaturants, which will be beneficial for further modification or conjugating with other carriers, especially under harsh reaction conditions. Nevertheless, their transfection efficiencies are still lower than polymeric gene carriers, and thus, it is necessary to construct a thermophilic histone−polymer hybrid gene carrier for improving the transfection efficiency and combining their individual advantages.

In the present research, a histone gene GK2215 was cloned from thermophilic bacterium *Geobacillus kastophilus* HTA426,

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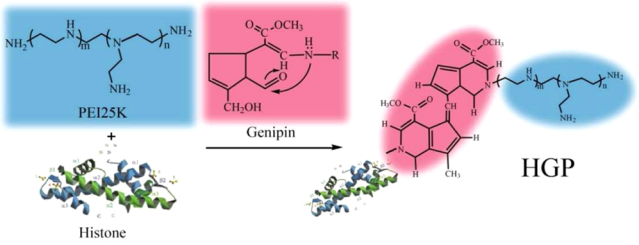
which was first isolated from the deepest sea mud of Mariana Trench[25] and has been sequenced for its genome.[26] The gene was then overexpressed in *Escherichia coli* BL21, and the purified thermophilic histone was chemically cross-linked with PEI25K by genipin to construct the thermophilic histone−PEI25K hybrid gene carrier, namely, HGP (Scheme 1). The

Scheme 1. Synthetic Route of Genipin-Cross-Linked Thermophilic Histone−PEI25K Hybrid Gene Carrier (HGP)



hybrid gene carrier, HGP, was expected to possess the advantages of both thermophilic histone and PEI25K and exhibit improved transfection efficiency due to the synergistic effects between these two components.

In the genome sequence data of *G. kastophilus* HTA426, GK2215 was identified to encode a thermophilic histone of 90 amino acids. Its molecular weight and isoelectric point were predicted to be 9716 Da and 9.53 via an online tool (http://au.expasy.org/tools/pi_tool.html), and thus, it was expected to form stable nanocomplex with pDNA. Through PCR technique, GK2215 gene was successfully cloned with a size of 270 bp (Figure S1). After the successful construction of recombinant *E. coli* BL21 strain harboring GK2215, thermophilic histone was purified through ultrasonication, thermal treatment (75 °C for 30 min), and affinity column chromatography. The purified thermophilic histone exhibited a single band with a molecular weight of 9−10 kDa through Tricine-SDS-PAGE analysis (Figure S2). Gel retardation assay indicated that thermophilic histone exhibited a certain DNA binding ability and could achieve a complete retardation at a carrier/pDNA ratio of 6:1 (w/w; Figure 3F). Meanwhile, thermophilic histone could protect pDNA against high temperatures with favorable pDNA binding and package ability after thermal treatment (Figure S3), which was probably attributed to its relatively stable conformation at high temperatures as shown in circular dichroism analysis (Figure S4). The particle size and zeta potential of thermophilic histone/pDNA nanocomplexes at different mass ratios were then measured (Figure S5). With an increasing ratio of thermophilic histone, zeta potential of nanocomplexes showed a charge reverse of negative charge to positive charge. At a ratio of 1:15, the particle size and zeta potential of nanocomplexes were 71 nm and +6.8 mV, which were suitable for achieving a stable gene transfection.

The in vitro cytotoxicity of thermophilic histone was assessed in HeLa and A549 cells by MTT assay (Figure S6). Thermophilic histone showed almost no effects on the cell viability in the concentration of 0−500 μg/mL, indicating its low cytotoxicity. The in vitro transfection efficiency was then assessed in HeLa cells in the absence of serum (Figure 1). Under an unoptimal transfection ratio (6:1, wt/wt), thermophilic histone exhibited no gene transfection, and a similar profile was observed using PEI25K as the carrier (0.3:1 or 0.6:1,

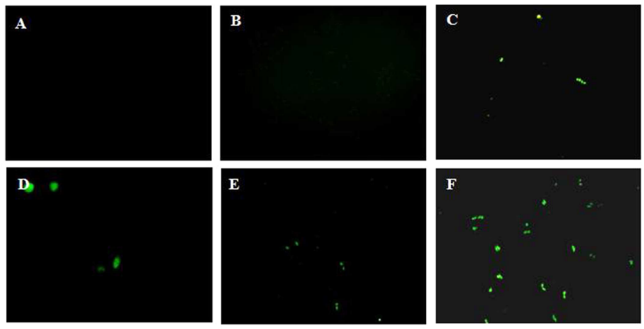


Figure 1. In vitro transfection assay of thermophilic histone and PEI25K, using plasmid pEGFP-N3 as a model: (A) control; (B) histone/pEGFP-N3 (6:1, wt/wt); (C) PEI25K/pEGFP-N3 (0.3:1, wt/wt); (D) PEI25K/pEGFP-N3 (0.6:1, wt/wt); (E) PEI25K/histone/pEGFP-N3 (0.3:6:1, wt/wt); and (F) PEI25K/histone/pEGFP-N3 (0.6:6:1, wt/wt).

wt/wt). The result was a usual phenomenon and probably caused by the fact that the optimal transfection ratio was generally much higher than the critical ratio for a complete pDNA retardation, as free polycation chains (not complexed with pDNA) played an important role for gene transfection.[27] Remarkably, a synergistic effect between thermophilic histone and PEI25K was obviously observed, as the mixture of thermophilic histone and PEI25K could achieve an enhanced gene transfection.

Through the genipin-mediated cross-linking of thermophilic histone and PEI25K, a series of hybrid gene carriers were constructed, namely, HGP (1:10, 1:5, 1:1, 5:1, and 10:1), which represented the molar ratios of thermophilic histone to PEI25K. Genipin is the enzymatic product of geniposide isolated from the fruit of gardenia plane and has been widely used as a cross-linking agent.[28−30] After the reaction, a dark blue was observed in the system, indicating the successful cross-linking of thermophilic histone and PEI25K, as genipin could spontaneously react with amino acids or proteins to form dark blue pigments.[31] The structure of HGP was characterized by FT-IR (Figure S7), and it possessed the characteristic bands of both PEI25K and histone: 3278 cm^{-1} (-N-H- stretching vibration), 2938 and 2815 cm^{-1} (-C-H- stretching vibration), 1124 and 1056 cm^{-1} (-C-N- stretching vibration), and 1461 cm^{-1} (-C-H- scissoring bending vibration) in PEI25K, 3342 cm^{-1} (-N-H- stretching vibration), and 1650 cm^{-1} (amide I vibration) in histone. The intensity at 1241 cm^{-1} (amide III) and 1103 cm^{-1} (-C-N- vibration) in HGP decreased and even disappeared, which was probably caused by the decrease of free ε-NH_2 of lysine during the genipin-mediated cross-linking reaction. Compared with no absorption of thermophilic histone and PEI25K in the UV−vis spectra, HGP showed a strong absorption at 295 nm due to the introduction of genipin (Figure S8). Moreover, HGP exhibited a strong red fluorescence due to the intrinsic red fluorescence of genipin (Figure 2), illustrating that it could keep the unique property of genipin after cross-linking. These results provided direct evidence for the success of genipin-mediated cross-linking of thermophilic histone and PEI25K.

The DNA condensation capacity of HGP was determined by gel retardation assay (Figure 3). All the HGP showed much higher pDNA binding and package ability than thermophilic histone, with critical mass ratios for complete pDNA retardation of 0.4−1.0:1. In addition, lower mass ratio for

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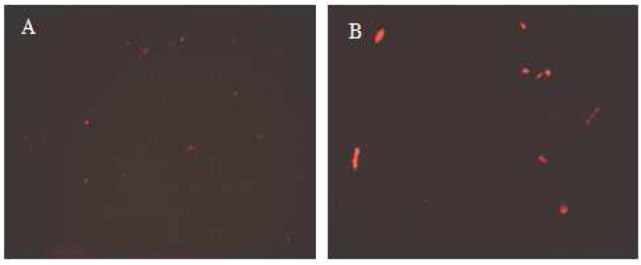

Figure 2. Fluorescence microscopic observation of genipin (A) and HGP 1:1 (B).

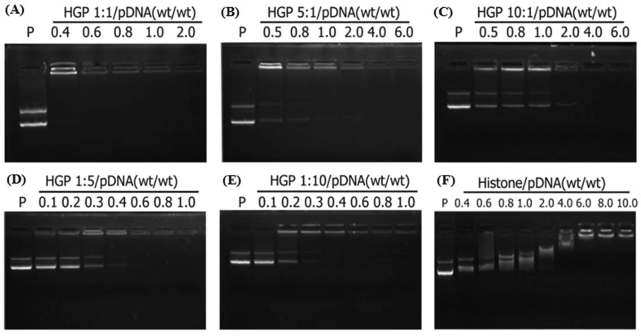

Figure 3. Gel retardation assay for HGP with plasmid pEGFP-N3 at different mass ratios: (A) HGP 1:1; (B) HGP 5:1; (C) HGP 10:1; (D) HGP 1:5; (E) HGP 1:10; and (F) thermophilic histone.

complete pDNA retardation would be observed with the increasing ratio of PEI25K, for example, HGP 1:10 could achieve a complete pDNA retardation at a mass ratio of 0.4:1, which was similar to PEI25K (0.3:1, wt/wt).[7] Thus, the favorable DNA condensation ability of HGP was mainly attributed to the introduction of PEI25K.

The in vitro cytotoxicity of HGP was assessed in HeLa cells by MTT assay (Figure 4). Compared with PEI25K, HGP showed obvious decreased cytotoxicities, for example, the cell viability was maintained to be >50% after the HGP 1:1 treatment at a concentration of 20 μg/mL. Meanwhile, the cytotoxicity showed a decreasing tendency with the increasing ratio of thermophilic histone in HGP, due to the introduction

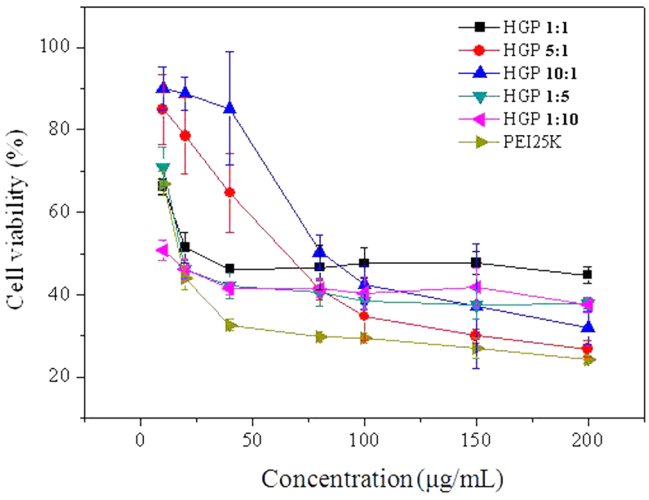

Figure 4. In vitro cytotoxicity analysis of HGP in HeLa cells. The data were expressed as mean value ± SD of three experiments.

of nontoxic component thermophilic histone. Furthermore, in contrary to PEI25K, both thermophilic histone and HGP 1:1 showed no obvious hemolysis in the concentration range of 0–400 μg/mL (Figure S9). Thus, HGP fully integrated the advantages of these two components, higher DNA condensation ability of PEI25K and lower cytotoxicity and favorable hemocompatibility of thermophilic histone.

Finally, in vitro transfection of HGP was assessed in HeLa cells using the plasmids pEGFP-N3 and pGL-3 as models. In the absence of 10% FBS, HGP 1:1 could achieve high transfection efficiency especially at a transfection ratio of 1:2 or 1:4, even higher than Lipofectamine^{2000} (Figure 5). As

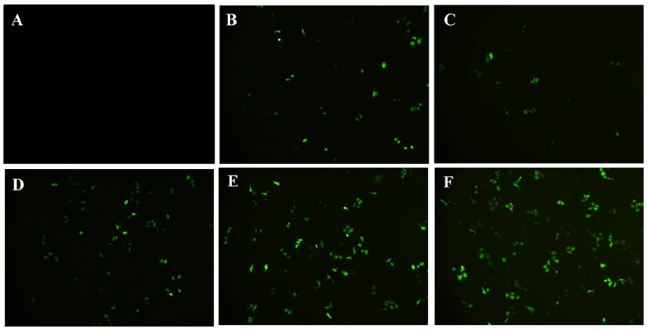

Figure 5. In vitro transfection assay of HGP in the absence of serum: (A) naked plasmid pEGFP-N3; (B) Lipofectamine^{2000}/pEGFP-N3; (C–F) pEGFP-N3/HGP 1:1 at mass ratios of 1:1, 1:1.5, 1:2, and 1:4, respectively.

described above, the addition of histone into polymeric transfection system could enhance the transfection efficiency, due to the synergistic effects between these two components. In this hybrid gene carrier, PEI25K mainly contributed to the condensation and transfer of pDNA, while thermophilic histone could enhance the endosomal escape and further nuclear location. However, in the presence of 10% FBS, the transfection efficiency of HGP would be dramatically decreased (Figure S10). The transfection efficiency of HGP was then quantified using flow cytometry and luciferase activity analysis (Figures S11 and S12). In terms of cell population expressing EGFP in flow cytometry analysis, higher ratio was achieved in HGP with a mass ratio of 1:4 than Lipofectamine^{2000}. Similarly, the highest luciferase activity was also obtained in the same transfection group.

In summary, a protein–polymer hybrid gene carrier was successfully constructed through the genipin-mediated cross-linking of thermophilic histone and PEI25K. The carrier exhibited superior transfection efficiency due to the synergistic effects between these two components. The introduction of hydrophilic histone would not only for decreasing the cytotoxicity but also be favorable for achieving a long circulation time during in vivo applications. Additionally, HGP showed a strong intrinsic red fluorescence owing to the presence of genipin. Thus, these characteristics made HGP a potential theranostic delivery system for realizing the combination of gene therapy and in vivo imaging.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, purification and characterization of thermophilic histone, FT-IR, UV–vis spectra, hemolysis, and in vitro transfection assay of HGP. The Supporting Information

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Notes

The authors declare no competing financial interest.

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