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Branched co-polymers of histidine and lysine are efficient carriers of plasmids

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ABSTRACT

We previously determined that a linear co-polymer of histidine and lysine (HK) in combination with liposomes enhanced the transfection efficiency of cationic liposomes. In the current study, we designed a series of HK polymers with increased branching and/or histidine/lysine ratio to determine if either variable affects transfection efficiency. In the presence of liposomes, the branched polymer with the highest number of histidines, HHK4b, was the most effective at enhancing gene expression. Furthermore, when serum was added to the medium during transfection, the combination of HHK4b and liposomes as a gene-delivery vehicle increased luciferase expression 400-fold compared to liposomes alone. In contrast to linear HK polymers, the higher branched HHK polymers were effective carriers of plasmids in the absence of liposomes. Without liposomes, the HHK4b carrier enhanced luciferase expression 15-fold in comparison with the lesser branched HHK2b carrier and increased expression by 5-logs in comparison with the HHK or HK carrier. The interplay of several parameters including increased condensation of DNA, buffering of acidic endosomes and differential binding affinities of polymer with DNA have a role in the enhancement of transfection by the HK polymers. In addition to suggesting that branched HK polymers are promising gene-delivery vehicles, this study provides a framework for the development of more efficient peptide-bond-based polymers of histidine and lysine.

INTRODUCTION

Successful *in vivo* gene therapy depends on the development of efficient, non-toxic gene delivery systems. Although viral-based gene therapy is efficient in transducing cells (1), safety concerns with respect to the use of viral vectors (2) have increased the attractiveness of non-viral carriers. Non-viral methods of gene delivery often include cationic liposomes and polymers. Efficient gene expression with these non-viral carriers depends on a number of interactions between the cell and the transfection complex. These interactions include binding to the cellular membrane and uptake of the complex by endocytosis (3), translocation of DNA from the endosomes to the cytosol (4) and nuclear transport of DNA. Although the mechanism of nuclear transport is not clear, several studies suggest that nuclear localization signal-mediated pathways are involved (5). To increase the transmembrane passage of DNA from endosomes into the cytoplasm, numerous helper molecules including lipids (6), lysosomotropic agents (7,8), glycerol (9) or fusogenic peptides (10) have been used. Moreover, cationic polymers including polyethylenimine (PEI), histidylated polylysine, polyamidoamine and Superfect (11–15) often increase transfection because they buffer acidic endosomes, which facilitates DNA release from endosomes. In addition to endosomal buffering, cationic polymers are frequently branched to increase transfection. The branched polymers effectively condense DNA more tightly (16,17), a property associated with increased uptake.

Similar to polymers, cationic liposomes are commonly used as non-viral carriers (18). Cationic liposome–DNA complexes (lipoplexes) have been used in several clinical trials for the treatment of cancer and cystic fibrosis (19,20), and are safe when administered locally in low dosages. Nevertheless, the efficiency of cationic liposomes needs to be improved (21,22). One limiting factor to using liposomes as carriers of DNA is that the DNA is not tightly packaged (23). In order to increase the condensation of DNA and transfection efficiency of liposomes, basic proteins with a high lysine content have been mixed with liposome and DNA complexes. Basic proteins such as protamine or poly-L-lysine have been found to reduce the size of liposome–DNA complexes 6-fold (24). Concomitant with the reduction in size, such proteins increase transfection of liposome–DNA complexes by as much as 10-fold (25,26). To augment further transfection efficiency of liposomes, our laboratory recently determined that a linear histidine and lysine (HK) co-polymer combined with liposomes increased gene expression compared to either the combination of liposome and polylysine carriers or to liposome carriers alone (27). The HK co-polymer with condensing and buffering properties, in combination with liposomes, increased luciferase expression in the presence of serum by >100-fold compared to the liposome–DNA complex alone. In contrast, luciferase expression was not measurable when the linear HK polymer alone was used as a carrier.

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In this proposal, we modified the HK carrier by varying the complexity and the histidine/lysine ratio. We found that branched HK polymers with a higher percentage of histidines, in combination with liposomes, enhanced gene expression. Furthermore, one polymer, HHK4b, was an effective carrier of plasmids even without liposomes.

MATERIALS AND METHODS

Cells

MDA-MB-435, MDA-MB-231 and MCF7 (three breast cancer cell lines), as well as CRL-5800 (a non-small cell lung cancer cell line; ATCC, Manassas, VA) and Chinese hamster ovary (CHO) were maintained in DMEM containing 10% fetal calf serum (FCS) and 20 mM glutamine.

Polymers

The biopolymer core facility at the University of Maryland synthesized the polymers on a Ranin Voyager synthesizer (PTI, Tucson, AZ). The following polymers were made: (i) HK (19mer; molecular weight 2454), |K-H-K-H-K-H-K-H-K-G-K-H-K-H-K-H-K-H-K|; (ii) HK2b (39mer; molecular weight 4758), |K-H-K-H-K-H-K-H-K-G-K-H-K-H-K-H-K-H-K|^2 K; (iii) HK3b (59mer; molecular weight 7654), |K-H-K-H-K-H-K-H-K-G-K-H-K-H-K-H-K-H-K|^3 K-K; (iv) HK4b (79mer; molecular weight 9684), |K-H-K-H-K-H-K-H-K-G-K-H-K-H-K-H-K-H-K|^4 K-K-K; (v) HHK (20mer; molecular weight 2608), |K-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-H-K|; (vi) HHK2b (41mer; molecular weight 5232), |K-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-K|^2 K; (vii) HHK3b (62mer; molecular weight 7900), |K-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-K|^3 K-K; and (viii) HHK4b (83mer; molecular weight 10 570), |K-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-H-K-H-K|^4 K-K-K. The polymers were then purified on an HPLC (Beckman, Fullerton, CA) and analyzed by mass spectroscopy (Perseptive Biosystems, Foster City, CA) to verify the predicted molecular mass. The branched polymers consist of polymers emanating from a lysine core in which the lysine core is uncharged (Fig. 1).

Preparation of liposomes

Preparation of the liposome–plasmid complexes has been previously described. In brief, DH5α bacteria (Life Technologies, Gaithersburg, MD) containing the plasmids were grown in Superbroth to mid-log phase. The plasmids were then purified with Qiagen columns. An analytical gel of each plasmid (cut and uncut) ensured that there was no contamination with other nucleic acids. Liposomes were composed of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Avanti, Birmingham, AL). After hydration of the lipids, the liposomes were sonicated until clear with a Branson 1210 bath sonicator in the presence of argon. The liposomes were then extruded through 50 nm polycarbonate membranes with LipsoFast-Basic (Avestin Inc., Ottawa, Canada). The final liposome concentration was 1 μg/μl. Several cationic liposomes were also purchased including lipofectin (Life Technologies), lipofectamine (Life Technologies) and 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propylamid (DOSPER) (Roche).

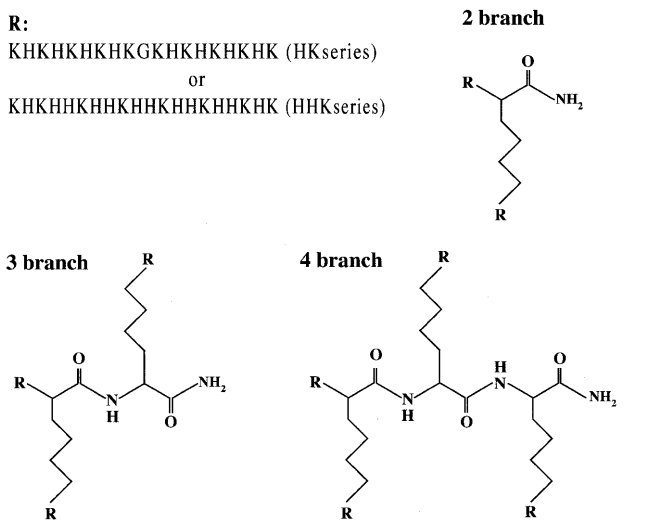

Figure 1. Schematic structure of HK branched polymers. The branched polymers consist of polymers emanating from a lysine core. Depending on the complexity of branching, the lysine core is composed of one, two or three lysines. R represents KHKHKHKHKGKHKHKHKHK for the HK series or KHKHHKHHKHHKHHKHHKHK for the HHK series.

In vitro transfection studies

Initially, 4.5×10^4 cells of MDA-MB-435 cells were plated into a 24-well plate. After 48 h, when the cells were 60 and 80% confluent, cells were transfected with a plasmid-encoding luciferase (PCI-luc). In transfection experiments, the co-polymer was initially incubated with 0.75 μg of PCI-luc for 30 min in OptiM. The amount of co-polymer varied from 0.06 to 7.5 nmol. Then, 1.5 μg (2.15 nmol) of cationic liposomes were added to the polymer–DNA mixture, and allowed to stand for an additional 30 min. The total volume for the polymer–liposome–DNA complex was 120 μl. The polymer–liposome–DNA complex was then diluted with either 200 μl of OptiM or OptiM + 15% serum. In experiments with lysosomotropic agents, bafilomycin A_1 (Sigma) was added to the medium at concentrations of 10 ng/ml. Four hours after transfection, the complexes were removed and DMEM with 10% serum was added. Forty-eight hours later, luciferase levels were measured with the direct current TD 20/20 luminometer (Turner Design, Sunnyvale, CA) as previously described. Relative light units were converted to protein (pg) of luciferase with recombinant luciferase (Promega, Madison, WI) as a standard. Duplicates were done for each concentration and each experiment was performed twice.

Measurement of particle size of polymer–DNA–liposome complexes

PCI-luc (2 μg in 200 μl of 10 mM HEPES pH 7.4) was mixed with 21 nmol of linear polymer or 1.05 nmol of branched polymer in 200 μl HEPES for 30 min. Then, 7 μg of liposome in 200 μl HEPES was added for another 30 min. After adding 1900 μl of HEPES to the complexes, a total volume of 2.5 ml was used for the measurement, which was carried out with a N4 Submicron Particle Sizer (Coulter, Miami, FL).

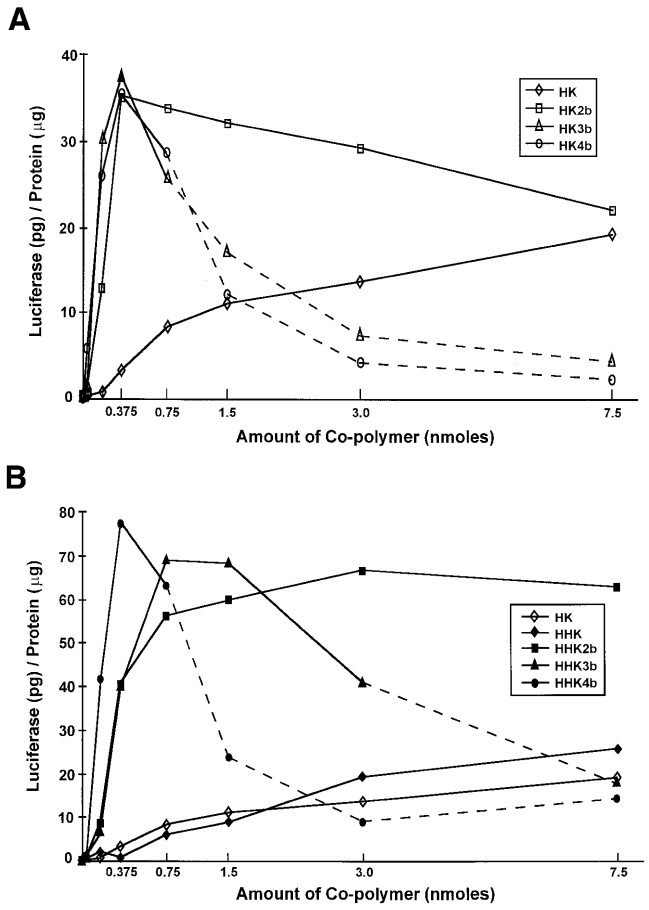

Figure 2. Effect of HK and HHK branching polymers on transfection efficiency of liposome. (**A**) Comparison of HK with branched polymers. Four polymers (HK, HK2b, HK3b and HK4b) at various concentrations (0.06, 0.18, 0.375, 0.75, 1.5, 3.0 and 7.5 nmol) with liposomes were examined for their ability to increase luciferase expression in MDA-MB-435 cells. The polymer–liposome–plasmid DNA complexes were prepared as described in Materials and Methods. The dashed lines represent the polymer dosage toxic to cells. (**B**) Five polymers (HK, HHK, HHK2b, HHK3b and HHK4b) were compared at various concentrations as described in (A).

RESULTS

Comparison of the linear and branched HK polymers for their ability to increase transfection efficiency

We have previously shown that a linear HK co-polymer markedly enhances transfection of liposomes. To investigate if branched polymers enhance transfection efficiency more effectively than the linear HK polymer (Fig. 1), we designed several branched HK polymers. We compared the following four HK polymers in order of increasing complexity for their ability to improve transfection: HK (linear), HK2b, HK3b and HK4b. At their respective optimal concentrations, the branched HK polymers increased gene expression ~2-fold more than the linear HK polymer ($P < 0.05$, HK2b, HK3b and HK4b versus HK) (Fig. 2A). Furthermore, at a low dose of polymer (0.18 nmol), the branched polymers stimulated transfection 20-fold more than the HK polymer ($P < 0.001$, HK2b, HK3b and HK4b versus HK) (Fig. 2A). At the respective polymer concentration that enhanced gene expression optimally, the stoichiometric binding of DNA for HK was 67.5, for HK2b it was 7.0 and for HK4b it was 3.32. Notably, the higher branched HK3b and HK4b polymers, in contrast to HK2b, exhibited more toxicity at higher dosages as indicated by decreasing luciferase and cellular protein values.

Polymers with an increased histidine/lysine ratio influence transfection

Since polymers with increased buffering capacity may increase gene expression, we examined whether increasing the ratio of histidine to lysine would enhance the efficacy of the HK polymer. We designed polymers with an increased histidine/lysine ratio derived from the linear polymer HHK (Fig. 1). Notably, even though there was little difference in the transfection enhancement between the linear HK and HHK polymers, the HK branched polymers were significantly less effective transfection agents than the branched HHK agents (Fig. 2A and B). In general, the more branched the HHK derivative, the greater was its ability to augment transfection (Fig. 2B). Compared to HK, branched polymers at their optimal concentration increased stimulation by the following amounts: HHK4b, 4-fold; HHK3b, 3.8-fold; and HHK2b, 3.7-fold. Furthermore, HHK4b was >2-fold more effective than HK4b in enhancing liposome-mediated luciferase expression (Fig. 2A and B). Similar to branched derivatives of HK, branching of the HHK polymer reduced the amount of polymer required to achieve optimal transfection (Fig. 2B). When a low dose of polymer (0.375 nmol) was used, HHK4b enhanced gene expression 40-fold more than the HK polymer ($P < 0.001$). Although HHK2b was slightly less effective than the more branched derivatives, one advantage of this polymer is that it stimulates transfection over a wide range of concentrations with little toxicity. Similar to the branched HK derivatives, the stoichiometry of binding DNA decreased as the branching and complexity of the HHK derivative increased. At the optimal concentration for transfection, stoichiometric binding was 50.4 for HHK, 5.15 for HHK2b and 2.54 for HHK4b. For subsequent experiments, we have focused on comparing branched polymers (HK4b and HHK4b) with linear polymers (HK and HHK) using their respective optimal dose.

Branched HHK4b polymer enhances gene expression in several cell lines

In addition to MDA-MB-435 cell lines, we also determined that the branched HHK4b polymer in combination with liposomes were more effective than the linear HK polymers in several other cell lines including MDA-MB-231, CHO, CRL-5800 and MCF7 cell lines (Fig. 3).

Influence of linear and branched polymer on the size of DNA–liposome complexes

Agents that condense DNA and reduce the size of lipoplex particles are considered to be critical for increasing transfection. We previously determined that addition of HK reduced the size of DNA–liposome complexes by ~1.7-fold in the absence of serum (27). Here we compared the effect of linear and branched polymers on the particle size of the liposome–DNA complex with an N4 Submicron Particle Sizer. The size of the liposome–DNA complex was significantly reduced by the addition of the branched polymers, HK4b or HHK4b (Fig. 4).

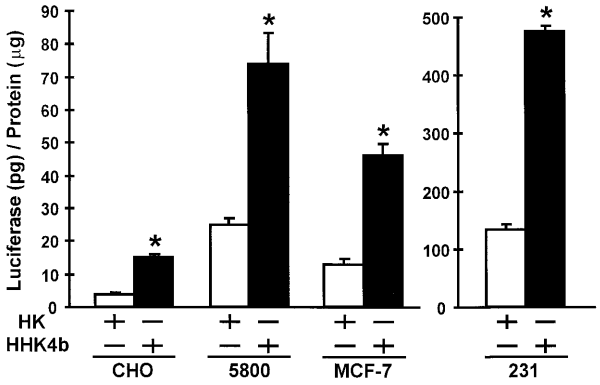

Figure 3. Comparison of gene expression in several cell lines with HK or HHK4b in combination with liposomes. The HK (7.5 nmol) or HHK4b (0.375 nmol) co-polymer was initially mixed with 0.75 μg of DNA (PCI-luc) before adding 1.5 μg of DOTAP liposomes. After transfection of either MDA-MB-231, CHO, CRL-5800 or MCF7 cells, luciferase activity was determined 48 h later. Due to marked differences in transfection, the scale for MDA-MB-231 varies from other cells. *, $P < 0.05$, HHK4b–liposome versus HK–liposome in CHO, CRL-5800, MCF7 cells and MDA-MB-231.

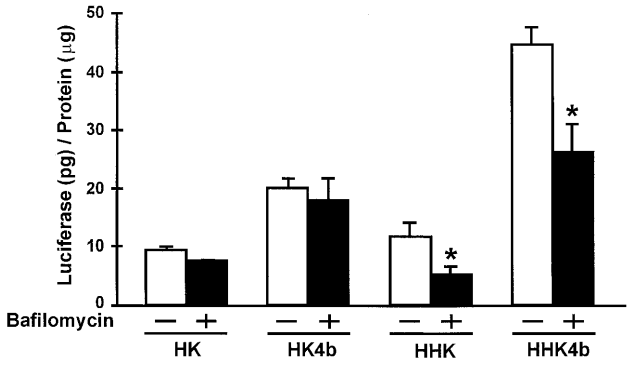

Figure 5. Effect of bafilomycin A_1 on the transfection efficiency of polymers. Either 7.5 nmol of HK and HHK or 0.375 nmol of HK4b and HHK4b was mixed with DNA and liposome. After the polymer–liposome–PCI-luc was prepared, the complexes and 10 ng/ml of bafilomycin were added to the cells for 4 h. The cells were then washed, and luciferase activity was measured 48 h later. *, $P < 0.05$, HHK with bafilomycin versus HHK without bafilomycin, HHK4b with bafilomycin versus HHK4b without bafilomycin, Mann–Whitney rank-sum test.

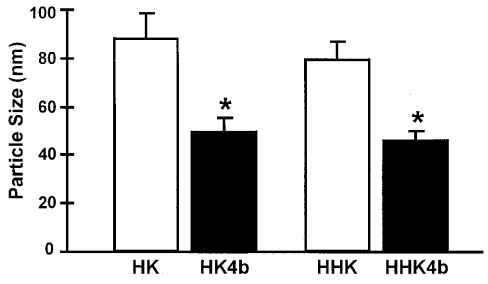

Figure 4. Effect of linear and branched polymers on particle size of complexes. Two micrograms of PCI-luc in 200 μl of 10 mM HEPES pH 7.4 were mixed to form complexes with 21 nmol of HK and HHK or 1.05 nmol of HK4b and HHK4b in 200 μl HEPES for 30 min. Then, 7 μg of liposome in 200 μl HEPES were added for another 30 min. After adding 1900 μl of HEPES, the complex particle size was measured by the N4 Plus Submicron Particle Sizer as described in Materials and Methods. *, $P < 0.05$, HK versus HK4b, HHK versus HHK4b, Mann–Whitney rank-sum test.

Thus the branched polymer reduced the particle size, probably by enhancing the condensation of DNA.

Effects of bafilomycin A_1 on DNA transfection mediated by the HK and HHK series

The buffering capacity of histidine may have a significant role in augmenting the transfection efficiency of the polymer. Since HHK4b is more effective than the HK4b co-polymer, the number of histidines and consequently the ratio of histidines to lysines may be important. In HHK4b, the ratio of histidines to charged lysines is 1.5, whereas in HK4b, the histidine to lysine ratio is 0.8. As a result, there are 16 more histidines per molecule of HHK4b than there are in HK4b. In order to determine if the buffering capacity of the various HK and HHK derivatives increased transfection, we asked if the lysosomotropic buffering agent bafilomycin A_1 would affect polymer-mediated transfection (14,27,28). By buffering acidic endosomes, bafilomycin A_1 most effectively reduces gene expression of carriers with the greatest buffering capacity. In the current study, bafilomycin A_1 had little effect on liposome-mediated gene expression with the linear HK or branched HK4b polymer (Fig. 5). In contrast, bafilomycin A_1 reduced liposome-mediated gene expression significantly in the presence of HHK or HHK4b polymer.

Branched polymer enhances transfection of several liposome preparations

We examined if the HK and HHK4b co-polymers increased gene expression in MDA-MB-435 cells with various liposomal preparations. Three of these cationic liposomes were commercially obtained (lipofectin, lipofectamine and DOSPER) and the DOTAP liposomes were made in our laboratory. With these four liposomal preparations, both HK and HHK4b markedly increased transfection more than liposomes alone as carriers (Fig. 6). Similar to DOTAP liposomes, the HHK4b stimulated gene expression more than HK with the other three liposomal preparations.

In the absence of liposomes, the branched polymer enhances transfection efficiency of plasmid

Without liposomes, the HK polymer had little effect on transfection efficiency. In contrast, branched polymers increased gene expression in the absence of liposomes, and efficiency was positively correlated with the degree of branching. Furthermore, the transfection enhancement was significantly greater with the HHK series than with the HK series. Without liposomes, luciferase activity was in many cases not detectable with the HK, HHK or HK2b polymers. In contrast, HHK4b, the most effective polymer without liposomes, increased luciferase expression by 15- and 10^5-fold (Fig. 7A) when compared to HHK2b and HK polymers, respectively. The luciferase values for HK, HHK2b and HHK4b were 1.7×10^{-4}, 1.45 and 37.4 luc (pg)/lysate protein (μg), respectively.

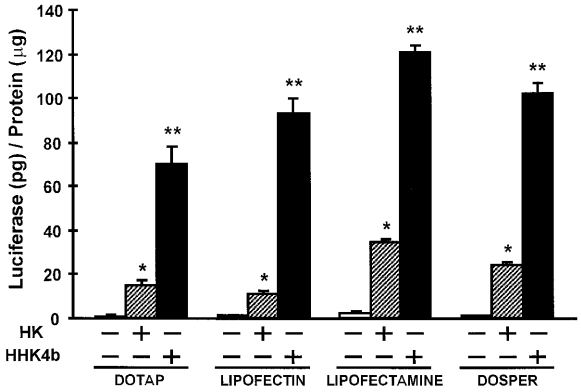

Figure 6. Branched polymer enhances transfection of several liposome preparations. HK (7.5 nmol) or HHK4b (0.375 nmol) co-polymer was initially mixed with 0.75 μg of DNA (PCI-luc) for 30 min, various liposome preparations were then added for an additional 30 min. Forty-eight hours later luciferase activity was measured. The following liposome amounts were added: DOTAP, 1.5 μg; lipofectin, 1 μg; lipofectamine, 5 μg; and DOSPER, 1.5 μg. *, $P < 0.05$, HK versus no polymer with all liposome preparations; **, $P < 0.05$, HHK4b versus HK with all liposome preparations.

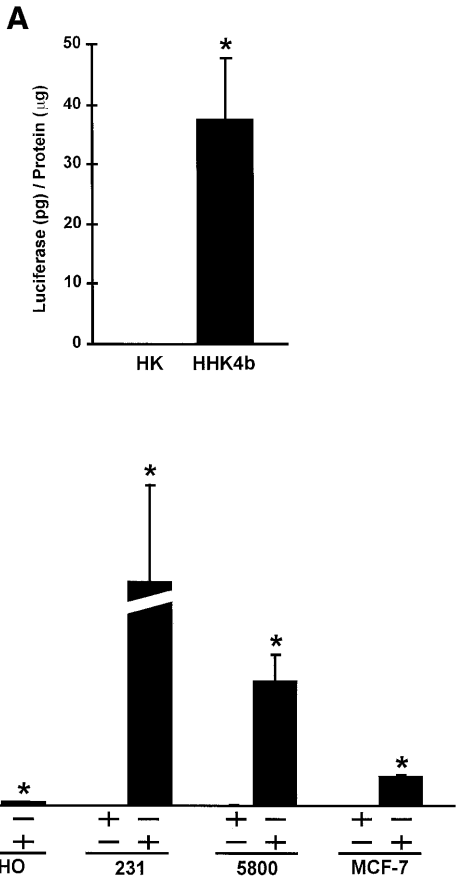

Figure 7. Branched polymer increases transfection efficiency of plasmid. (**A**) 7.5 nmol of HK or 0.375 nmol of HHK4b was mixed with PCI-luc, and luciferase activity was measured in MDA-MB-435 cells as described in Materials and Methods. *, $P < 0.05$, HHK4b versus HK, Student's *t*-test. (**B**) In addition to MDA-MB-435 cells, HHK4b as a sole carrier was significantly better in augmenting gene expression compared to HK in CHO, MDA-MB-231, CRL-5800 and MCF7 cells. *, $P < 0.05$, HHK4b versus HK in CHO, MDA-MB-231, CRL-5800 and MCF7, Student's *t*-test.

Similarly, HHK4b as a sole carrier enhanced gene expression significantly more than the HK carrier in several cell lines in addition to the MDA-MB-435 cells (Fig. 7B).

Influence of serum on the transfection of polymer–DNA–liposome

A significant problem with non-viral liposomal delivery systems is their low transfection efficiency, particularly in the presence of serum. We have shown previously that, in the presence of serum, the HK co-polymer increased transfection of DNA–liposome up to 100-fold compared to liposome–DNA complexes (27). When used in combination with liposomes, the HK4b or HHK4b polymers enhanced luciferase activity by 2- and 4-fold, respectively, over the HK polymer in the presence of serum (Fig. 8) ($P < 0.05$, HK4b or HHK4b versus HK polymer). Furthermore, the combination of HHK4b and liposomes as a gene-delivery vehicle increased luciferase expression by 400-fold compared to liposomes alone when serum was added to the medium during transfection. With serum, HK4b and HHK4b were not cytotoxic at 0.7 nmol, a concentration markedly above the dose required for maximal transfection efficiency.

DISCUSSION

We previously demonstrated that linear HK co-polymers, when combined with cationic liposome, were highly efficient carriers of plasmids. There are several possible mechanisms by which the HK co-polymer may increase transfection of liposome carriers. For example, the lysine moiety of the co-polymer is thought to neutralize, in part, the negative charge of the plasmid DNA, the histidine buffers and aids in the release of plasmid DNA from the endosomal vesicle, and the cationic liposomes neutralize the remainder of DNA charge and provide a scaffold for the polymer–DNA complex. The linear HK has little effect on transfection without liposomes, however, indicating that cationic liposomes may aid in compacting

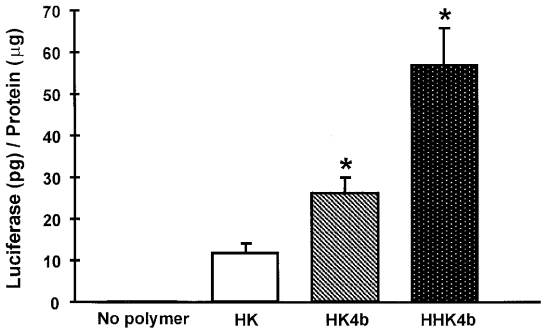

Figure 8. Branched polymers increase the transfection efficiency in the presence of serum. The optimal dosage of HK (7.5 nmol), HK4b (0.375 nmol) or HHK4b (0.75 nmol) was mixed with 0.75 μg of PCI-luc and 1.5 μg of liposome. The complex was added to the cells for a 4 h incubation in the presence of 10% FCS. *, $P < 0.05$, HK4b versus HK or HHK4b versus HK. Anova on Ranks with Dunnet's multiple comparison.

DNA efficiently (27). Although investigators have reported that a histidylated polylysine polymer by itself was an efficient carrier of plasmids, it was significantly less effective than PEI in most cell lines tested (14). In contrast, the linear HK copolymer combined with liposomes was a markedly better carrier than PEI in MDA-MB-435 cells (27).

In the present study, we determined that both the degree of branching and an increased ratio of histidine to lysine significantly affect the ability of HK polymers to enhance transfection. Increased gene expression from either the HK4b or HHK4b polymer compared to their linear counterparts appears to correlate with a decreased size of liposome–DNA complexes. The condensed structure of the carrier and DNA may undergo endocytosis by the cell more readily, resulting in augmentation of transgene expression. Although reduction in the size of liposome–DNA complexes was similar with the HHK4b and HK4b polymers, HHK4b significantly enhanced luciferase expression compared to HK4b. As a result, reduction in size of the transfection complex is not the sole determinant of HHK4b's ability to enhance transfection.

Luciferase expression depends on uptake, endosomal release and nuclear transport of DNA (3–5). Thus, although cellular uptake of plasmids may be enhanced similarly by these two branched polymers because of the condensed carrier–DNA complex, the marked differences between HK4b and HHK4b may also be due to augmentation of endosomal buffering with resultant DNA release from endosomes (3–5). Since HHK4b has a higher percentage of histidines per molecule than HK4b, it is likely that the HHK4b polymer is more effective at buffering the endosomal pH of cells. Enhanced buffering of endosomal pH by the carrier has been associated with a higher transfection (11–14). Consequently, the ability of HHK4b to enhance transfection through increased buffering is consistent with inhibition by the pH buffering agent, bafilomycin, on HHK4b-mediated transfection. In addition to buffering and condensation, we are currently investigating if binding affinities of the polymers and DNA play a role in transfection efficiency.

We are particularly encouraged by the results obtained with the HK2b and HHK2b polymers. Both of these polymers in combination with liposomes had a broad therapeutic range with little toxicity, and the maximal enhancement of gene expression with 2b polymers is similar to that of the higher branched polymers. In contrast to the 2b series, polymers with greater complexity from either series (HK3b, HHK3b, HK4b and HHK4b) when used in combination with liposomes had a more narrow therapeutic range with cellular toxicity evident at the higher concentrations. We think that it is unlikely that the histidine moiety contributes significantly to the toxicity due to the similar toxicity of the branched HK3b, HK4b, HHK3b and HHK4b series. It is more probable that lysines in a branched configuration increase toxicity. Although we have found that serum significantly reduces the toxicity of these complexes, it is not clear what additional factors may further mitigate toxicity. Nevertheless, when used in combination with liposomes, the 2b series may be useful *in vivo* due to their low toxicity exhibited *in vitro*.

To optimize further HK polymers as carriers, we believe that peptide synthesizers will be critical to titrate specific ratios and sequence order of histidines and lysines. This study underscores the hypothesis that relatively small differences in the complexity and composition of HK polymers profoundly influence their ability to stimulate gene expression. In future studies, we will continue to modify the design of the HK polymer to further improve their properties as carriers.

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