

Rapid Communication

**A BIOREDUCIBLE POLYPEPTIDE FOR EFFICIENT GENE TRANSFECTION
BOTH *IN VITRO* AND *IN VIVO****

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Abstract A disulfide bond containing polypeptide, PolyK₆-R₈, was designed and prepared by oxidative polymerization between terminal cysteinyl thiol groups of CHK₆HC and CR₈C. The molar ratio between CHK₆HC and CR₈C within obtained PolyK₆-R₈ was 9:1, and PolyK₆-R₈ could combine DNA compactly when weight ratio reached 5. Through *in vitro* investigation, it was found that PolyK₆-R₈ was an efficient gene vector with low cytotoxicity for delivering DNA in both COS-7 and HeLa cells. Cellular uptake of DNA mediated by PolyK₆-R₈/DNA complexes was promoted after incubation for 4 h. Moreover, by examining the histological sections of hindlimb ischemia rats through immunohistochemistry, PolyK₆-R₈/VEGF complexes were proved to be effective in both VEGF protein expression and succeeding vessel formation. The results indicated that polypeptide-based PolyK₆-R₈ is a promising gene vector for the limb ischemia treatment.

Keywords: Gene vector; Polypeptide; Angiogenesis; Limb ischemia.

As a potential lethal disease caused by peripheral circulation obstacle, critical limb ischemia (CLI) have attracted increasing research interests in recent years. In America, limb ischemia diseases result in over 150000 amputation operations each year^[1]. In the past decades, traditional treatments such as molecular and cell-based therapies made no remarkable progress in hindlimb ischemia animals by collateral artery enlargement^[2]. Currently, the novel approach of clinical CLI study is therapeutic vessel formation based on promoting of angiogenesis^[3,4]. Vascular endothelial growth factor (VEGF) is a specific mitogen for endothelial cells, and its improvement effects of angiogenesis have been widely confirmed^[5]. By utilizing gene therapy involved naked VEGF plasmid DNA or adenovirus/VEGF complexes, it has been proved to be positive effects in peripheral artery diseases and clinical ischemic heart trials^[6,7].

Viral vectors and non-viral vectors are two types of vehicles for gene delivery^[8,9]. Despite their outstanding transfection efficiency, viral vectors are highly infective and inclined to cause immunological responses. Therefore, non-viral vectors with good biocompatibility are more feasible to apply as a safer carrier for gene therapy^[10–12]. Among non-viral vectors, cationic peptides with inherent biodegradability are newly developed for gene delivery^[13]. Lysine-rich sequence is regarded as a DNA condensing peptide which is effective for DNA combination. Arginine-rich sequence is frequently used as a cell penetration peptide (CPP) for facilitating the endocytosis of gene vectors. Both peptides have been proved to be contributed to overcoming the barriers in

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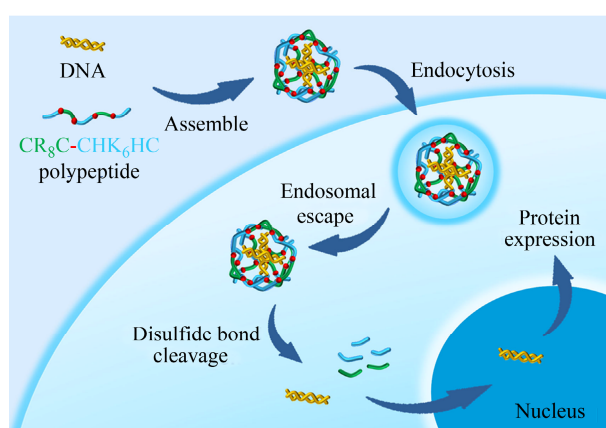
Invited paper dedicated to Professor Fosong Wang on the occasion of his 80th birthday

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gene delivery effectively^[14–16]. After gene vectors entering the cell membranes, endosomal escape and nuclear transport proceed subsequently. Meanwhile, glutathione (GSH) is a reductant existing in cytoplasm with a certain concentration. It was found that intracellular glutathione could react with disulfide bonds and induce bond cleavage between vector molecules, which reduce endocellular accumulation of vectors and facilitate cellular transfection^[17]. Furthermore, gene vectors containing disulfide bonds are stable outside cellular environment. These vectors can bind DNA more effectively, which keeps DNA from being degraded by enzymes^[18].

To utilize the transfection bioactivity of peptides and the sensitivity advantage of disulfide bonds, we designed a polypeptide-based vector fabricated from lysine-rich sequence, CHK_6HC , and arginine-rich sequence, CR_8C , for gene delivery. Through oxidative polymerization between terminal cysteinyl thiol groups of CHK_6HC and CR_8C , a bio-reducible polypeptide containing disulfide bonds was obtained. The schematic illustration of protein expression mediated by polypeptide vector is shown in Scheme 1.



Scheme 1 Schematic illustration of protein expression mediated by polypeptide vectors

By using standard solid phase peptide synthesis technique, CHK_6HC and CR_8C were prepared manually according to classical Fmoc chemistry^[19]. The molecular weights of the peptides were confirmed by electrospray ionization mass spectrometry (ESI-MS): $[\text{M}+\text{H}]^+$ of CHK_6HC was found 1269.5, while calculated was 1269.6; $[\text{M}+\text{H}]^+$ of CR_8C was found 1473.8, while calculated was 1473.8. The polypeptide, $\text{PolyK}_6\text{-R}_8$, was obtained by oxidative polymerization of CHK_6HC and CR_8C in DMSO solution^[20–22]. The structure of the polypeptide was analyzed by $^1\text{H-NMR}$ by a Varian Unity 300 MHz spectrometer using D_2O as the solvent. The molar ratio was 9:1 between CHK_6HC and CR_8C . Gel permeation chromatography and multiangle laser light scattering (GPC-MALLS) analysis were employed to assess the molecular weight distribution of $\text{PolyK}_6\text{-R}_8$. A dual detector system consisting of a MALLS device (DAWNEOS, Wyatt Technology) and an interferometric refractometer (Optilab DSP, Wyatt Technology) was adopted. 0.1 mol/L NaNO_3 solution and 0.1 mol/L HAc/NaAc (pH 4.2) buffer solution were used as the eluent at a flow rate of 0.3 mL/min. The MALLS detector was operated at a wavelength of 690.0 nm. The molecular weight (M_w) and polydispersity (M_w/M_n) of $\text{PolyK}_6\text{-R}_8$ was 9200 and 1.46, respectively. Through agarose gel retardation assay, the $\text{PolyK}_6\text{-R}_8/\text{DNA}$ complexes could combine DNA compactly when the weight ratio reached 5 (Fig. 1a). The result indicated that cationic $\text{PolyK}_6\text{-R}_8$ could bind negatively charged DNA effectively. As shown in Fig. 1(b), after treated within reducing conditions of 20 mmol/L DTT for 15 min, $\text{PolyK}_6\text{-R}_8/\text{DNA}$ complexes (8, W/W) could not combine DNA firmly, and smeared DNA band was observed. This outcome represented the successful break of disulfide bonds within $\text{PolyK}_6\text{-R}_8$, which might improve DNA release and decrease cytotoxicity of $\text{PolyK}_6\text{-R}_8/\text{DNA}$ complexes in cytoplasm.

The cytotoxicity of $\text{PolyK}_6\text{-R}_8$ was evaluated with COS-7 and HeLa cells by MTT assay. 25 kDa PEI was used as a control. As illustrated in Fig. 2(a) and Fig. 2(b), the cell viability of $\text{PolyK}_6\text{-R}_8$ was higher than 80% in

both COS-7 and HeLa cells. Compared with 25 kDa PEI, PolyK₆-R₈ exhibited no obvious cytotoxicity, which could be attributed to the good biocompatibility of peptide. In addition, the endocellular disruption of disulfide bonds within PolyK₆-R₈ could decrease the surface positive charge of polypeptide, which might reduce the cytotoxicity of PolyK₆-R₈ as well. The cytotoxicity of PolyK₆-R₈/DNA complexes was also studied in COS-7 and HeLa cells. The cell viability of PolyK₆-R₈/DNA complexes was higher than 90% in both cells (Fig. 2c and Fig. 2d). The results indicated that PolyK₆-R₈/DNA complexes were safe for *in vitro* transfection study.

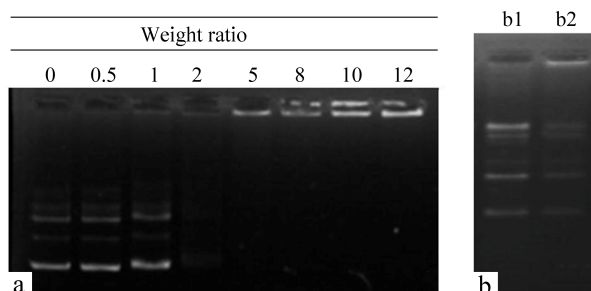


Fig. 1 Agarose gel electrophoresis retardation assay of (a) PolyK₆-R₈/DNA complexes at weight ratios ranging from 0 to 12, and (b) PolyK₆-R₈/DNA complexes at weight ratio of 8 incubated with 20 mmol/L DTT: (b1) Naked DNA and (b2) DTT treated PolyK₆-R₈/DNA complexes

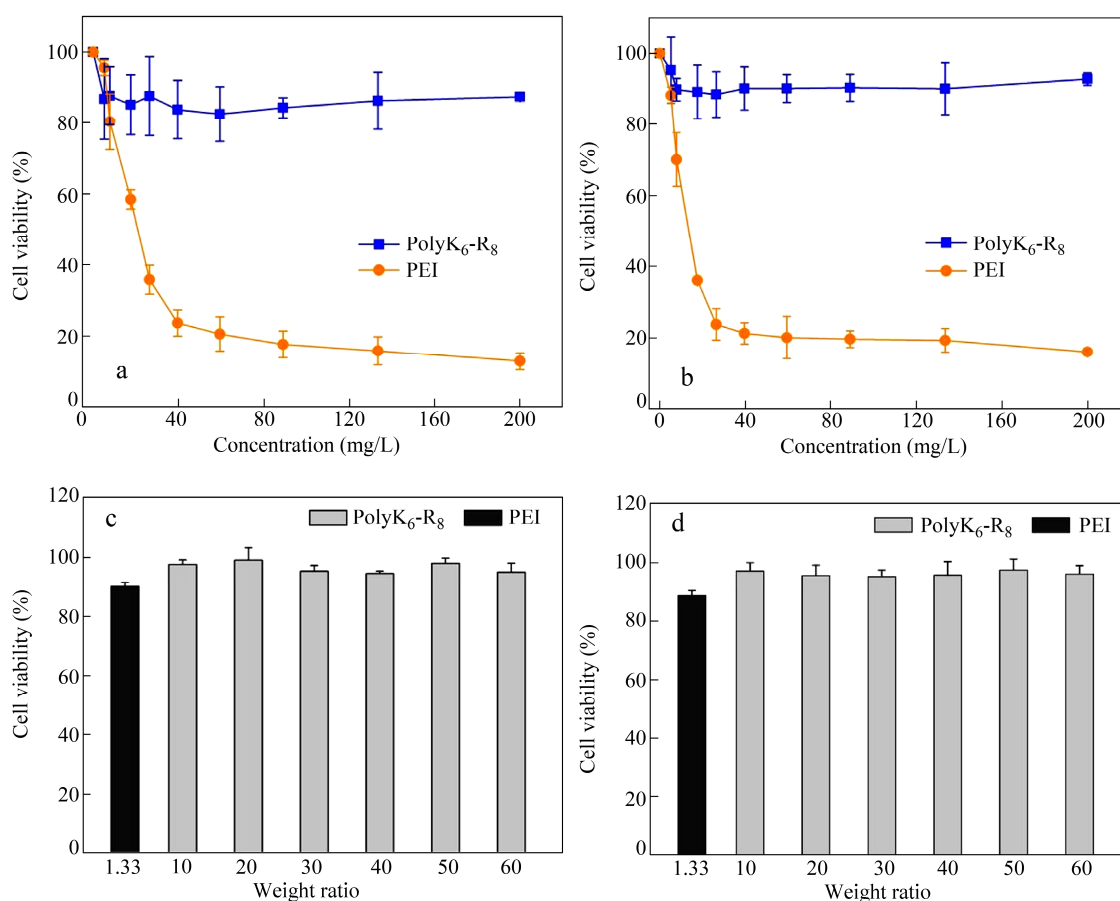


Fig. 2 Cytotoxicity of PolyK₆-R₈ in (a) COS-7 and (b) HeLa cells and cytotoxicity of PolyK₆-R₈/DNA complexes in (c) COS-7 and (d) HeLa cells. 25 kDa PEI was used as the control. Data were shown as mean $\pm$ SD ($n = 3$).

The transfection efficiency of PolyK₆-R₈/DNA complexes was evaluated with pGL-3 plasmid. As shown in Fig. 3(a) and Fig. 3(b), PolyK₆-R₈/DNA complexes exhibited similar transfection efficiency in both COS-7 and HeLa cells. At the ratio of 30, the luciferase expression of PolyK₆-R₈/DNA complexes reached the maximum value as 7.16×10^7 RLU/mg protein in COS-7 cells. PolyK₆-R₈/DNA complexes with the ratio of 20 reached a peak value of 9.2×10^7 RLU/mg protein in HeLa cells. Compared with PolyK₆/R₈ (physical blend of CHK₆HC and CR₈C), PolyK₆-R₈/DNA complexes exhibited a much higher transfection efficiency, which might be attributed to the effective release of DNA in endocellular reductive conditions. The result indicated that PolyK₆-R₈/DNA complexes could promote the transfection in COS-7 and HeLa cells *in vitro*. Furthermore, the cellular uptake of PolyK₆-R₈/DNA complexes at the ratio of 20 in HeLa cells was studied. PolyK₆-R₈/DNA complexes were stained green by YOYO and observed under a confocal laser scanning microscopy (Nikon C1-si TE2000, Japan) after incubating with cells for 4 h. As observed in Fig. 3(c), cell uptake of PolyK₆-R₈/DNA complexes was obvious in cytoplasm, and some complexes entered nuclei. It represented that PolyK₆-R₈/DNA complexes could facilitate the endocytosis of delivered DNA, which was considered to be crucial for subsequent transfection. These results further proved the excellent transfection ability of PolyK₆-R₈/DNA complexes *in vitro*.

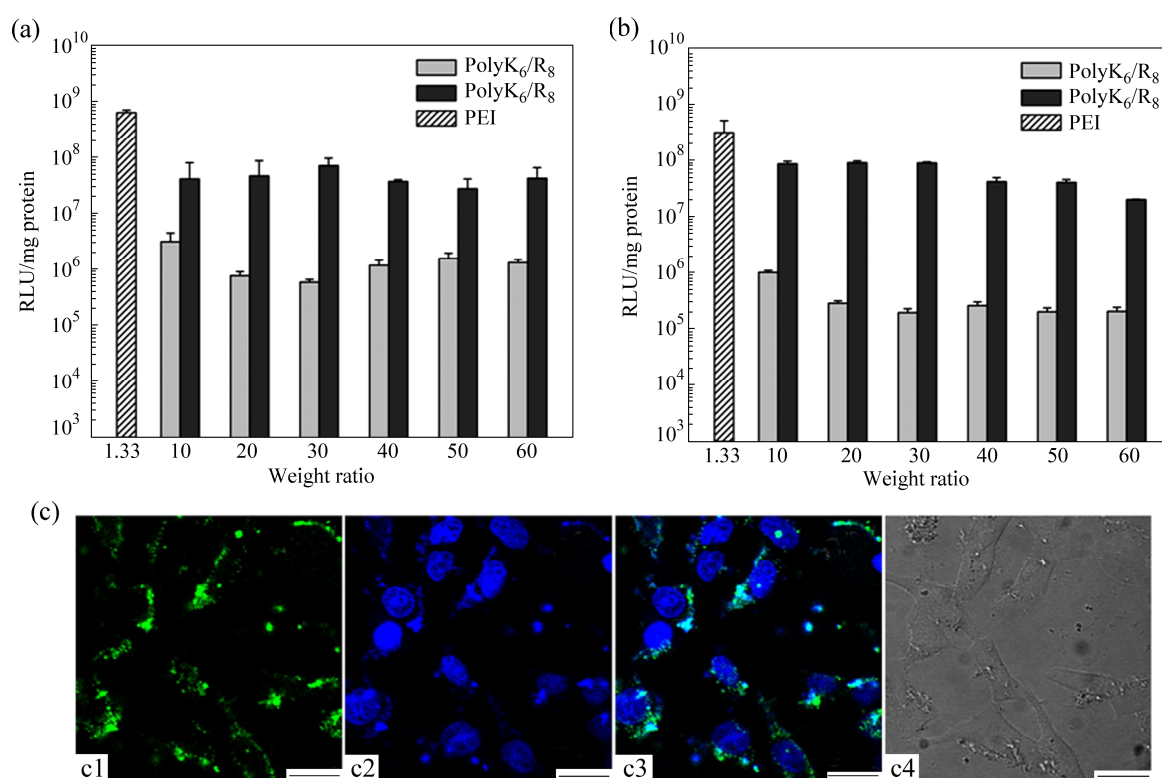


Fig. 3 *In vitro* transfection efficiency of PolyK₆-R₈/DNA complexes at weight ratios ranging from 10 to 60 in (a) COS-7 and (b) HeLa cells, respectively (PolyK₆/R₈ (physical blend of CHK₆HC and CR₈C with a molar ratio of 9) and 25 kDa PEI were used as the control. Data were shown as mean $\pm$ SD ($n = 3$.); (c) Cell uptake of PolyK₆-R₈/DNA complexes (20, *W/W*) in HeLa cells recorded by confocal laser scanning microscopy: (c1) the green fluorescence image of PolyK₆-R₈/DNA complexes, (c2) the blue fluorescence image of nuclei, (c3) the overlapped image of endocytosis of PolyK₆-R₈/DNA complexes and (c4) the light image of endocytotic cells (Scale bars represent 20 μ m.)

To investigate the *in vivo* transfection efficiency of PolyK₆-R₈/VEGF complexes, the angiogenic effects were assessed in hindlimb ischemia rats. For detecting the expression of VEGF protein in ischemia muscle

tissues, immunohistochemistry was adopted to examine the histological sections of investigated groups. As shown in Fig. 4(a), both PolyK₆-R₈/VEGF complex group and VEGF plasmid group obtained positive results in immunohistochemistry assay. PolyK₆-R₈/VEGF complex group presented a much more distinct over expression of VEGF protein in ischemia hindlimb muscle than that of VEGF plasmid group. In contrast, no obvious VEGF protein expression was observed in the histological section of PBS group. The results indicated that PolyK₆-R₈/VEGF complexes could improve local VEGF protein expression effectively, which might induce the formation of vessels subsequently. Moreover, the histological sections of investigated groups were evaluated with α -SMA and CD31 staining, respectively. Through the specific markers in tissue sections, the latent vessels were figured out by arrows directly. As presented in Fig. 4(b) and Fig. 4(c), the vessel number of PolyK₆-R₈/VEGF complex group was the highest compared with other groups. While in PBS group, there were only a few or even no vessels were distinguished from α -SMA and CD31 staining, respectively. The VEGF plasmid group exhibited medium vessel density in both stainings. Therefore, these results demonstrated that PolyK₆-R₈/VEGF complexes could up-regulate the expression of VEGF protein, which in turn promoted angiogenesis in ischemic hindlimbs, and for further ameliorating blood circulation efficiently.

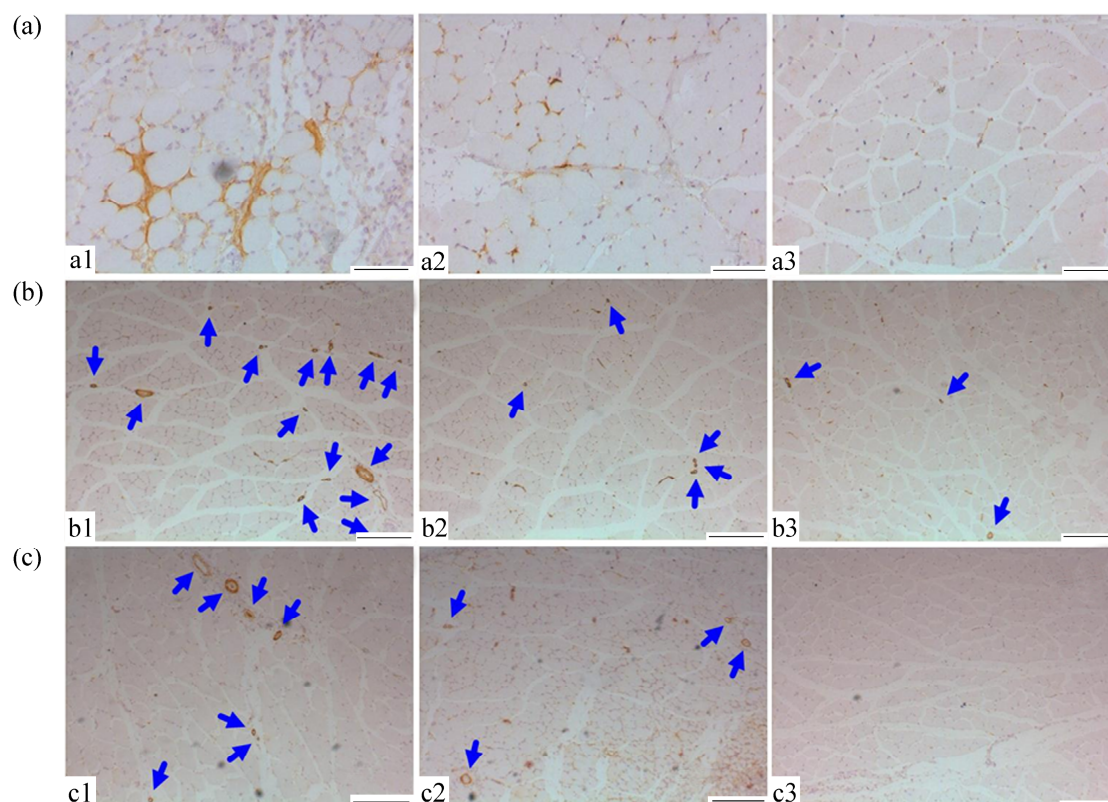


Fig. 4 (a) VEGF protein expression, (b) α -SMA and (c) CD31 staining in histological sections of hindlimb ischemia rats by immunohistochemistry assay: PolyK₆-R₈/VEGF complex group (a1, b1, c1), VEGF plasmid group (a2, b2, c2) and PBS group (a3, b3, c3) (Scale bars represent 100 μ m in (a) and 250 μ m in both (b) and (c), respectively.)

In summary, a bio-reducible polypeptide, PolyK₆-R₈, consisting of CHK₆HC and CR₈C was prepared by oxidative polymerization between terminal cysteinyl thiol groups for gene delivery. PolyK₆-R₈ was demonstrated to be an effective gene vector with low cytotoxicity in *in vitro* transfection assay. In addition, PolyK₆-R₈/VEGF complexes could efficiently mediate VEGF protein expression in hindlimb ischemia rats, which subsequently induced an obvious angiogenesis in tissues. The results indicated that polypeptide-based PolyK₆-R₈ is a promising gene vector for the limb ischemia treatment.

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