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Improved Gene Expression Using Low Molecular Weight Peptides Produced from Protamine Sulfate

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Abstract—DNA condensation plays a key role in non-viral gene delivery by affecting gene transfection, nuclear targeting, and eventual gene expression efficiency. Theoretically, a DNA condenser with the appropriate DNA condensation ability but without affecting DNA dissociation from DNA condensates inside the cytoplasm should be a perfect carrier for gene delivery. Protamine is a natural DNA condensation agent and has been widely used in gene delivery. In this work, protamine was selectively digested enzymatically to produce low molecular weight protamine fragments (LMWPs) of various lengths and amino acid compositions. The DNA condensation ability and gene transfection efficiency of these LMWP peptides were tested. Compared to protamine, all the LMWP peptides showed lower DNA binding strength. However, some LMWP peptides demonstrated excellent DNA condensation ability and could form very compact DNA condensates with small particle size (~100 nm). More interestingly, LMWP peptide-mediated *in vitro* gene delivery showed prolonged (up to 12 days) gene expression. Results from this study suggest that designing DNA condensers with appropriate and tunable DNA binding strengths and condensation abilities would be an effective means to improve gene expression and thus gene therapy efficiency. Since LMWP peptides have low immunogenicity, they would be safer than protamine for use in gene therapies.

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Key words: peptide, enzyme digestion, DNA condensation, controlled gene expression

Gene delivery has become a powerful and popular tool for both basic and pharmaceutical research. There are two general gene delivery systems—viral and non-viral systems. Although viral vector-mediated systems are by far the most effective means (>90%) of DNA delivery, this method is limited only to basic research because of its toxicity, restricted targeting of cells, and limited DNA carrying capacity. For these reasons non-viral systems have become increasingly desirable in both basic research laboratories and clinical settings. Non-viral gene delivery includes three steps: DNA condensation, endocytosis, and nuclear targeting. Among these, DNA condensation plays a key role and determines gene endocytosis, nuclear targeting, and eventual gene expression efficiency [1, 2]. Because of the anionic nature of DNA molecules, a variety of cationic materials such as synthesized polymers (polyethyleneimine) and peptides (poly-L-lysine, protamine) have been applied in DNA condensation [3, 4]. Extensive research has been conducted on DNA condensate formation. Facts such as small particle size of DNA condensates associated with high endocytosis and resistance of highly compacted DNA condensates to DNase digestion have been well studied [5, 6]. However, the fate of DNA condensate after entering into the cell is poorly studied. There is little knowledge on how DNA dissociates from DNA condensates and gets expressed in the cell. Since the dissociation of DNA from DNA condensation complex in cytoplasm or in the nuclei is a critical step for gene expression, the strong binding between DNA and cationic peptides that is favorable for high endocytosis efficiency and DNA stabilization seems not to be appreciated here. In this regard, materials with appropriate DNA binding strength, i.e. enough for efficient DNA condensation but without affecting DNA dissociation from the DNA complex in the cells, should be the ideal carrier for gene delivery.

Protamine, a cationic peptide isolated from the salmon sperm, is a natural DNA condensation agent and has been widely used in gene delivery [7, 8]. In this work, salmon protamine was digested by lysosomal elastase to

Abbreviations: EB) ethidium bromide; LMWPs) low molecular weight protamines.

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produce low molecular weight fragments of various lengths and amino acid compositions. We tested the DNA condensation abilities and the gene delivery efficiency of these low molecular weight peptides *in vitro* and then compared them with the parent protamine. By performing studies of this type, our goal is to understand more about the interaction of DNA and peptides, and to determine the critical factors that control gene delivery and expression.

MATERIALS AND METHODS

Salmon protamine sulfate and lysosomal elastase (EC 3.4.21.37) were from Sigma (USA). Peptides P-1 and P-2 were synthesized by Genscript Inc. (USA). Double-labeled (maleimide/rhodamine and GFP (green fluorescent protein)) plasmid (pGeneGrip™) was purchased from Gene Therapy Systems (USA). HeLa and CHO cells and culture media were from the American Type Culture Collections (ATCC) (Manassas, VA).

Production of low molecular weight protamines (LMWPs). Salmon protamine and elastase were mixed in (1 : 20)-(1 : 200) ratios in phosphate-buffered saline (PBS) containing 20 mM $CaCl_2$. The reaction mixture was incubated at room temperature for 3 h. The reaction mixture was immediately mixed with 50 mM EDTA (0.2 ml) and then incubated in boiling water for 10 min to stop the protease reaction. Digestion products were first fractionated by HPLC with a heparin affinity column (HiTrap) (Amersham, Great Britain). A linear NaCl gradient (buffer A: 50 mM Tris-HCl, pH 7.2; buffer B: 50 mM Tris-HCl, pH 7.2 + 2 M NaCl) was used for peptide separation. LMWP fractions with high heparin binding ability were collected. After desalting, the molecular weight and purity of the obtained LMWP peptides were analyzed by MALDI mass spectrometry as described previously [9].

Preparation and characterization of DNA condensates. DNA condensates were prepared at a DNA concentration of 20 μg/ml in Hanks' solution using a stoichiometry of 0.1-1.2 nmol of protamine or LMWP per μg of DNA. DNA condensates formed instantly, although physical measurements were carried out after 120 min incubation to allow the stabilization of formed DNA condensates. The formation of DNA complexes was confirmed through turbidity assay by measuring the solution absorbance at 320 nm. After centrifugation at 13,000 rpm for 20 min, free DNA concentrations in the supernatant were determined using a microplate reader by measuring absorbance at 260 nm. Particle sizes of DNA condensates were determined by quasi-elastic light scattering on a particle sizer equipped with an avalanche photodiode detector.

Stability of DNA condensates. Formed DNA condensates (peptide/DNA, 1 nmol/μg) were suspended in NaCl solutions of various concentrations (0.8-1.4 M). After vortexing, the DNA/peptide condensate solutions were sonicated at amplitude 6.0 for 30 sec [10]. Solutions were applied to a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Gel retardation was assayed at 70 V for 80 min [10], and gels were destained in deionized water for 12 h.

***In vitro* transfection efficiency.** HeLa and CHO cells were plated on a slide chamber 24 h before each experiment. Cells were exposed to DNA (pGeneGrip™) condensates suspended in Hanks' solution (equivalent to 0.5 μg DNA per well) at 37°C for 60 min. After removal of the culture medium, cells were washed with Hanks' solution and fixed with methanol at 4°C for 15 min. For acid washing experiments, cells were incubated with an acidic solution (28 mM Na-acetate, 120 mM NaCl, 20 mM barbital, pH 2.5) at 0°C for 20 min, followed by three-fold washing using the same acidic solution [11]. Then the cells were fixed at room temperature by dipping the slide into a methanol solution. The cells were analyzed on a FACScaliber flow cytometer (Becton Dickinson, USA) equipped with a 550 nm air-cooled argon laser. The filter settings were 580 nm bandpass (FL2) for emission from rhodamine-labeled GFP plasmids. The fluorescence of 10,000 live cells was acquired and the data were visualized in logarithmic mode [12].

Gene expression assay using flow cytometry. HeLa and CHO cells were cultured in 24-well format with media containing 10% fetal bovine serum and antibiotics. Cells were grown to 85% confluence before transfection. For transfection, cells were washed twice with PBS and exposed to LMWP/pGeneGrip™ condensates suspended in Hanks' solution (equivalent to 3 μg DNA per well). The plates were incubated at 37°C for 4 h. Then the wells of plates were rinsed thoroughly with PBS and filled with 1 ml of culture medium. At different culture time points, cells were harvested through trypsin digestion. After the same acidic wash as described above, gene expression was monitored using flow cytometry. Analysis was conducted on a FACScaliber flow cytometer (Beckton Dickinson) equipped with a 488 nm air-cooled argon laser. The filter settings were 530 nm bandpass (FL1) for emission from expressed GFP proteins. The fluorescence of 10,000 live cells was acquired and the data were visualized in logarithmic mode [13].

Immunogenicity assay. The immunogenicity of protamine and LMWP fragment V was examined in mice. Polyclonal antibodies were produced according to the method of Cooper and Paterson [14]. Twenty-six ICR mice (6-7-week-old), 12 for protamine and 14 for LMWP, were included in this study. Each mouse was immunized with 50 μg of protamine or LMWP in complete Freund's adjuvant. The first booster was given at the fourth week after primary immunization with 5 μg of protamine (or LMWP) in incomplete Freund's adjuvant. Blood was collected eight weeks after the primary immunization,

allowed to clot, and centrifuged to collect serum. The production of antibodies in the sera was measured by ELISA. Briefly, high binding 96-well EIA plates were first coated with 100 µl of equal weight concentration of protamine and LMWP (100 µg/ml) in PBS/Tween 20 (pH 7.5) and allowed to incubate for overnight at 4°C. The unbound protamine or LMWP was removed by draining the plate and washing four times with PBS/Tween 20. The remaining binding sites in the wells were blocked by incubating the plates with 120 µl/well PBS/Tween 20 containing 1% human serum albumin for 1 h at 37°C. Anti-protamine and anti-LMWP antibodies in diluted serum of immunized mice were detected by the routine ELISA method using goat anti-mouse IgG-alkaline phosphatase as the detection antibody. In competitive ELISA, diluted serum was replaced with a fixed dilution of serum (100 times) containing increasing concentrations (1 to 1000 µg/ml) of free protamine or LMWPs.

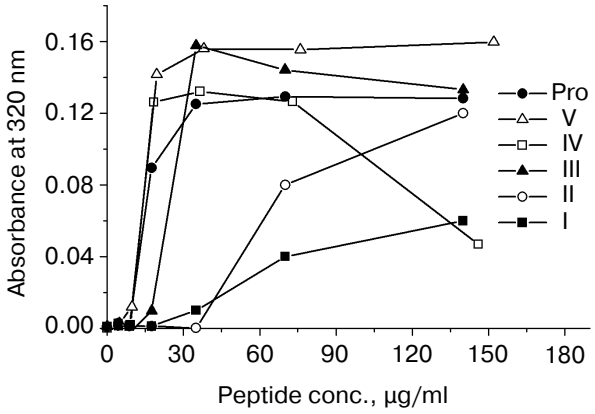

Fig. 1. Formation of DNA/peptide condensates. Peptides of various concentrations were mixed with DNA solution with fixed concentration (20 µg/ml). Formation of DNA condensates was estimated by measuring the solution turbidity changes at 320 nm.

RESULTS AND DISCUSSION

Production of low molecular weight protamine (LMWP). Five fractions were collected from the HPLC elution of elastase-digested protamine. Fractions were numbered based on elution time. There is a close correlation between the molecular weights of LMWP fragments and their affinity to heparin (Table 1). Among all collected fractions, fraction V has the strongest (eluted at 1.2-1.3 M NaCl) while fraction I has the weakest (eluted at 0.2 M NaCl) heparin binding ability. Since heparin is a rigid and anionic macromolecule like DNA, the affinity of LMWP fractions to a heparin column may reflect the electrostatic interaction strength of these peptides to DNA. According to mass spectrometric results (data not shown) none of the five fractions are pure peptides, and they contain more than two LMWP fragments.

DNA condensation by protamine and LMWPs. DNA condensates were prepared by mixing 20 µg/ml DNA with protamine or LMWPs of various concentrations. The DNA condensation ability of the LMWPs was estimated by measuring solution turbidity at 320 nm and particle sizes of formed DNA condensates. As shown in Fig. 1, all the LMWP fragments are able to form complexes with DNA as indicated by the increased solution turbidity. There is a correlation between the molecular weights (Table 1) and DNA complexing abilities (Fig. 1) of LMWP fragments, suggesting that peptide length rather than amino acid sequence determines LMWP-mediated DNA complexing. Since protamine is a very basic peptide, which contains 77% (mol/mol) arginine in its sequence, this result also implies that the interaction between DNA and LMWP fragments is mainly controlled by electrostatic force. Fractions IV and V are as effective as protamine and exhibit almost identical turbidity curves to that of protamine. However, LMWP fractions I-III show low DNA condensation ability, and large amounts of the peptides are required to achieve the same DNA complexing results as that of protamine.

Table 1. Low molecular weight protamine fragments from enzymatic digestion

Elution fraction	Eluent NaCl concentration, M	Molecular weight, kD
I	~ 0.2	<0.6
II	0.3 ~ 0.4	0.8 ~1.1
III	0.6 ~ 0.8	1.2 ~ 1.5
IV	0.9 ~ 1.1	1.6 ~ 2.4
V	1.2 ~ 1.3	2.8 ~ 3.0
Protamine	1.6-1.8	4.0 ~ 4.3

The particle sizes of DNA condensates from all LMWP fractions at fixed peptide/DNA ratio (0.5 nmol peptide/µg DNA) were measured and compared (Table 2). DNA condensates from fractions I to IV have large particle sizes, ranging from 1000 to 3000 nm, which is much larger than the DNA condensates (278 nm) from the intact protamine. However, DNA condensates from fraction V have the smallest particle size of about 142 nm. It has been well documented that DNA condensates within a size range 50-300 nm can be favorably taken up by cells [5, 6]. In this regard, only LMWP fraction V may be suitable for efficient DNA condensation and effective gene delivery.

***In vitro* gene transfection and expression.** The gene transfection and expression efficiency of LMWP fraction V and protamine were compared *in vitro*. Double-labeled (maleimide/rhodamine and GFP) plasmid (pGeneGrip™) was selected as the reporter gene for

Table 2. Particle sizes of DNA condensates formed from protamine and LMWPs

Elution fraction	I	II	III	IV	V	Protamine
Particle size, nm	2692	2196	2840	1064	142	278

this purpose. DNA condensates from protamine and LMWP fraction V were prepared at a fixed peptide/DNA ratio (0.8 nmol peptide/μg DNA). HeLa cells were transfected with prepared pGeneGrip condensates and GFP expression was monitored for a period of 10 days using FACS. The average fluorescence intensity of GFP-expressing cells (cells in M1 area in the FACS histogram of Fig. 2a) was calculated and used to represent the gene expression level. As shown in Fig. 2b, no GFP expression can be observed in naked DNA treated cells for the entire experimental period. On the contrary, significant GFP expression was found in the DNA condensates-treated cells. GFP expression can be detected as early as 48 h after the transfection. Protamine-mediated gene expression reaches a maximal at 72 h after transfection. After that gene expression decreases rapidly and no GFP expression can be detected after eight days. In contrast, LMWP fraction V-mediated gene transfection seems to be a long-lasting process, reaching a maximum at 72 h and then maintaining this level for another 48 h. After that, GFP expression decreases gradually and significant GFP expression (at about 50% of the maximal gene expression level) is still maintained 10 days after transfection.

To examine if this long-lasting gene expression was from the high gene transfection efficiency of LMWP fraction V, the DNA (pGeneGrip) uptake efficiency of cells treated with LMWP fraction V or protamine-condensed DNA were compared. Cells were incubated with GFP condensates from fraction V or protamine at 37°C for 20 min. Then the cells were washed with acidic solution (28 mM Na-acetate, 120 mM NaCl, 20 mM barbital, pH 2.5) at 0°C three times [11] to remove cell surface-bound condensates. Cell uptake of rhodamine-labeled GFP plasmid was examined using FACS by counting GFP plasmid positive cell numbers. The results show that there is no significant gene transfection efficiency difference between protamine and LMWP fraction V-mediated gene transfection. The gene transfection efficiency is 89 ± 9 and 86 ± 4% for protamine and LMWP fraction V, respectively. Obviously, gene transfection efficiency cannot be used to explain prolonged gene expression period in LMWP fraction V-treated cells.

Characterization of DNA condensates. According to the mass spectrometry results, fraction V is composed of two peptides with molecular weight of 2871.79 and 2914.15 daltons. We separated these two peptides (P-1 and P-2) using reverse-phase HPLC and determined their sequences through amino acid analysis (peptide P-1, PRRRRSSSRPIRRRRPRRASRR; peptide P-2, RRRRPRRVSRRRRRRGGRRRR). We characterized the DNA condensates from these two peptides and compared the results with condensates from parent protamine to study the gene delivery mechanisms.

First, the DNA complexing ability of protamine and LMWPs were compared. To DNA solutions (20 μg/ml), peptides of various concentrations were added. The mixtures were incubated at room temperature for 60 min and then centrifuged at high speed to removed formed DNA condensates. Free DNA concentrations in the supernatants of various samples were measured and compared. There is a reverse correlation between the final peptide

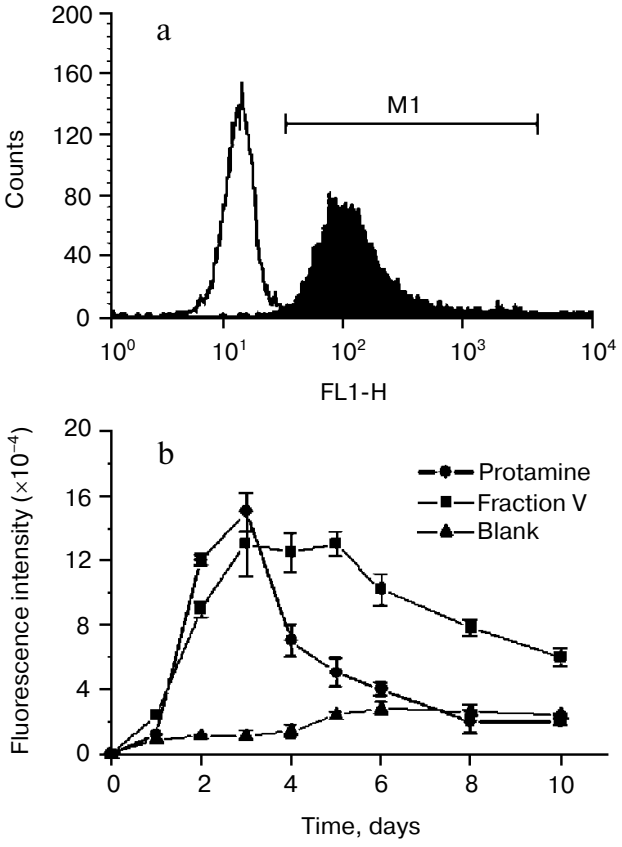

Fig. 2. *In vitro* GFP expression profiles of protamine and LMWP-mediated DNA transfection in HeLa cells. GFP expression was estimated by measuring the average fluorescence intensity (b) of transfected cells in a fixed area (M1) in the FACS diagram (a). Blank is a control in the absence of protein.

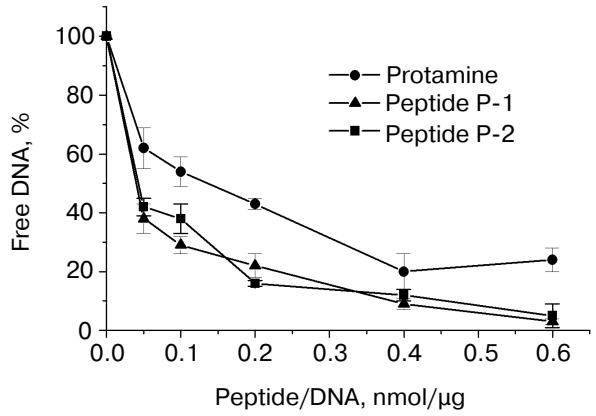

Fig. 3. Comparison of DNA condensation capability of protamine and peptides P-1 and P-2 by measuring free DNA concentrations in the solution at various peptide concentrations.

concentrations of the solutions and free DNA concentrations in the supernatants (Fig. 3). Increased peptide concentrations are associated with decreased free DNA concentrations in the supernatants. Both peptide P-1 and peptide P-2 have excellent DNA complexing ability and are able to complex all DNA in the solutions once the LMWP/DNA ratios (nmol peptide/μg DNA) are higher than certain values (0.6 and 0.4 for peptides P-1 and P-2, respectively). However, protamine has a limitation in DNA complexing. Despite the use of high concentrations of protamine (up to 1.2 nmol protamine/μg DNA), only about 80% of DNA can be complexed by protamine, leaving about 20% of the free DNA in the supernatants.

The high DNA complexing ability of LMWPs was further confirmed by agarose gel retardation experiments. No significant amounts of free or partially complexed DNA can be found when the LMWP/DNA ratios are at and higher than 0.1 nmol peptide/μg DNA (Fig. 4a). On the contrary, free or partially complexed DNA exists in the protamine group even when the protamine/DNA ratio reaches 0.6 nmol peptide/μg DNA. It is very interesting to observe that when the peptide/DNA ratios of DNA/LMWP condensates reach 0.6-0.8 nmol peptide/μg DNA, the DNA/LMWP condensates retarded in the sample loading wells can hardly be stained by ethidium bromide (EB) dye. A reasonable explanation for this phenomenon should be that DNA/LMWP condensates at these LMWP/DNA ratios are tightly compacted so that EB molecules cannot access the DNA molecules inside the condensates to stain them.

We further examined the effects of protamine or LMWP concentration on particle sizes of DNA condensates. As shown in Table 3, increased protamine concentrations have very limited effects on DNA condensate sizes. The smallest particle size of DNA/protamine condensates is 213 nm. However, there is a close correlation between LMWPs concentrations and DNA condensate sizes. High LMWP concentrations are associated with small DNA condensate sizes. The smallest particle sizes for DNA/P-1 and DNA/P-2 condensates are 76 and 81 nm, respectively. This particle size difference between protamine and LMWP condensed DNA is consistent with the DNA condensation capability results (Fig. 3) and the EB staining results observed in the gel retardation experiments (Fig. 4a), confirming that LMWPs have better DNA condensation ability than protamine.

According to the elution results of LMWP and protamine from a heparin affinity column (Table 1), protamine has stronger affinity than LMWPs to anionic macromolecules. Although both heparin and DNA are rigid and anionic macromolecules, it is unclear if protamine will also have stronger affinity than LMWPs to DNA. The DNA binding strength of protamine and LMWPs were compared using a well-established sonication assay [10]. First, prepared DNA condensates were suspended into solutions with different salt concentrations. The suspensions were then subjected to a moderate sonication, which can break down free DNA in the solution without affecting the DNA integrity in the condensates. If DNA/peptide complexes are unstable and dissociate in a solution with certain salt concentration, free DNA released into the solution will be chopped by sonication, and DNA fragmentation can be observed in a the agarose gel electrophoresis. On the contrary, if DNA/peptide complexes are stable in a salt solution, all DNA will exist in the form of condensates and no DNA fragmentation can be detected in the sonication assay. As shown in Fig. 4b, DNA fragmentation of DNA/protamine complexes occurs at 1 M NaCl, and the salt con-

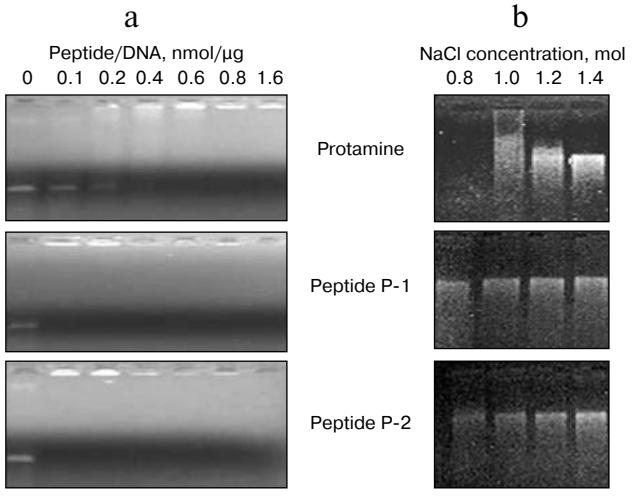

Fig. 4. a) Agarose gel retardation assay. DNA and peptides were mixed and then loaded onto 1% agarose gel for binding assay. b) DNA–peptide strength assay. DNA condensates were suspended in solutions of different NaCl concentrations. After sonication, DNA fragmentation was analyzed using 1% agarose gel electrophoresis.

Table 3. Particle size (nm) analysis of DNA/LMWP complex

Peptides	Peptide/DNA ratio, nmol/μg				
	0.1	0.2	0.4	0.6	0.8
Protamine	226	268	429	213	383
P-1	265	115	100	82	76
P-2	286	206	105	78	92

centration for complete DNA fragmentation of DNA/protamine condensate is 1.4 M. In contrast, the complete fragmentation of DNA/LMWP condensates happens when salt concentrations reach 0.8 M NaCl. Obviously, protamine has strong DNA affinity compared to peptide P-1 and peptide P-2.

Despite its nearly universal use in clinical practice, protamine induces adverse reactions ranging from mild hypotension to idiosyncratic fatal cardiac arrest [15-19]. We compared the immunogenicity of protamine and LMWP fraction V on mice by monitoring the production of polyclonal antibodies. As shown in Fig. 5, anti-LMWP antibodies could be detected in the serum of 8 out of 14 LMWP-immunized mice (57%) after eight weeks of primary immunization. This result was comparable with anti-protamine antibody production where 8 of 12 protamine-immunized mice (67%) produced anti-protamine antibody. However, the antibody titers in pooled sera from protamine-immunized mice are much higher than that from LMWP-immunized mice. Therefore, LMWP exhibited lower immunogenicity than that of protamine.

Protamine is a natural and excellent DNA condensation agent. We digested protamine into small fragments with different molecular weights and DNA affinity. The DNA condensation, gene transfection, and gene expression efficiency of these LMWPs were tested and compared. The studies showed that DNA/LMWP condensates had some extraordinary properties including small particle size (Tables 2 and 3), high DNA condensation capability (Fig. 3), strong DNA compacting ability (Fig. 4a), and moderate DNA binding strength (Fig. 4b). The *in vitro* results demonstrated that compared to protamine, LMWP-condensed DNA showed prolonged gene expression period, from the ordinary 5-7 days to more than 10 days (Fig. 2).

As we know, DNA condensation is a vital step for gene transfection and expression. It has been proven that small particle size (usually <100 nm) of DNA complex is essential for high gene transfection efficiency through the endocytosis mechanism. In addition, since DNA in compacted DNA condensates will become resistant to DNase degradation, DNA condensation is also critical in order to achieve high gene expression.

Most positively charged polymers are able to condense DNA and thus their use has been attempted for gene delivery [3-6]. However, results regarding gene transfection and gene expression efficiency are not that great.

Extensive research has been conducted on DNA condensate formation. Facts such as small particle size of DNA condensates associated with high endocytosis and highly compacted DNA condensates resistance to DNase digestion have been well studied [5, 6]. However, there is little knowledge on how DNA dissociates from DNA condensates and gets expressed in the cell. Since the dissociation of DNA from DNA condensation complex in cytoplasm or in the nuclei is a critical step for followed gene expression [20, 21], stronger binding between DNA and cationic carriers that is favorable for high endocytosis efficiency and DNA stabilization seems not to be favored here. Theoretically, a carrier with appropriate DNA/cationic peptide binding strength, i.e. enough for efficient

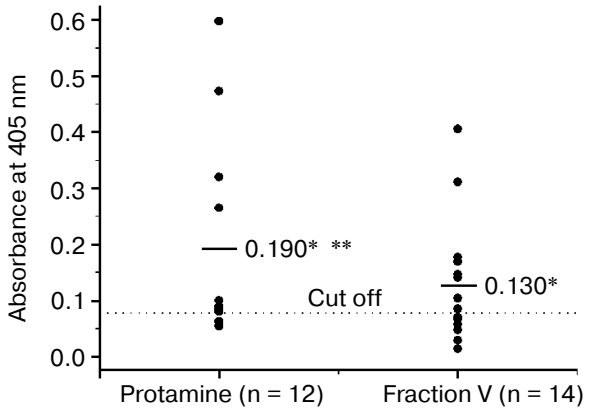

Fig. 5. Induction of antibody production in mice immunized with protamine (12 mice) and LMWP fraction V (14 mice). Mice received protamine (50 μg) or LMWP (50 μg) in primary inoculation, and then were boosted with protamine (10 μg) or LMWP (10 μg) at four weeks after primary immunization. Blood samples were collected eight weeks after immunization, and antibodies were detected on protamine- or LMWP-coated plates using ELISA. The cut-off line represents OD ± 3 SD of normal mouse sera. Data with ** represent $p < 0.01$ in comparison with LMWP group. Data with * represent $p < 0.01$ in comparison with untreated group.

DNA condensation but without affecting DNA dissociation from the DNA complex in the cells, should be the perfect carrier for gene delivery.

In most cases, synthesized cationic polymers and peptides only make use of ionic interactions for molecular recognition and binding of DNA, and thus their bindings to DNA are solely dependent on charge interactions. In contrast, for natural peptides which are originally from nuclei, such as protamine and LMWPs, the process of selective molecular recognition and binding of DNA by peptides is governed by a hierarchy of interactions involving amino acid side chains, including stacking interactions, hydrogen bonding, and ionic interactions [22]. Results from this study suggest that the specific interactions between DNA and LMWPs are responsible for the effective gene transfection and gene expression abilities of LMWPs.

Further studies on the mechanisms of less toxic LMWP-mediated effective gene delivery are of great interest. The toxicity of protamine is mediated through two pathways: (i) non-immunological pathway, and (ii) immunoglobulin-mediated pathway. The mechanisms of protamine-induced adverse responses via the non-immunological pathway are attributed to the "cross-linking" ability of protamine due to its polycationic and polymeric nature [20]. The anaphylactoid type of reactions produced via this mechanism, which are manifested by complement activation, thromboxane generation, and histamine release, are more common. However, they can normally be aborted with slow administration of protamine and thus less dangerous. On the contrary, anaphylactic types of responses produced via the immunoglobulin-mediated pathway are unpredictable, not preventable, and always life threatening [18, 19]. Another advantage of using LMWP peptides for gene therapy is its low toxicity. The low immunogenicity of LMWP will enable protamine challenged patients to be freed from risks of the immunoglobulin-mediated, severe protamine responses in protamine-mediated gene therapies.

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