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Efficient cleavage of RNA, enhanced cellular uptake, and controlled intracellular localization of conjugate DNAzymes

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Abstract—Conjugate DNAzymes with polyamines and peptides were successfully prepared by solid phase fragment condensation (SPFC) and showed up to 4.2 times higher catalytic efficiency (k_{cat}/K_m) and enhanced tolerance against DNase 1digestion. To be pointed out, intracellular localization of DNAzymes could be controlled by conjugated with naturally occurring signal peptides responsible for nuclear cytoplasmic transport of proteins.

DNAzymes have been engineered by SELEX and have attracted much attention from biological and medical points of view.[1] Although their RNA cleaving activities are attractive so much as a controlling tool of genetic expression, their application to cellular system or in vivo is strictly limited because of their deficient stability against nuclease digestion. Chemical modifications are also limited because their catalytic activities are largely dependent on their chemical structures. For example, Tien et al. reported that introduction of phosphorothioate backbone in the flanking sequence of an DNAzyme increased its stability in cells but was accompanied by a severe decrease of catalytic efficiency in vitro.[2] As well as chemical modifications, conjugation of DNAs with other functional molecules is a fascinating way to add some intelligent properties to them. For example, conjugate DNAs can be expected to have higher binding affinities to target RNA, enhanced resistances against nuclease digestion, and increased permeability through cellular membrane. The present study describes on synthesis and characterization of conjugate DNAzymes.

Sequence of catalytic loop of DNA enzymes Dz**1**–Dz**9** was the same as reported by Joyce et al., and the recognition sequence was complimentary to HIV-1 gag mRNA.[1] The substrate RNA was labeled with FITC (Fig. 1).

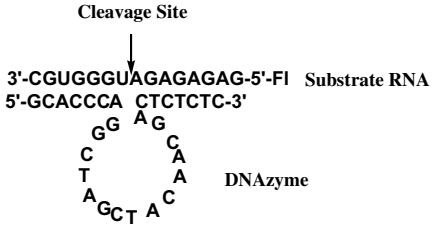

Figure 1. DNAzyme targeted to HIV-1 gag mRNA.

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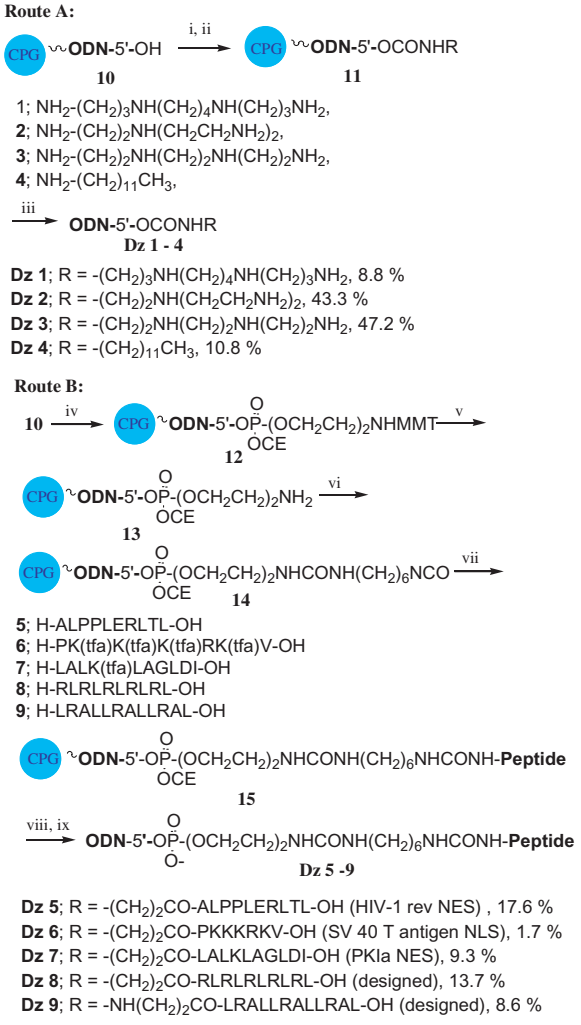
Conjugate DNAzymes (Dz**1**–Dz**4**) with amines **1**–**4** were prepared by solid phase fragment condensation (SPFC) through Route A and those with peptides (Dz**5**–Dz**9**) through Route B as shown in Scheme 1.[3]

Syntheses of oligonucleotides were performed in one micromole scale by an automated DNA/RNA synthesizer on CPG support by using standard cyanoethyl-phosphoramidite chemistry. In Route A, 5′-hydroxyl groups of oligonucleotides were reacted with 50 μmol of carbonyldiimidazole (CDI) in 1 mL of anhydrous CH_3CN at rt for 1 h. After washing with anhydrous CH_3CN (1 mL × 5), 10 μmol of amines **1**–**4** and 12 μmol of diisopropylethylamine (DIEA) in 1 mL of anhydrous CH_3CN were reacted at rt for 12 h. Cleavage and deprotection was performed with 1 mL of saturated aqueous ammonia at 55 °C for 5 h. Purification by RPHPLC (ODS 4 × 125 mm, buffer A: 100 mM TEAA, buffer B: 30% 100 mM TEAA in CH_3CN, a linear gradient of buffer B from 10 to 100% in 60 min) gave DNAzyme–amine conjugates (**Dz1**–**Dz4**) in good yields and the products were fully characterized by MALDI-TOF MS (Table 1). In Route B, oligonucleotides were modified at 5′-end with *N*-monomethoxytrityl-2-aminoethoxyethylcyanoethyl-phosphoramidite (5′-Amino Modifier 5, Glenn Research). After removal of MMT group with 3% trichloroacetic acid in 1 mL of anhydrous CH_3CN at rt for 5 min, 1,6-diisocyanatohexane (50 μmol) in 1 mL of anhydrous CH_3CN was injected into the reaction column via a syringe. After shaking the reaction column at 20 °C for 12 h, CPG support was washed with anhydrous CH_3CN (1 mL × 5) to remove the excess diisocyanate. Peptide fragments **5**–**9** were independently prepared by automated peptide synthesizer using standard Fmoc chemistry on Wang resin and cleaved by trifluoroacetic acid (95%) and thioanisol. Hydroxyl, carboxyl, guanidinyl groups on peptide side chains were deprotected simultaneously when cleaved, whereas ε-amino groups of lysines were still protected with trifluoroacetyl group. β-Alanine was attached at N-terminus of each peptide and its amino group was deprotected by treatment with piperidine prior to cleavage reaction. Partially protected peptide fragments were obtained by HPLC purification in satisfactory yields and characterized by a MALDI TOF mass spectrometer (Supporting Information). Peptide **5** is a nuclear export signal (NES) sequence of HIV-1 Rev protein,[4] peptide **6** is a nuclear localization signal (NLS) sequence of SV-40 large T antigen[5] and peptide **7** is NES of PKIα.[6] The peptides **8**[7] and **9**[8] are artificially designed cationic ones which form amphiphilic α-helical structure and β-sheet structure, respectively, when they bind to dsDNA. A solution of thus obtained partially protected peptide (10 equiv) in DMF was introduced into the reaction column using a syringe. The reaction column was shaken at 20 °C for 24 h, washed with anhydrous CH_3CN (1 mL × 5) and treated with aqueous ammonia at 55 °C for 4 h for cleavage and deprotection. Solid support was filtered off and the obtained crude mixture was purified by reversed phase HPLC (ODS 4 × 125 mm, buffer A: 100 mM TEAA, buffer B: 30% 100 mM TEAA in CH_3CN, a linear gradient of buffer B from 10% to 100% in 60 min).



Scheme 1. Synthesis of conjugate DNAzyme by SPFC reaction conditions; (i) CDI (50 equiv), CH_3CN, rt, 1 h; (ii) RNH_2 (10 equiv), CH_3CN, rt, 10 h; (iii) concd NH_3, 55 °C, 6 h; (iv) 2-cyanoethyl *N*-monomethoxytrityl-2-aminoethoxyethyl diisopropylphosphoramidite, CH_3CN, rt, 15 min; (v) 3% TCA, CH_3CN, rt, 5 min; (vi) OCN-$(CH_2)_6$NCO (50 equiv), CH_3CN, rt, 2 h; (vii) Peptide–NH_2 (10 equiv), rt, 12 h; (viii) concd NH_3, 55 °C, 6 h; (ix) RPHPLC, ODS, TEAA/CH_3CN.

Table 1. Characterization of **Dz 1–9** by MALDI TOF-MS matrix

	MALDI TOF-MS	
	Found, *m/z*	Calcd
Dz 1	8988.31	8993.34
Dz 2	8940.17	8921.39
Dz 3	8948.41	8921.40
Dz 4	8975.20	8974.35
Dz 5	10,308.40	10,307.17
Dz 6	10,071.63	10,065.09
Dz 7	10,199.38	10,197.18
Dz 8	10,556.79	10,535.83
Dz 9	10,549.97	10,550.16

Matrix: solution A: solution B = 1:1, solution A; saturated 2′,6′-dihydroxyacetophenone in 50% CH_3OH/H_2O, solution B; saturated diammonium hydrogen citrate in 50% CH_3OH/H_2O.

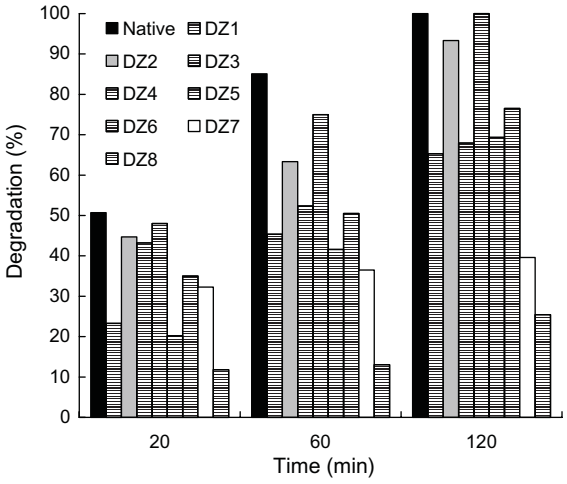


Figure 2. Degradation of **Dz 1–8** by DNase 1. Conditions: 0.15 M NaCl, DNase 1 = 160 Kunitz/unit, [Dz] = 1 mM, incubate for 120 min at 37 °C (pH 7.5).

Resistances of **Dz1**–**Dz9** against DNase 1 digestion were evaluated (0.15 M NaCl, 37 °C) and the results were summarized in Figure 2. **Dz1**–**Dz9** amines and peptides at their 5′-end bearing were found more resistant to DNase 1 digestion than native phosphodiester DNAs and phosphorothioate DNAs. Interestingly **Dz5**–**Dz9** conjugated with peptides were more resistant that **Dz1**–**Dz4** conjugated with polyamines. **Dz8** conjugated with a designed amphipathic cationic peptide was only digested in 20% while phosphodiester one was completely decomposed and phosphorothioate one was decomposed in 80% in 2 h under these conditions.

Kinetic parameters for catalytic cleavage of RNA by **Dz1**–**Dz9** were determined under physiological conditions (2 mM $MgCl_2$, 150 mM NaCl, pH 7.5) by Lineweaver–Burk plot and listed in Table 2. K_m values showed that binding affinities of DNAzymes were increased by conjugation with polyamines (**Dz1** and **Dz2**) and cationic peptide (**Dz6**) and decreased by conjugation with hydrophobic amine (**Dz4**) and peptides (**Dz5**, **Dz7**). **Dz8** and **Dz9** were conjugated with basic peptides with amphiphilic character. Peptides in **Dz8** and **Dz9** were shown to form antiparallel β-sheet and α-helix structure, respectively, and bind to stabilize dsDNA. But in this case, they slightly destabilized Dz-RNA hybrid. Catalytic turn over numbers (k_{cat}) were increased in **Dz4**, **Dz6**, and **Dz9** by two to three times. It is interesting that DNAzymes conjugated with hydrophobic amines or peptides have decreased K_m values and increased k_{cat} values. **Dz1**, **Dz3**, and **Dz5** have slightly increased k_{cat} values and **Dz2**, **Dz7**, and **Dz8** have slightly decreased k_{cat} values. As a result, catalytic efficiencies (k_{cat}/K_m) of **Dz1**, **Dz6**, and **Dz9** were 2.0, 4.2, and 2.6 times higher than those of the native DNAzyme, respectively. As a whole, it can be said that catalytic activities were increased or not influenced so much by conjugation with amines or peptides at the 5′-end.

Table 2. Kinetic parameter of **Dz 1–9** by MALDI TOF-MS

Dz	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($10^7 M^{-1} min^{-1}$)
Native	3.22	0.119	0.36
Dz 1	1.84	0.133	0.73
Dz 2	2.19	0.094	0.43
Dz 3	3.50	0.130	0.37
Dz 4	7.11	0.213	0.29
Dz 5	9.84	0.132	0.13
Dz 6	1.12	0.171	1.52
Dz 7	5.74	0.109	0.19
Dz 8	4.27	0.057	0.13
Dz 9	3.61	0.331	0.92

Reaction conditions: 2 mM $MgCl_2$, 150 mM NaCl, 50 mM Tris, pH 7.5, 37 °C, [Dz] 1.0 μM.

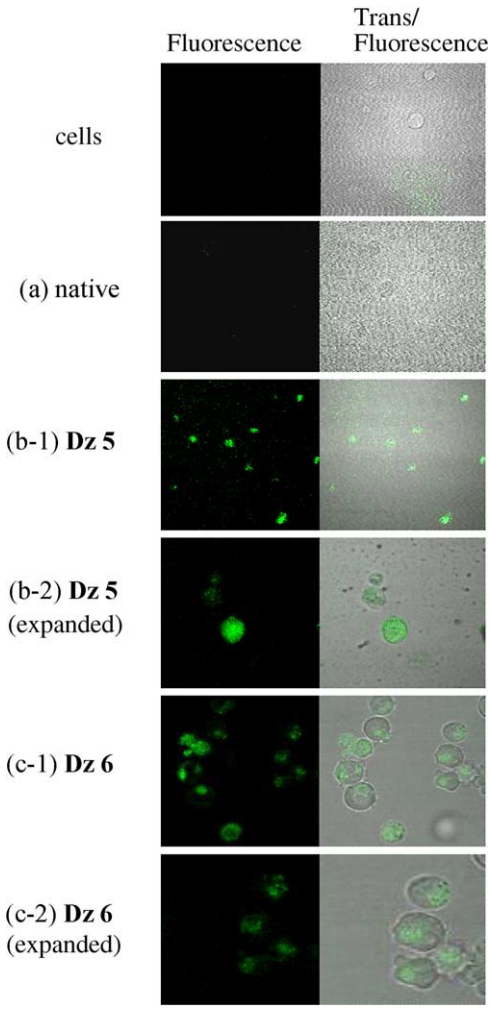


Figure 3. Cellular uptake of **Dz 5** and **Dz 6**.

Finally, in order to demonstrate that conjugate DNAzymes with polyamine and cationic peptides can be effective in cellular system and in vivo, cellular uptake of DNAzymes using human leukaemia cell line Jurkat by confocal laser scanning microscope (Fig. 3).

FITC labeled DNAzyme–peptide conjugates **Dz5-f**, **Dz6-f** were prepared in a similar manner.

Dz 5-f: R=–$(CH_2)_2$CO–k(**fl**)ALPPLERLTL-OH (HIV-1 rev NES)
Dz 6-f: R=–$(CH_2)_2$CO–k(**fl**)PKKKRKV-OH (SV 40 T antigen NLS)

Dz5-f and **Dz6-f** were incubated with Jurkat cells (1×10^6 cells/ml) in RPMI-1640 medium supplemented with 10% fetal calf serum (FBS) and streptomycin (100 μg/ml) and penicillin (100 U/ml) for 24 h at 37 °C over 5% CO_2. Although native DNAzyme was not taken up into cells (A), **Dz5-f** which is conjugated with a hydrophobic NES peptide derived from HIV-1 Rev protein was taken up effectively and localized all over the cytoplasm (B). It is to be noted that **Dz6-f** conjugated with a cationic NLS peptide derived from SV-40 large T antigen was also taken up effectively and localized in cellular nucleus (C). This successful controlled intracellular delivery of DNAzymes will be quite important for the DNAzymes to act in cells or in vivo because Taira et al. clearly showed that ribozymes could act more efficiently when localized in the cytoplasm rather than in the nucleus.[9]

As a summary, these results strongly suggested that conjugate DNAzymes which have increased resistance against DNase 1 digestion, enhanced permeability through cellular membrane, controlled intracellular localizability and increased catalytic activities can be applied to cellular system or in vivo.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2004.10.009.

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