Controlled Intracellular Localization of Oligonucleotides by Chemical Conjugation

Takanori Kubo[1], Rumiana Bakalova[2], Zhivko Zhelev[2], Hideki Ohba[2], and Masayuki Fujii[1,3]

1 Introduction

Artificial control of genetic expression by oligonucleotides is a powerful tool for biological studies and medical therapies. Nucleic-acid drugs, such as antisense oligonucleotides, ribozymes, decoys and siRNAs, have attracted much attention and have been intensively studied for the past two decades (Crooke et al. 2004; Tung et al. 2000; Fischer et al. 2001; Eisele et al. 1999; Zubin et al. 2002; Antopolsky et al. 1999). Difficulties in using oligonucleotides as therapeutic agents involve their transport through the cell membrane, delivery and localization in the targeted cellular structure, and targeting of the specific mRNA or DNA sequence with sufficient affinity and specificity. For these reasons, DNA-peptide conjugates have been attracting intensive attention as alternative and advanced materials for the technology of genetic medicines and novel functional nucleic acids (Stetsenko et al. 2000, 2002; Soukchareum et al. 1995; Haralambidis et al. 1987; Antopolsky et al. 2002). In this chapter, the cellular uptake and controlled localization of oligonucleotide-peptide conjugates are discussed.

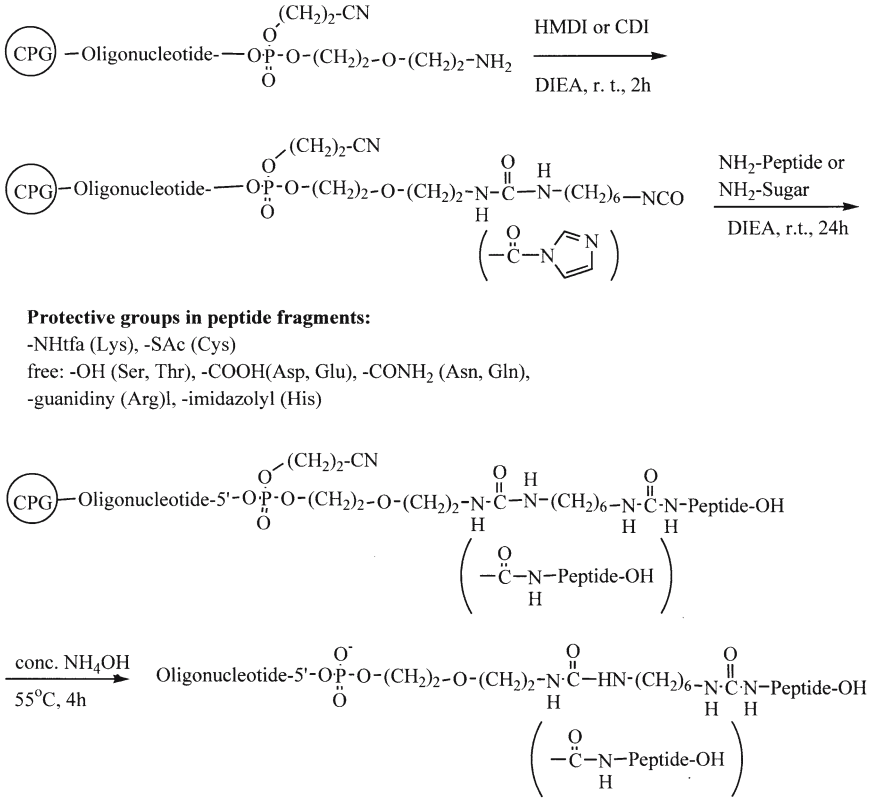
2 Synthesis of Oligonucleotide-Peptide Conjugates by Solid-Phase Fragment Condensation

Synthetic methods of oligonucleotide-peptide conjugates so far studied can be classified into two categories: solution-phase synthesis and solid-phase synthesis. The former mostly involves coupling procedures of oligonucleotide and peptide fragments using small linker molecules having two different functionalities (Antopolsky et al. 1999; Stetsenko et al. 2000).

[1] Department of Biological and Environmental Chemistry, Kyushu School of Engineering, Kinki University, 11-6 Kayanomori, Iizuka, Fukuoka 820-8555, Japan
[2] Kyushu National Industrial Research Institute, Agency of Industrial Science and Technology (AIST-Kyushu), Ministry of International Trade and Industry, 807-1 Shuku-machi, Tosu, Saga 841-0052, Japan
[3] Molecular Engineering Institute, Kinki University, 11-6 Kayanomori, Iizuka, Fukuoka 820-8555, Japan

Tandem synthesis of oligonucleotide-peptide conjugates on solid phase has been intensively studied (Haralambidis et al. 1987; Soukchareum et al. 1995; Antopolsky et al. 2002; Stetsenko et al. 2002; Zubin et al. 1999). One of the major problems is how to prepare fluorenylmethoxycarbonyl (fmoc) derivatives of all amino acids having protective groups on side chains labile under treatment with ammonia. Another problem of solid-phase synthesis of oligonucleotide-peptide conjugates, in which first peptides are usually prepared first and then oligonucleotides are prepared, is the low coupling efficiencies of peptide synthesis. Insufficient coupling yields of peptides will result in lowering overall yields of the desired products. In order to overcome such problems as (1) limitation of amino acid components in peptides and (2) low overall yields of the products, fragment coupling on solid support has been attempted (Zubin et al. 1999; Zatsepin et al. 2002).

Recently, we reported a universal method to prepare oligonucleotide-peptide conjugates by solid phase fragment condensation (SPFC) (Scheme 1) (Kubo et al. 2001, 2003). The strategy of SPFC is that a DNA fragment having a free amino group prepared on CPG support is reacted with α,ω-diisocyanatoalkane or carbonyl diimidazole (CDI) and then with a peptide fragment bearing a single reactive amino group. The resulting oligonucleotide-peptide conjugate covalently linked to solid support is



Scheme 1. Synthesis of Oligonucleotide-Peptide Conjugates by SPFC

cleaved from CPG and deprotected by treatment with ammonia. The isolated yields were 10%–30% and the products were well characterized by MALDI-TOF MS and RPHPLC.

As shown in Table 1, peptide moieties in the conjugates could contain aspartic acid (D), glutamic acid (E), lysine (K), methionine (M), aspergine (N), glutamine (Q), arginine (R), serine (S), threonine (T), and tryptophan (W) as well as other non-protected hydrophobic amino acids. Except for lysine, all the side chain residues were deprotected prior to the coupling reaction. ε-Amino groups of lysine were protected with trifluoroacetyl groups, which were removed at the final treatment with ammonia. It is advantageous to use amino groups of peptide fragments for SPFC because these can be easily introduced at desired positions of peptides and selectively protected by both acid- and base-labile protective groups. SPFC will allow the selective linkage of oligonucleotides to peptides at the desired positions. Peptide moieties in the conjugates synthesized are derived from the nuclear localization signal (NLS) of SV40 T-antigen (Newmeyer et al. 1986), the NLS of HIV-1 Tat protein (Dingwell et al. 1986), a nuclear export signal (NES) sequence of HIV-1 Rev protein (Dingwell et al. 1986), NES of MAPKK protein (GoldFarb et al. 1986) as well as designed amphiphilic peptides (Fujii et al. 2001; Kubo et al. 2001, Yokoyama et al. 2001, Yano et al. 2003). Peptides in **C4** and **C5** (also in **C15** and **C16**) were proven to form an antiparallel β-sheet and an amphipathic α-helical structure in the presence of DNA, respectively, and both could bind to and stabilize dsDNA (Fujii et al. 2000; Kubo et al. 2001). Galactosamine was also successfully conjugated to oligonucleotides without any protection (**C6**, **C17**).

3 Nuclear and Cytoplasmic Localization of Oligonucleotide-Peptide Conjugates

Recently, the mechanisms of intracellular transport of proteins have been revealed in detail, and some of the signal sequences responsible for nuclear-cytoplasmic transport of proteins have been identified (Kim et al. 1994; Goerlich et al. 1999).

Heitz and colleagues showed that oligonucleotide conjugated with a hydrophobic peptide derived from HIV-1 gp41 protein was effectively taken up into human fibroblast H9C2 cells (Chaloin et al. 1998). Behr and co-workers showed that plasmid DNA conjugated with a NLS peptide derived from SV40 large-T-antigen was effectively taken up into cells and localized in the nucleus (Zanta et al. 1999).

Our group also studied cellular uptake and intracellular localization of DNA-signal peptide conjugates in detail by confocal laser fluorescence microscopy and flow-cytometry (Figs. 1, 2) (Kubo et al. 2003, 2005). Jurkat human leukemia cells were incubated with fluorescently labeled oligonucleotide-peptide conjugates **C1F**-**C6F** (Scheme 2) in 10% serum for 48 h. Microscopy clearly showed that cellular uptake of all of the oligonucleotide-NLS conjugates **C1F**-**C3F** was enhanced without any transfection reagents, whereas natural oligonucleotide **N1F** was hardly taken up into cells (Fig. 1a). It can be assumed that the basic characteristic features of NLSs enhanced ability of the conjugates to penetrate the cellular membrane. As expected, conjugates bearing NLS sequences of SV40 large-T-antigen (**C1F**) and HIV-1 Tat protein (**C2F**) were taken up and localized into the nucleus (Fig. 1b,c). The conjugate of phosphorothioate oligonucleotide and SV40 large-T-antigen NLS (**C3F**) was taken up much better and localized throughout the large nuclei of Jurkat cells (Fig. 1d).

Table 1. MALDI TOF-MS Data of Oligonucleotide-Peptide Conjugates
N1: 5′-TTTTTCTCTCTCTCT-3′
N2: 5′-CAGTTAGGGTTAG-3′
S1: 5′-CsAsGsTsTsAsGsGsGsTsTsAsG-3′

#	Nucleotide	Peptide/Sugar (Origin)	Linker	Y (%)[a]	MALDI TOF-MS Experimental/calculated
C1	**N1**	-βA[b]PKKKRKV-OH (SV40 T antigen NLS)	HMDI	6.5	5637.77/5636.10
C2	**N1**	-βA[b]NSAAFEDLRVLS-OH (Influenza V nucleoprotein NLS)	CDI	10.7	6003.97/6001.78
C3	**N1**	-βA[b]LPPLERLTL-OH (HIV-1 Rev NES)	CDI	10.7	5799.53/5798.03
C4	**N1**	-βA[b]LRALLRALLRAL-OH (designed)	CDI	10.6	6064.46/6061.29
C5	**N1**	-βA[b]RLRLRLRLRL-OH (designed)	CDI	15.7	6051.81/6050.38
C6	**N1**	galactosamine	CDI	27.0	59322.67/5930.19
C7	**N2**	-βA[b]NSAAFEDLRVLS-OH (Influenza V nucleoprotein NLS)	CDI	7.3	5602.72/5600.56
C8	**N2**	-βA[b]PKKKRKV-OH (SV40 T antigen NLS)	HMDI	21.5	5505.97/5505.34
C9	**N2**	-βA[b]RKKRRQRRRPP-OH (HIV-1 Tat NLS)	HMDI	10.7	6303.02/6301.98
C10	**N2**	-βA[b]LPPLERLTL-OH (HIV-1 Rev NES)	CDI	12.7	5614.30/5613.21

C11	**N2**	Ac-GPKKKRKVGK[c]- (SV40 T antigen NLS)	CDI	18.2	5384.26/5390.51
C12	**N2**	Ac-GRKKRRQRRRPPGGK[c]-CDI (HIV-1 Tat NLS)	10.1	6226.18/6226.40	
C13	**N2**	-βA[b]NSAAFEDLRVLS-OH (Influenza V nucleoprotein NLS)	CDI	7.3	5602.72/5613.30
C14	**N2**	Ac-LPPLERLTLGK[c]- (HIV-1 Rev NES)	CDI	20.7	5461.60/5460.59
C15	**N2**	-βA[b]LRALLRALLRAL-OH (designed)	CDI	15.4	5815.13/5814.89
C16	**N2**	-βA[b]RLRLRLRLRL-OH (designed)	CDI	19.3	5659.38/5657.86
C17	**N2**	galactosamine	CDI	27.9	4400.03/4401.69
C18	**S1**	Ac-GPKKKRKVGK[c]- (SV40 T antigen NLS)	CDI	30.5	5642.63/5599.25

[a] Isolated yield determined by absorbance at 260 nm after HPLC purification.
[b] β-amino group is linked to oligonucleotide.
[c] ε-amino group of Lys is linked to oligonucleotide.

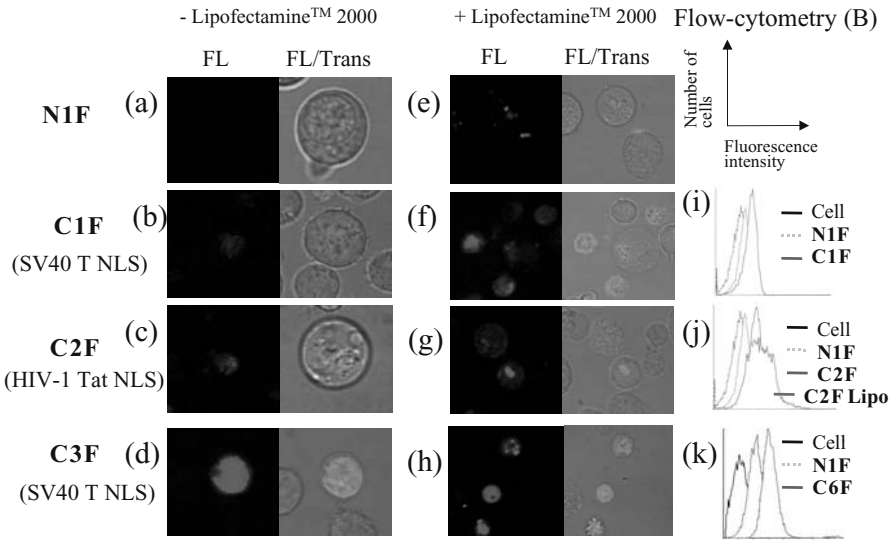


Fig. 1. Nuclear localization of oligonucleotide-peptide conjugates

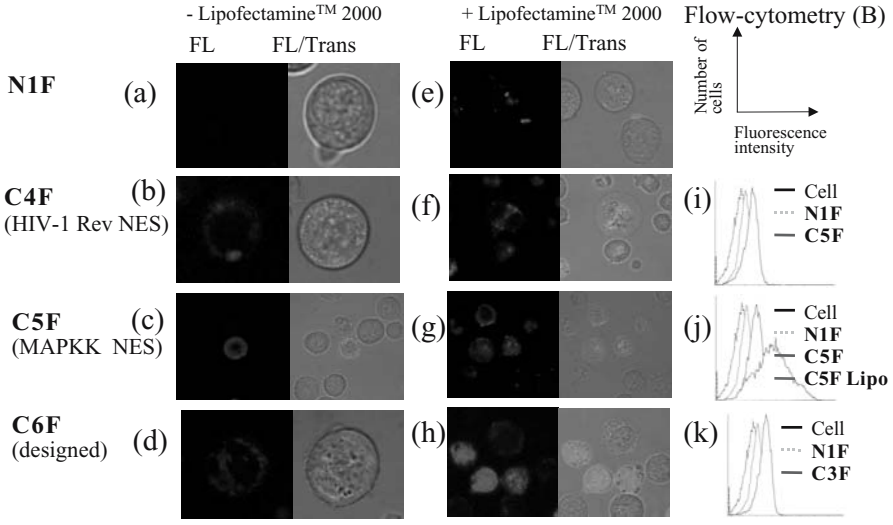


Fig. 2. Cytoplasmic localization of oligonucleotide-peptide conjugates

N1F: FITC-5'-CAGTTAGGGTTAG-3'

C1F: **N1**-SV40 T ant NLS

3'-GATTGGGATTGAC-5'-$OPO_3CH_2CH_2OCH_2CH_2NHCONH(CH_2)_4$CHCONH-**PKKKRKV**-OH
NHCOCH$_2$CH$_2$NH-FITC

C2F: **N1**-HIV-1 Tat NLS

3'-GATTGGGATTGAC-5'-$OPO_3CH_2CH_2OCH_2CH_2NHCONH(CH_2)_4CH(COOH)NH$-**QPPRRRQRRK KRG**-$COCH_2CH_2NH$-FITC

C3F: **S1**-SV40 T ant NLS

3'-s(GATTGGGATTGAC)-5'-$OPO_3CH_2CH_2OCH_2CH_2NHCONH(CH_2)_4CH(COOH)NH$-**GVKRKKKPG** -$COCH_2CH_2NH$-FITC

C4F: **N1**-HIV-1 Rev NES

3'-GATTGGGATTGAC-5'-$OPO_3CH_2CH_2OCH_2CH_2NHCONH(CH_2)_4$CHCONH-**LPPLERLTL**-OH
NHCOCH$_2$CH$_2$NH-FITC

C5F: **N1**-MAPKK NES

3'-GATTGGGATTGAC-5'-$OPO_3CH_2CH_2OCH_2CH_2NHCONH(CH_2)_4CH(COOH)NH$-**GEDLELEELKK QA**-$COCH_2CH_2NH$-FITC

C6F: **N1**-designed peptide

3'-GATTGGGATTGAC-5'-$OPO_3CH_2CH_2OCH_2CH_2NHCONH(CH_2)_4$CHCONH-**LRALLRALLRAL**-OH
NHCOCH$_2$CH$_2$NH-FITC

Scheme 2. Structures of FITC Labeled Oligonucleotide-Peptide Conjugates

When the conjugates were mixed with Lipofectamine 2000 and incubated with cells in 10% serum for 48 h, different features of cellular uptake were observed (Figure 1e–h). The natural olignucleotide **N1F** was taken up into cells but to a lesser extent (Fig. 1e). Small fluorescent particles could be observed in the cells, indicating that a large portion of the oligonucleotides remained in the endosomes after entry into cells. By contrast, oligonucleotide-NLS conjugates (**C1F-C3F**) were taken up much more efficiently than **N1F** and escaped from endosomes very rapidly. Flow-cytometric analysis also showed that use of the transfection reagent slightly enhanced cellular uptake of the conjugate **C2F** (Fig. 1j). It should be pointed out that use of the transfection reagent seemed to disturb control of the localization of the conjugates in the nuclei, perhaps by disturbing the interaction of importin-α with NLS peptides that are rich in basic amino acids.

Cellular uptake and intracellular localization of oligonucleotide-NES peptide conjugates **C4F-C6F** were analyzed by microscopy and flow cytometry (Fig. 2). It can be clearly seen that cellular uptake of the oligonucleotide-NES conjugates was greatly enhanced without any transfection reagents compared with uptake of the natural oligonucleotide **N1F** (Fig. 2a). It is likely that the hydrophobic characteristics of NES peptides facilitated penetration of the conjugates through the cellular membrane. It should be noted that conjugates bearing either a NES sequence of HIV-1 Rev protein (**C4F**) or MAPKK (**C5F**) localized throughout the cytoplasm (Fig. 2b, c). Curiously, conjugate **C6F**, bearing a designed peptide with cationic amphipathic features, localized in the cytoplasm, just like the oligopnucleotide-NES conjugates **C4F** and **C5F** (Fig.

2d). It can be assumed that the sequence of the designed peptide, with leucine residues every three amino acids, was recognized as a NES signal by an exportin. The use of the transfection reagent Lipofectamine 2000 enhanced cellular uptakes of the conjugate (Fig. 2j) but seemed to disturb the precise control of intracellular localization of the conjugates (Fig. 2f–h).

Thus oligonucleotide-peptide conjugates were shown to be taken up effectively into cells in the absence of any transfection reagents. Controlled nuclear localization was achieved by oligonucleotide-NLS conjugates and cytoplasmic localization was achieved by oligonucleotide-NES conjugates.

4 Mechanisms of Cellular Uptake of Oligonucleotide-Peptide Conjugates

To investigate the mechanisms of the entry of the oligonucleotide-peptide conjugates, the effect of an endocytosis inhibitor cytochalasin B on cellular uptake were studied (Simeoni et al. 2003). The results are shown in Fig. 3. Cellular uptake of **N1F** using Lipofectamine 2000 was inhibited by the addition of cytochalasin B (Fig. 3a–c), which indicated that natural oligonucleotide was taken up into cells by an endocytotic mechanism. By contrast, the entries of both the oligonucleotide-NLS conjugate **C2F** and the oligonucleotide-NES conjugate **C5F** were not affected by the addition of cytochalasin B in the presence or absence of the transfection reagent (Fig. 3d–f and g–i, respectively). These results strongly indicate that entry of the oligonucleotide conjugates is not dependent on an endocytotic mechanism. Further investigations to identify the detailed mechanism involved in this process are now in progress in our laboratory.

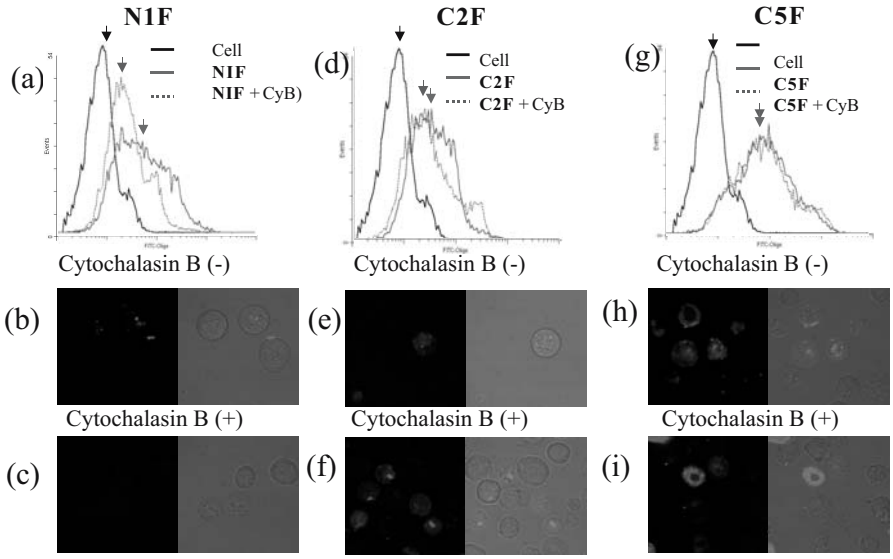


Fig. 3. Inhibitory effect of endocytotic entry by cytochalasin

We have already found that an antisense oligonucleotide-NLS conjugate inhibited telomerase in human leukemia cells by 98% while an antisense oligonucleotide-NES conjugate did not inhibit it at all. We also have found that an antisense oligonucleotide-NES conjugate suppressed the expression of the gene *bcr/abl* on Philadelphia chromosome by 86%, while an oligonucleotide-NLS conjugate suppressed expression by only 20% (Bakalova et al. 2004). These results prove that intracellular localization of antisense oligonucleotides is extremely important for their activities (Kuwabara et al. 1999; Warashima et al. 2001). Furthermore, we confirmed that oligonucleotide-peptide conjugates were much more resistant against cellular nucleases, that they could bind to target RNA with higher affinities, that the hybrids between them and complimentary RNAs could activate RNase H, and that they showed little cytotoxicity. The detailed data will be published elsewhere in the near future.

These findings strongly suggest that oligonucleotide-peptide conjugates are promising candidates for effective genetic medicines of the next generation. We believe that new, intelligent oligonucleotides can be created by linking oligonucleotides to natural and unnatural molecules, forming compounds that are never found in nature.

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