Drug Delivery System Based on Responses to an HIV Infectious Signal

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Abstract: Gene therapy is a growing topic in the medical arena. Since the safety system of gene therapy has not been sufficiently established, its clinical use is limited. Recently, we developed a cell-specific gene regulation system based on a new concept, D-RECS, or Drug and Gene Delivery System Responding to Cellular Signals. We hoped here to apply this D-RECS concept to gene therapy for virus infections. In the present study, we report the design, synthesis and characterization of the functional polymers, which are able to discriminate normal and human immunodeficiency virus type 1 (HIV-1) infected cells. In the D-RECS concept, certain intracellular signals, which are extraordinary activated in the target disease cells specifically, are used as a trigger to activate a transgene expression. Thus, we paid attention to HIV protease as a target signal in this case, because HIV protease is essential for the proliferation of HIV. This protease is therefore an indicator of HIV infection. Two types of polymers were designed and synthesized using methacryloyl peptide and acrylamide with radical copolymerization as a functional gene regulator. The grafted peptide possesses a cationic protein transduction domain (PTD) sequence of HIV-Tat protein, GRKKRRQRRRPPQ for cell permeation, which are connected with polyacrylamide backbone *via* a consensus substrate sequence for HIV protease, SQNY/PIVQ. At first, the polymers were evaluated to see whether they possess DNA binding ability and HIV protease responsibility using gel retardation assay. The results suggested that a polymer could form a stable complex with DNA and release the DNA specifically responding to HIV protease activity. Furthermore, it was shown that this controlled release of DNA by the HIV protease signal-responsive intelligent polymer actually regulated the gene expression in the cell-free system. This system would be a useful tool for gene therapy in HIV infection, and this methodology will be applicable if the cationic peptide is replaced by another virus-specific protease, which is critical for the replication of a corresponding virus.

Key Words: HIV, HIV protease, D-RECS, gene delivery system, gene therapy.

INTRODUCTION

Gene therapy is a growing topic in medicine today. Clinical trials of several gene therapeutic strategies for HIV infection have been developed since the 1990s, but these methods are still inadequate [1-3]. In addition, development of highly active antiretroviral therapy (HAART) [4-6] that blocks virus replication led to a major breakthrough in the treatment of HIV infection, which discouraged the incentive to study genetic approach to combat HIV infection. However, the most critical limitation of HAART is its failure to eradicate the virus even in the lymph nodes of patients in prolonged HAART therapies with undetectable HIV in the blood [7], such that the patient is chronically infected. This failure is mainly due to the existence of drug resistant strains, which are probably able to survive for the life of the patient [8, 9]. In many cases, the real problem to eradicate the virus is the existence of viral reservoirs. Recently, several approaches for eradication of latent HIV have reported toward the development of alternative strategy [10-12]. Thus, development of new therapies to solve these HAART problems is urgently needed.

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At this moment, though a number of transgenes acting *via* diverse mechanisms have been devised [13], therapeutic transgene products that achieve a HIV-infected cell-specific expression have not been established. Currently, most of the effective gene carriers are based on viruses, but their clinical use is limited [1]. Non-viral vectors such as cationic polymers are more advantageous than viral vectors in terms of safety, immunogenicity and simplicity of use. A target cell-specific delivery or gene expression using active targeting method has reported, however, it has not worked effectively. Giving more accuracy, we recently reported cell-specific gene delivery systems based on a new concept, D-RECS, or Drug and Gene Delivery System Responding to Cellular Signals [14-16]. Here, we applied the D-RECS concept to gene therapeutic strategies for HIV infection (Fig. **1**). Because the production of HIV protease is one of the key indicators of HIV infection, we designed and synthesized a new peptide-polymer conjugate, which can activate a transgene expression in response to HIV protease activity. Characterization of the HIV protease-responsive DNA release from the polymer/DNA complex and its ability to regulate gene expression were also investigated. The results encouraged us to achieve HIV-infected cell-specific transgene expression.

MATERIALS AND METHODS

Materials

Fmoc-SAL-PEG-resin and all Fmoc-protected amino acids for peptide synthesis were obtained from Watanabe

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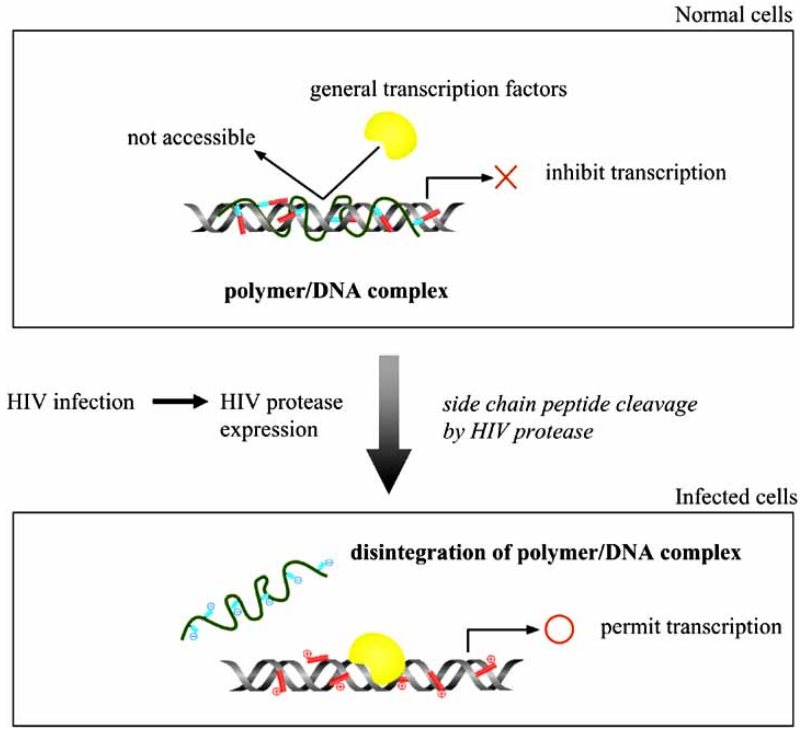


Fig. (1). Mechanistic scheme of an artificial gene regulation responding to HIV-1 protease.

When the CPCHIVtat forms a complex with the DNA, gene transcription is suppressed, probably due to steric hindrance of the polymer chain prohibiting RNA polymerase movement along the DNA strand. In the case of HIV-infected cells, HIV protease is expressed, and the cationic portion of the peptide in the CPCHIVtat is cleaved with HIV protease. These events cause a disintegration of the complex and release the DNA to activate transcription.

Chemical Industry Co. Ltd. (Hiroshima, Japan). Coupling reagents such as 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyuronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were also purchased from Watanabe Chemical Industry Co. Ltd. All other chemicals were of the best grade available from Wako Pure Chemical Industries, Co. Ltd. (Osaka, Japan). The recombinant HIV protease-1 was purchased from BACHEM Bioscience Inc. (King of Prussia, PA, USA).

Synthesis of Polymers for Gene Regulator

Substrate peptides were synthesized by an automatic peptide synthesizer according to an Fmoc synthetic strategy. Peptides were liberated from the resin by treatment with trifluoroacetic acid (TFA) containing 2.5% triisopropylsilane and 2.5% water, and purified by reversed-phase HPLC (Inertsil ODS-3 column, 250 × 20 mm, 3.5 μm, GL Sciences Inc., Tokyo, Japan). The measurements of mass spectra were done on a MALDI-TOF-MS spectrometer (Voyager^{TM} DE RP Biospectrometry Workstation, Applied Biosystems, Framingham, MA, USA) to verify the peptides synthesized. Polymers were synthesized in a manner similar to that described previously [14]. Typically, a corresponding methacryloyl-peptide (1.0 μmol), in which the methacryloyl group was attached at the amino-terminus of the peptide, and the acrylamide (7 mg, 99 μmol) were dissolved in degassed water and allowed to stand at room temperature for 90 min after the addition of ammonium persulfate (0.65 mg, 2.8 μmol) and N,N,N',N'-tetramethyl-ethylenediamine (0.86 μl, 5.7 mmol) as the redox initiator couple. The product was then purified by overnight dialysis against water using a semipermeable membrane bag (with a molecular-weight cutoff of 50,000), followed by lyophilization to obtain a white powder. The content of the peptide was calculated from elemental analysis.

MALDI-TOF-MS Based HIV Protease Assay

Fragment peptides digested by HIV protease were evaluated using MALDI-TOF mass spectrometry. A solution (1.0 μl) of recombinant HIV protease at a concentration of approximately 100 ng/μl was added in 35 μl of acetic acid/sodium acetate buffer (pH 4.7) containing 100 mM NaOAc, 300 mM NaCl, and 4 mM EDTA. After the synthetic substrate peptide solution (4 μl) was added, the solution (40 μl) was incubated for 1 h at 37°C. The reaction was quenched by addition of 4 μl of 0.1% TFA containing a α-cyano-4-hydroxycinnamic acid (CHCA) matrix (10 mg/ml) in 50% water/acetonitrile to 4 μl of the reaction mixture. The mixture (1 μl) was applied on the sample plate and allowed to dry to induce crystallization and then analyzed by MALDI-TOF mass spectrometry.

Gel Retardation Assay

Polymers were assessed in a manner similar to that described above [17]. Briefly, 1234 bp linear DNA (0.1 μg) was dissolved in 2 μl of sterile water. The polymer was then

added to the solution at various concentrations. The net charge of the polyplex was calculated from the pendant peptide assuming at +8.0. All solutions were diluted to 4.0 µl with sterile water and incubated for 15 min at room temperature for the DNA/polymer polyplex formation, and then the solutions were subjected to gel electrophoresis on 1% agarose. For the HIV protease digestion, HIV protease-1 (0.42 µg) was added to each solution and incubated for 120 min at 37˚C prior to gel electrophoresis experiment.

Ethidium Bromide Displacement Assay

Plasmid DNA (0.3 µg, pEGFP-C1, CLONTECH Laboratories, Inc.) was mixed with 0.05 µg of ethidium bromide (EtBr) in 8.0 µl of sterile water, and the solution was incubated for 5 min at room temperature. Then the CPCHIVtat or poly-L-lysine (PLL) was added, and the reaction mixture was incubated for additional 15 min, and then the volume was adjusted to 100 µl by addition of 10 mM Tris-HCl (pH 7.5) followed by the measurement of fluorescence intensity (λex/λem = 531/590 nm, ARVO SX (Wallac Inc.)). The recorded fluorescent intensities were expressed relative to the fluorescence intensity of the plasmid DNA/EtBr solution in the absence of the polymer.

Luciferase Assay in Cell-Free System

The luciferase assay was carried out essentially as described above [17]. In brief, all experiments were performed using a cell-free expression system (T7 S30 Extract System for Circular DNA, Promega Corp., WI, USA). After the complexation of plasmid DNA with the polymer at a charge ratio of 7.5 as described in the section on gel retardation assay, recombinant HIV protease-1 (0.42 µg) was added to the polymer-DNA solution, the reaction mixture was incubated at 37˚C for 2 hr. "T7 Quick Master Mix" (16 µl) and 1 mM methionine (0.5 µl) was added to the reaction mixture and was incubated at 30˚C for 30 min. A luciferine solution (100 µl) was then added to 10 µl of the reaction mixture, and the chemiluminescence was measured using a multilabel counter ARVO SX (Wallac Inc.).

RESULTS AND DISCUSSION

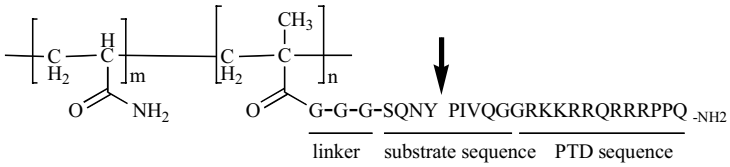
Concept of the HIV Protease-Responsive Gene Regulation

Fig. **1** shows a schematic illustration of the target protease specific D-RECS concept. In the polymer-DNA complex, CPCHIVtat (cationic polymer possessing the cleavage site for HIV protease-1) suppresses the accessibility of RNA polymerase to the DNA strand. In contrast, when the intracellular HIV protease is continuously activated, this complex is disintegrated due to cleavage of the cationic portion of the peptide side chain from the main polymer chain. In this case, the cationic charges are completely removed from the polymer chain, and free DNA is released from the polymer-DNA complex due to the disappearance of the electrostatic interaction. As a result, HIV protease signaling should accelerate the gene expression.

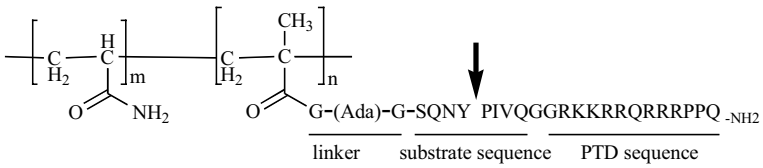
Design and Synthesis of Cationic Peptide Substrate for HIV Protease-1 Responsible Gene Regulator

Sequences known as substrates for HIV protease are not cationic, therefore a cationic portion was added to the peptide substrate to interact with plasmid DNA. So the cationic protein transduction domain sequence of HIV-Tat protein, GRKKRRQRRRPPQ, was incorporated into the substrate peptide at the C-terminus *via* glycine residue. This sequence renders not only DNA-binding ability to the polymer, but also cell permeability to the material. On the other hand, tri-glycine and/or glycil-8-amino-3,6-dioxaoctanoyl-glycine were incorporated into the peptide at the N-terminus as a link to polymer backbones (Fig. **2**), and represented as CPCHIVtat-G3 and CPCHIVtat-G(Ada)G, respectively.

First of all, we examined if the HIV protease was able to cleave the synthetic peptides using MALDI-TOF mass spectrometry. Fragment peptides (*m/z*: 2213.77 in CPCHIVtat-G3, and *m/z*: 2213.61 in CPCHIVtat-G(Ada)G) due to the cleavage with HIV protease were detected, suggesting that these peptides were sufficient to act as an HIV protease substrate.



CPCHIVtat-*G3*



CPCHIVtat-*G(Ada)G*

Fig. (2). Chemical structures of CPCHIVtat.

Both polymers are graft-type copolymers that were synthesized using each methacryloyl-peptide monomer and acrylamide with radical copolymerization. Arrows indicate the cleavage site for HIV-1 protease, and Ada means $-NH-(CH_2CH_2O)_2-CH_2CO-$.

Preparation of HIV Protease-Responsive Gene Regulator

The HIV protease-responsive polymers were synthesized using each methacryloyl-peptide monomer and acrylamide with radical copolymerization followed by dialysis. The resulting polymers (CPCHIVtat-G3 and CPCHIVtat-G(Ada)G) contained the peptide side chain at concentrations of 0.30 and 0.44 mol%, respectively. Since it was not clear if the peptide was also discriminated in the polymers by the protease as well as the peptide alone, we assessed the reactivity of the polymers with HIV protease using mass spectrometry. The cationic peptide fragment due to the cleavage with HIV protease (*m/z*: and 2213.51) was detected for CPCHIVtat-G(Ada)G, but the corresponding fragment was not seen in CPCHIVtat-G3. Presumably, HIV protease was not able to access to the substrate peptide in CPCHIVtat-G3 because of steric hindrance and/or poor flexibility of the substrate portion. In contrast, HIV protease was able to access and cleave the substrate in CPCHIVtat-G(Ada)G possessing 8-amino-3,6-dioxaoctanoic acid (Ada) into the link. Interestingly, this fragment peptide was detected even in the CPCHIVtat-G(Ada)G/plasmid DNA complex (*m/z*: 2213.55). This indicates that HIV protease recognizes the substrate sequence even in the polymer condensed with the plasmid DNA. Then we investigated whether the cleavage of the cationic portion of the pendant peptide in CPCHIVtat-G(Ada)G disintegrated the complex and released the plasmid DNA using gel retardation assay. Fig. (**3A**) compares the stability of polymer/DNA complexes subjected to gel electrophoresis in the presence or the absence of HIV protease. In the absence of HIV protease (lanes 5 and 6 in Fig. **3A**), the polyplex with C/A +1.0 remained stable, and no DNA was released from the complex. The polyplex with C/A ratio +0.5 showed a slight retardation with a smear band indicating a fraction of free DNA. On the other hand, in the presence of HIV protease (lanes 3 and 4 in Fig. **3A**), the polyplex with C/A ratio +1.0 showed the release of a fraction of DNA as indicated by a smear band. At C/A ratio +0.5, the brightness of the DNA band was detected more or less equal to lane 2, in which only naked DNA ran, indicating that most DNA was released from the polyplex. These indicate that the HIV protease actually disintegrates the complex to release the free DNA through the cleavage off the cationic peptide from the polymer backbone. In addition, the DNA binding ability of the polymer was assessed by fluorescence titration experiments employing ethidium bromide (EtBr) displacement assay. EtBr intercalates between the base pairs of the DNA double helix, yielding a highly fluorescent DNA/EtBr complex. When the cationic polymer binds to DNA, EtBr is expelled from the DNA/EtBr complex, resulting in a decrease in fluorescence intensity [18, 19]. Thus, the degree of displacement of the dye provides an indirect measure of the binding affinity, indicating the relative strength of the interaction between the DNA and the polymer. As shown in Fig. (**3B**), the addition of the polymer to the DNA/EtBr complex resulted in a displacement of EtBr, reaching a sharp saturation point around C/A 1. This result indicates that the synthesized polymer would make DNA compaction as poly-L-lysine did. Thus, the CPCHIVtat-G(Ada)G possesses sufficient basic property as a gene regulator specific to HIV infectious cells.

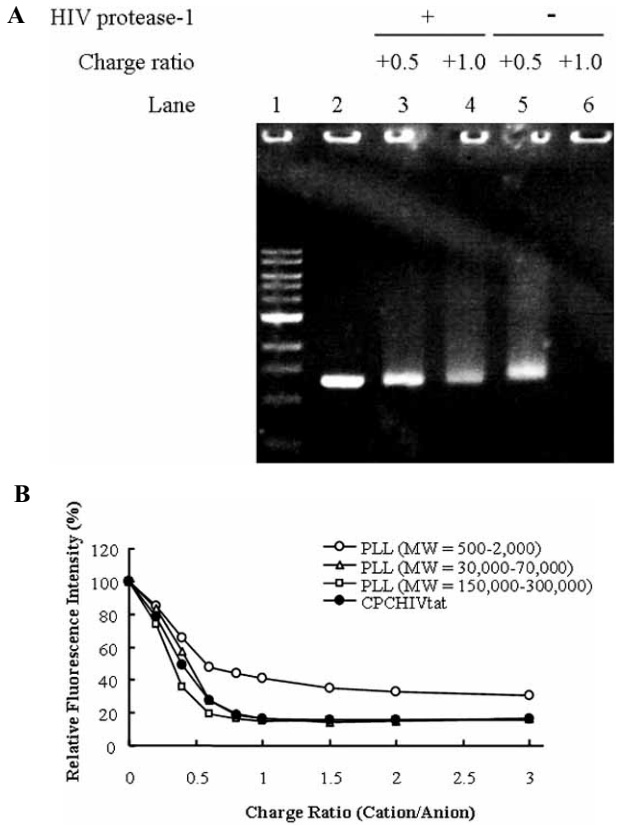


Fig. (3). Formation of CPCHIVtat/DNA complex and its disintegration with HIV protease signal. (**A**) CPCHIVtat was mixed with the plasmid DNA at various concentrations. After 15 min, the solution was subjected to 1% agarose gel electrophoresis and stained with ethidium bromide. Lane 1: 1kbp molecular marker, Lane 2: DNA alone, Lane 3-6: CPCHIVtat/DNA complex (Lane 3,4: in the presence of HIV protease, Lane 5,6: in the absence of HIV protease). + and -, presence and absence of HIV protease, respectively. Data shown are representative of three independent experiments. (**B**) The ability to condense DNA was assessed by ethidium bromide exclusion assay. Ethidium bromide was mixed with the plasmid DNA, and then the CPCHIVtat or poly-L-lysine (PLL) with various range of molecular weight was added, and the reaction mixture was incubated for 15 min followed by the measurement of fluorescence intensity (λex/λem = 531/590 nm).

Regulation of Gene Expression in Cell-Free Systems

The results from gel retardation assays indicate that the complex of CPCHIVtat-G(Ada)G/DNA releases the DNA by the treatment of HIV protease. This was supported by an *in vitro* transcription and translation system to evaluate the luciferase expression in cell-free systems. In this system, the interaction between the cationic polymer and the anionic DNA is not required to be specific because the luciferase expression plasmid was only driven by the T7 promoter, not by the HIV promoter. Consequently, when the polyplex disintegrates due to the cancellation of the cationic net charge of the polymer, the regulation of HIV protease responsible for gene expression was able to evaluate. Fig. (**4**) shows the regulation of the luciferase expression with an HIV protease

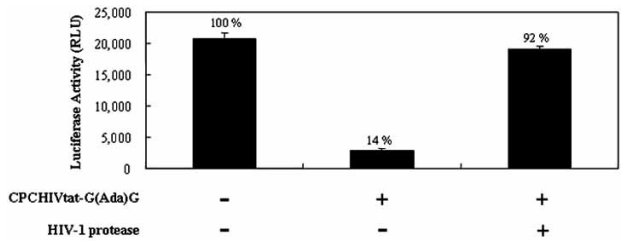


Fig. (4). Suppression of luciferase expression with the CPCHIVtat and its cancellation with HIV protease signaling in a cell-free system. CPCHIVtat was mixed with the luciferase expression plasmid. After 15 min, HIV-1 protease was added to the solution, and the reaction mixture was incubated at 37˚C for 2 hr. Then, the luciferase was expressed by the addition of "T7 Quick Master Mix", and the lusiferase activity was measured. The luciferase activity in the presence of CPCHIVtat-G(Ada)G with or without active HIV-1 protease was shown. + and -, presence and absence of CPCHIVtat-G(Ada)G or HIV protease, respectively. Data shown are the mean ± SEM of duplicate samples and are representatives of two independent experiments.

signal. The luciferase expression was significantly suppressed in the CPCHIVtat-G(Ada)G/DNA complex at a 7.5:1 charge ratio. However, the treatment of this complex with HIV protease recovered the expression ratio to approximately 100% compared to that of free DNA. In contrast, when we used another protease, caspase-3 and factor Xa, the gene expression did not recover (data not shown). CPCHIVtat-G(Ada)G has cationic peptide moieties corresponding to HIV-1 Tat protein, so that this cationic polymer interacts with anionic polymer DNA by electrostatic interaction. This interaction is not required to be specific as described above. The cationic peptide moiety responds to HIV protease in a sequence-specific manner, but the cationic peptide itself was not able to suppress the transgene expression (data not shown).

PERSPECTIVES

In the present study, design and synthesis of the substrate peptide for HIV protease, and HIV protease-responsive gene regulator possessing the peptide were prepared according to the D-RECS strategy. The relevant efforts to identify molecules, which play pivotal roles in virus replications, allow the possibility of the development of therapeutic reagents with molecular targeting. There are several molecular targeting agents approved to treat HIV/AIDS, for example, azidothymidine (AZT) as a nucleoside reverse transcriptase inhibitor (NRTI), nevirapine as a non-nucleoside reverse transcriptase inhibitor (NNRTI), and saquinavir as a HIV protease inhibitor (PI). More recently, the integrase inhibitors [20] and chemokine receptor CCR5 inhibitors [21] have been reported as new categories of anti-HIV drugs, and several of these drugs have been tested in the phase II/III clinical trials. As described in the introductory section, the new strategy to solve present problems such as latent HIV and drug-resistant strains should be urgently addressed. At present, most drugs have been designed to target a single molecule in a particular cellular signaling. If it is possible to convert an extraordinary cellular signal to an artificial and advantageous signal, but not to augment or inhibit a cellular signal, an effective and universal concept for drug design would be possible. Our D-RECS concept described here is quite different from the molecular targeting and DDS (drug delivery system) strategies, and could be applicable to other viral infections by utilizing their intrinsic proteases that are essential for their replications, *i.e.*, CPCHIVtat-G(Ada)G with a peptide responding to the protease would deliver a toxic gene only into the cells infected by the virus. Thus our D-RECS concept can be useful to establish the universal concept of drug design to activate particular DNA only in virus-infected cells. Although the development of HIV/AIDS gene therapy is not yet at a sufficient level, a functional gene regulator may be a powerful tool to overcome the series issue that underlies current gene therapy techniques. Recent studies have shown that several types of RNA gene therapies including RNA interference could inhibit HIV replication by targeting viral or cellular genes [22]. Our HIV protease-responsive DNA releasing system will also be applicable to those technologies because the data reported here suggests a specific transgenes expression only in HIV- infected cells. However, another carrier, which can encapsulate our polymer/DNA complex, should be needed for the application *in vivo*. Recently, we succeeded in delivering the D-RECS polymer/DNA complex into living cells using HVJ-E, an inactivated virus envelope [14, 15] and/or functional bio-nanocapsules (unpublished data). More recently, we confirmed that our system functioned and released DNA by responding to HIV protease in HIV-infected Jurkat cells (manuscript submitted). The gene delivery system using above tools will be reported in the future.

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ABBREVIATIONS

D-RECS	=	Drug and gene delivery system responding to cellular signals
HIV	=	Human immunodeficiency virus
PTD	=	Protein transduction domain
HAART	=	Highly active antiretroviral therapy
Fmoc	=	9-fluorenylmethoxycarbonyl
HBTU	=	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyuronium hexafluorophosphate
HOBt	=	1-hydroxybenzotriazole
TFA	=	Trifluoroacetic acid
HPLC	=	High-performance liquid chromatography
MALDI-TOF-MS	=	Matrix assisted laser desorption ionization time-of-flight mass spectrometry
CHCA	=	α-cyano-4-hydroxycinnamic acid

CPCHIV = Cationic polymer possessing the cleavage site for HIV-1 protease

Ada = 8-amino-3,6-dioxaoctanoic acid

EtBr = Ethidium bromide

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