Controlled Release of DNA Using Thermoresponsive Polymers

Masayuki Yokoyama

1 Significance of DNA Release from Synthetic Gene Carriers

The primary role of synthetic DNA vectors is their translocation through cellular membranes, since only a small amount of naked DNA goes through these membranes due to its large molecular weight and negative charge at very high density. Thus, in order to obtain high rates of uptake, the DNA is commonly complexed with positively charged lipids or polymers, yielding ion complexes packaged in synthetic vectors. The complexes usually have a net positive charge and thus interact with negatively charged cellular membranes, resulting in cellular uptake by endocytosis. Another advantage of packaging DNA within complexes is that the resistance to DNA hydrolytic enzymes is enhanced.

A large number of studies have focused on optimizing complex formation using various chemical structures of lipids and polymers to maximize the transfection efficiency of synthetic vectors. In contrast, only a few studies have examined the dissociation of the DNA-carrier complexes. In order for the transfected DNA to be recognized by transcription enzymes or form base pairs, thereby allowing, respectively, transcription or the formation of duplexes or triplexes via antisense DNA, it must be in the naked form. Therefore, both the formation and the dissociation of the complex are important to achieving highly effective synthetic DNA carrier systems.

As illustrated in Fig. 1, the optimization of DNA-carrier complex formation is important for the efficient entry of the complexes into cells and the protection of DNA from degradation. However, subsequent quick release of the DNA from the complex is necessary in order to accelerate the expression of DNA functions. In addition to the enhancement of transfection efficiency, controlled DNA release allows selective expression of DNA functions, such as gene expression and antisense action, which are important for medical applications. In many types of gene therapy, controlled gene expression is not strictly required for some genes. Adenosine deaminase is a typical example in that therapeutic effects are obtained over a wide range of adenosine deam-

Kanagawa Academy of Science and Technology, KSP East 404, 3-2-1 Sakado, Takatsu-ku, Kawasaki 213-0012, Japan

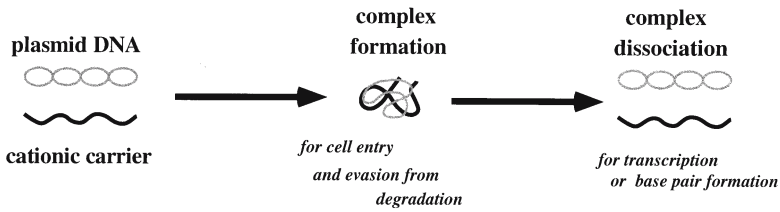


FIG. 1. Significance of complex formation and dissociation

TABLE 1. Methodologies for specific gene expression.

1. Specific promoters
* signal-inducible (e.g., heat shock, lactose, tertacycline)
* organ-, tissue-specific
2. Selective delivery to target with carriers
3. Stimuli-responsive gene carriers

inase concentrations in the blood. Additionally, the cell type and the location of protein production are not restricted as they are in nature if the produced protein is to be present in the blood. In contrast, the quantity, site, and timing of protein production must be strictly controlled in order to obtain the therapeutic effects of many other kinds of proteins, for example, cytokines for gene therapies of cancer. Naturally, the amount of cytokines, as well as the site, timing, and duration of production are strictly controlled by the immune system. Therefore, cytokine expression in an excess amount, at unwanted sites, or for too long causes severe toxic side effects.

If DNA release from the DNA-carrier complex is controlled by external stimuli or internal signals that are specific to the therapeutic sites, the desired amount of selective gene expression at a specific site, with the correct timing, and for the appropriate duration could be accomplished. This is analogous to drug targeting systems (for conventional drugs) that achieve selective drug action at the therapeutic sites without toxic side effects. In most gene therapy protocols that have been carried out to date, gene expression was not precisely controlled in terms of the parameters mentioned above. The specific regulation of gene expression, particularly in vivo, would substantially improve progress in gene therapy.

2 Stimuli-Responsive Synthetic DNA Carriers

Table 1 summarizes three approaches to obtaining site-, timing-, and duration period-specific gene expression,. Firstly, the use of specific promoters (Russel 1999) can initiate transcription of the gene of interest selectively in a specific environment. Physical signals, such as γ-ray (Stackhouse and Buchsbaum 2000; Nuyts et al. 2001) and temperature (Schweinfest et al. 1988), and chemical signals, such as lactose (Jacobsen and Willumsen 1995) and tertacycline (Gossen et al. 1995; Furth et al. 1994), induce transcription of genes placed downstream of the respective signal-inducible promoter. Alternatively, tissue- or organ-specific promoters have been used to obtain the corre-

sponding tissue- or organ-specific gene expression, even if the exact nature of the signal is not always known. Using these specific promoters, induction ratios, defined as the ratio of gene expression in the presence and absence of the signal, of up to several thousand-fold have been obtained. The utility of many of these promoters, however, is considerably limited, since their specificity is based on the the binding properties of naturally occurring transcription factors. For example, the heat-shock-protein promoter responds only to a temperature (42°C) that is dangerous to living bodies. Artificial protein engineering to change the signal temperature is very difficult using current technologies. Furthermore, the specific delivery of chemicals to therapeutic sites poses another problem for chemical-signal-inducible promoters, since, in general, the specific concentrations and duration of action of these chemicals at the target sites are not easily achieved in vivo.

The second approach to selective gene expression is delivery by carriers. Similar to the delivery of pharmaceutical drugs, several types of carriers, such as antibodies, liposomes, and synthetic polymers, have been studied with the goal of achieving the selective delivery of genes. Even without specific promoters, selective gene expression is possible by the selective delivery of genes. In nature, viral vectors work as selective carriers by utilizing their infection tropism for specific cell types.

The third approach to obtaining specific gene expression is by using stimuli-responsive gene carriers. Synthetic vectors induce transcription by releasing the DNA component of the DNA-carrier complex at specific environments. Thus, stimuli-responsive gene carriers not only allow site-specific gene expression, but they also contributes to greatly enhancing the efficiency of gene expression. As stated above, it is very difficult to optimize complex formation and dissociation using ion-complex-type synthetic gene carriers, since these two processes are in opposition to each other. In order to overcome this difficulty, intelligent systems to control complex formation/dissociation by external stimuli are being studied. By applying physical stimuli. such as light and temperature, complex formation/dissociation or tight/loose complex formation may be achieved.

One of the benefits of stimuli-responsive vector systems is high gene expression efficiency. As shown in Fig. 2, in conventional cationic vector systems, the complex must be formed with an intermediate tightness in order to simultaneously fulfill the two opposing requirements of protection from enzymatic degradation and free access to RNA polymerase. However, an intermediate tightness prohibits maximum efficiency for either function. Similarly, for stimuli-responsive vector systems, the avoidance of DNA degradation can be optimized by forming a very tight complex, while transcription efficiency is maximized by complex dissociation or loose complex formation. Gene expression efficiency (transfection efficiency) is a multi-step process that is dependent upon the efficiency of various cellular processes, including attachment to the cell surface, endocytosis, translocation from endosome to cytosol and then from cytosol to nucleus, and, finally, transcription. In contrast to cationic vector systems, efficiency may be maximized in stimuli-responsive gene carrier systems, since the efficiency of each cellular step can be increased without decreasing the efficiencies of the other steps.

The second benefit of stimuli-responsive vectors is that site-, timing-, and duration-specific gene expression may be achieved by releasing the DNA from the DNA-carrier complex in a stimuli-responsive manner. Compared with the other methodologies

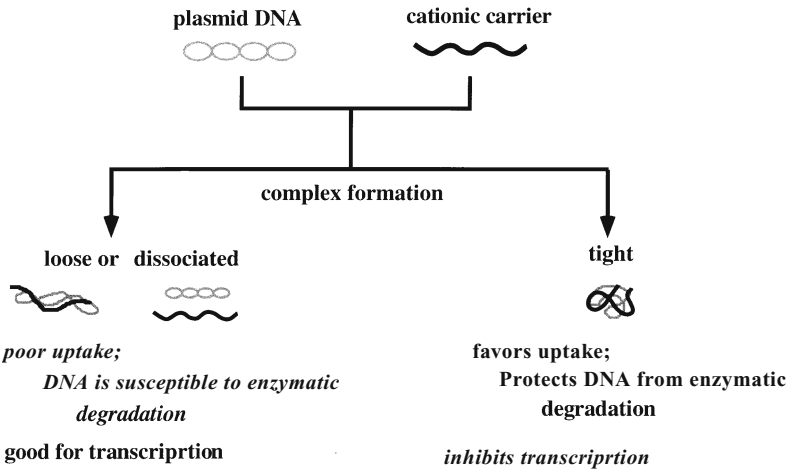


Fig. 2. The properties of tight vs. dissociated or loose DNA-cationic carrier complexes

listed in Table 1, stimuli-responsive synthetic vectors possess several advantages. First, fine adjustments in stimuli applied to living bodies are possible; for example, while in the heat-shock-protein promoter system, the inducing temperature is fixed for, in the stimuli-responsive synthetic systems it can be easily changed by designing appropriate chemical structures. Second, site specificity is easily obtained by the application of light and temperature. While light possesses advantages over temperature with respect to site precision, temperature can also be applied with considerable specificity, such as by using a 5-mm ultrasound device (Umemura et al. 1996; Kawabata and Umemura 1996). In addition, a temperature stimulus has advantages over light in terms of available depth from the surface. In particular, such site-specificity can be readily obtained in a uniform tissue (e.g. dermis and muscle) or organ.

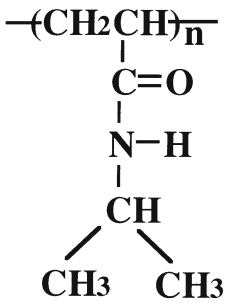
Nagasaki et al. developed novel synthetic light-inducible gene expression systems using dendrimers and lipids. These studies are described elsewhere in this book.

3 Temperature-Responsive Polymers and Their Applications

Temperature is one of the safest external signals that can be applied to a variety of sites of living bodies. Site-specific heating is now routinely carried out in hyperthermia therapy against solid tumors (Falk and Issels 2001). The hyperthermia, which is localized, is usually applied by microwave, which selectively heats the target site with a precision of approximately 1 cm. Furthermore, since microwave heating can reach a considerable depth from the body surface, this therapy can be used to treat most internal organs and tissues, even though the microwave radiation is applied from outside the body.

In a separate approach, thermoresponsive synthetic polymers have been actively studied due to strong interest in their unique physico-chemical characteristics as well

Fig. 3. Chemical structure of poly(*N*-isopropylacrylamide) (P(IPAAm))



as to their potential applications in medicine and biotechnology. Among these thermoresponsive polymers, poly(*N*-isopropylacrylamide) [P(IPAAm)] (Fig. 3) has received the most attention, mainly owing to the fact that its phase-transition of temperature 32°C is near body temperature (Heskins and Guillet 1968). P(IPAAm) has been applied extensively to biomedical applications, such as hydrogels (Yoshida et al. 1995; Kaneko et al. 1995), bioconjugates (Matsukata et al. 1996), and polymeric micelles as drug carriers (Chung et al. 1998, 1999; Kohori et al. 1998). The phase-transition temperature of P(IPAAm) is a lower critical solution temperature (LCST). Below the LCST, P(IPAAm) is water-soluble, hydrophilic, and assumes an extended chain form, while above the LCST, it undergoes a reversible phase transition to form an insoluble and hydrophobic aggregate. The other benefits of P(IPAAm) for biomedical applications are its simple chemical structure and sharp phase-transition behavior. The phase transition temperature of P(IPAAm) can be altered by copolymerizing IPAAm with another monomer unit. Copolymerization with hydrophilic and hydrophobic monomers results in an increase or decrease of the LCST, respectively. For example, in order to obtain a thermoresponsive drug releasing system, the phase-transition temperature was adjusted to 39°C by introducing a hydrophilic comonomer, *N,N*-dimethylacrylamide.

4 DNA Carrier Systems Using Temperature-Responsive Polymers: Temperature-Responsive Polymeric Vector Systems

A temperature-responsive polymeric vector system based on poly(*N*-isopropylacrylamide) was developed by Kurisawa, Takeda, and Okano (Takeda et al. 2004; Kurisawa et al. 2000; Yokoyama et al. 2001). Using this polymer as carrier, a tight complex with DNA formed above the transition temperature by hydrophobic aggregation of poly(*N*-

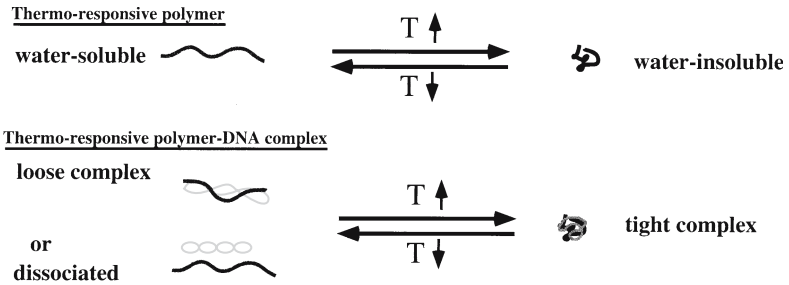


FIG. 4. Concept of thermoresponsive gene carriers. *T* Phase-transition temperature

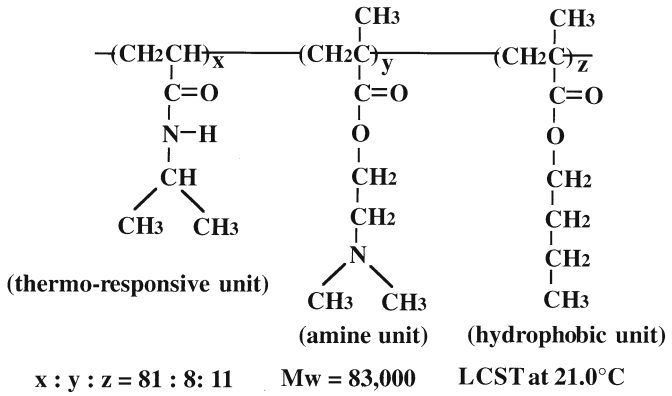


FIG. 5. Chemical structures of thermoresponsive gene carriers. *LCST* Lower critical solution temperature

isopropylacrylamide), as shown in Fig. 4. Below the transition temperature, the complex dissociated or loosened owing to the dissolution of the hydrophobic aggregate. A tightly formed complex is favorable for efficient cellular uptake and avoidance of enzymatic degradation of DNA, while free DNA released from the dissociated or loosely packed complex is favorable for transcription. Therefore, by switching from a tight complex and to a dissociated (or loosely formed) one contribute to optimizing the efficiency of those cellular processes needed for selective gene expression such as binding to cells, entry into cells, and transcription.

A temperature-responsive polymeric gene carrier was designed as shown in Fig. 5. This polymeric carrier was composed of three components; a temperature-responsive unit, a cationic unit, and a hydrophobic unit. The cationic unit interacts with anionic DNA to give high DNA yields in the polymer-DNA complex. The hydrophobic unit adjusts the phase-transition temperature. The poly(*N*-isopropylacrylamide) homopolymer possesses a LCST of 32°C in water. Introduction of an amine unit to homopolymer chain raises the LCST, whereas the addition of hydrophobic units lowers the LCST. The LCST of this copolymer was set below physiological body temperature in order to test temperature enhancement of gene expression. If the LCST is

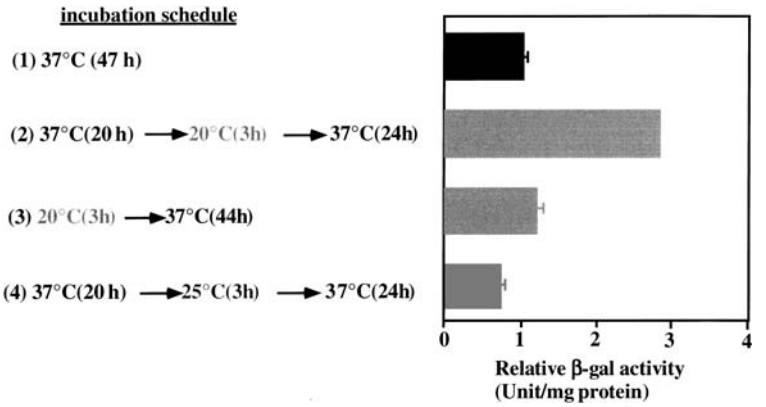


FIG. 6. Increase in β-galactosidase expression by lowering incubation temperature

set above 37°C, then enhanced gene expression following an increase in temperature can be considered to be due to both cell activation and formation/dissociation control of the complex. When the temperature is decreased to less than 37°C, then only enhanced gene expression occurs, since cell activities such as protein synthesis are reduced at low (<37°C) temperature.

The copolymer shown in Fig. 5 was mixed with plasmid DNA encoding the β-galactosidase gene at 37°C to form a polymer-DNA complex. The phase-transition temperature of this copolymer was 21°C, which was also the transition temperature after complex formation with DNA. This complex (charge ratio of polymer:DNA = 3:1) was added to COS-1 cells, and incubated for 1 h at 37°C. After removal of the complex solution, fresh DMEM medium containing 10% FBS was added to the cells, which were then cultured at various times and temperature schedules, as shown in Fig. 6. Compared with cells cultured according to incubation schedule (1), which lacked any cooling period at 20°C, β-galactosidase activity of cells cultured according to schedule (2) was significantly higher. Under this condition, the number of β-galactosidase-expressing cells also increased, as shown in Fig. 7. Generally, temperatures lower than 37°C are disadvantageous for transfection. In fact, the transfection efficiency of a homopolymer of the amine unit was found to decrease by half when the incubation temperature was lowered (Kurisawa et al. 2000). Interestingly, gene expression of the temperature-responsive vector system was controlled by the precise conditions of the incubation schedules. For example, when the cells were cooled immediately after the incubation period with the complex [schedule (3)], the transfection efficiency was almost the same in for schedule (1) cells. When the cells were cooled at 25°C, which is higher than the phase-transition temperature of this vector system, no increase in transfection efficiency was observed [schedule (4)]. These results indicate that the enhanced transfection efficiency obtained by lowering the temperature was most likely mediated by preferential DNA release during late cellular processes in the cytoplasm and nucleus.

In order to achieve an even greater enhancement of gene expression, the incubation period of the DNA-carrier complex with cells was varied (Yokoyama et al. 2001).

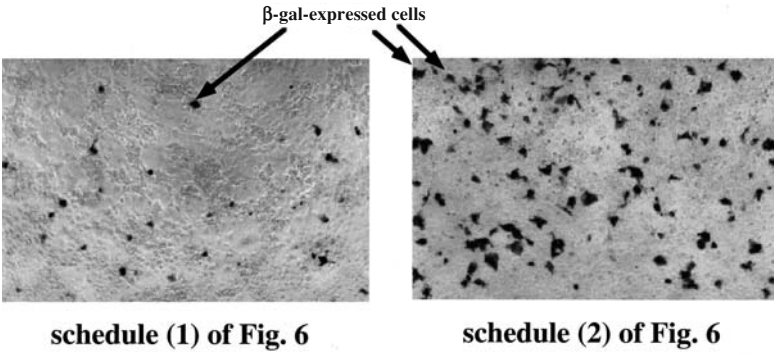


FIG. 7. Increase in the number of β-galactosidase-expressing cells by lowering incubation temperature

The enhancement ratio (activity with the cooling procedure/activity without the cooling procedure) reached 8.6 following a 3-h incubation period. The ability to selectively enhance expression may be very useful in obtaining site and time-specific expression of foreign genes in biological and medical applications.

While the above-mentioned copolymer showed thermoresponsive gene expression, the transfection efficiency was the same as that obtained with a homopolymer of the cationic unit used as the thermoresponsive copolymer, even when the appropriate temperature change was applied. Therefore, in order to raise the transfection efficiency, the amounts of both the amine and hydrophobic units were increased (Takeda et al. 2004). Figure 8 compares the transfection efficiency of two thermoresponsive copolymers and the amine homopolymer in the presence of a very small amount of DNA in order to evaluate transfection in the background of a high level of cell viability. Copolymer A indicates the copolymer whose composition is shown in Fig. 5, and copolymer B is the modified copolymer possessing more amine and hydrophobic units. Using a small amount of DNA per cell, only copolymer B exhibited prominent transfection activity (Fig. 8a) and maintained thermo-responsive transfection behavior (Fig. 8b). Consequently, a synthetic gene carrier possessing both thermoresponsive gene expression and greatly enhanced transfection efficiency was successfully synthesized by optimizing the content of both amines and hydrophobic units.

The above mentioned copolymer system was the first example of temperature-mediated control of gene expression using a synthetic vector. In this system, the phase-transition temperature was below 37°C, and expression of the target gene was enhanced by DNA release inside the cells. Alternatively, enhanced gene expression can be obtained by applying heat in the following way. If the phase-transition temperature of the DNA-carrier complex is set above that of body temperature, selective cellular uptake of the complex may be obtained by localized heating at a specific site and time. Subsequently, the complex in the cytoplasm or nucleus is dissociated at body temperature, which is below the phase-transition temperature, and gene expression occurs via DNA released from the complex. Thus, gene expression is selectively obtained at the heated site. The feasibility of this approach

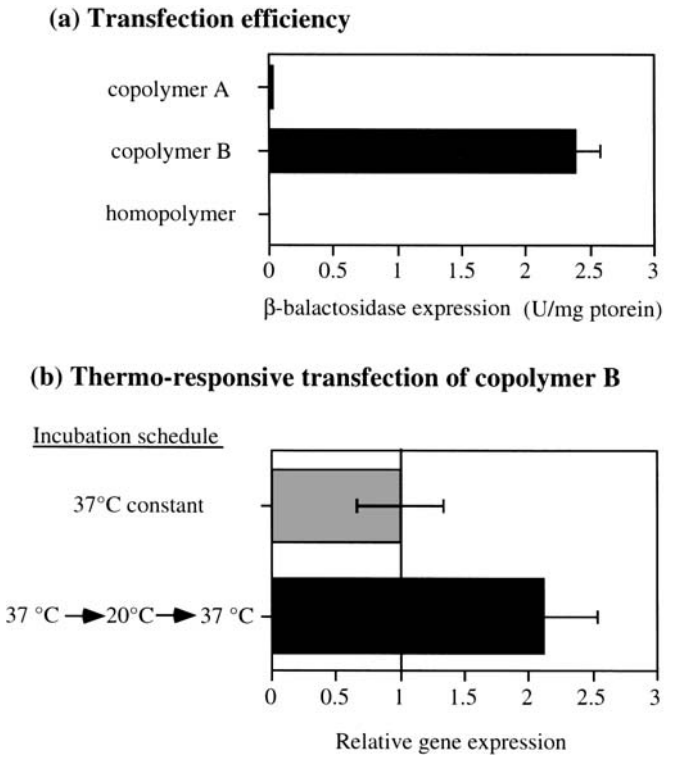


FIG. 8. A copolymer showing high transfection efficiency (**a**) while maintaining its thermoresponsive properties (**b**)

has been reported (Yokoyama et al. 2001). Taken together, the results show that temperature-responsive gene carrier systems may be widely applicable to in vivo gene therapies, both by heating and cooling the therapeutic sites to control gene expression.

Lastly, the possible importance of DNA release outside cells should also be pointed out. This chapter has focused on intracellular delivery of DNA to the nucleus, since in vivo applications (e.g. intravenous injection) using naked DNA are not possible, due to its negligible transfection efficiency. Intramuscular injection of naked DNA, however, is an exception. Additionally, high in vivo transfection efficiency of naked DNA using a hydrodynamics-based procedure was recently reported (Liu et al. 1999; Zhang et al. 2000). In this process, a large volume of DNA solution (1.5 ml to 3.0 ml per mouse) is injected over a short time period (several seconds). The naked DNA, without any vector, transfects cells at high efficiency. Gene expression efficiencies obtained by intramuscular injection or the hydrodynamic method are considerably lower than obtained with vector-based systems due to enzymatic degradation of naked DNA in muscular tissue fluid and the bloodstream (Miyao et al. 1995; Mahato

et al. 1995). Therefore, DNA release both inside and outside the target cells using temperature-responsive vector systems deserves further study.

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