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Gene delivery using temperature-responsive polymeric carriers

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Synthetic temperature-responsive polymers can be applied as gene carrier systems. For successful gene therapies, efficient and safe vectors are essential because they deliver genes to target cells and aid gene expression of therapeutic peptides. Vector systems that can control gene expression are favorable, especially for genes whose therapeutic effects are considerably dependent on quantity, site, duration and timing of their expression. Strategy and clinical feasibility of the temperature-responsive vector system are discussed with an example of gene expression enhancement by temperature stimuli.

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▼ By 1 February 2001, 3436 patients had received clinical trails of gene therapies from 532 protocols (http://www.wiley.com/legacy/wileychi/genmed/clinical/). One of the most important factors for successful gene therapies is a vector that delivers genes into cells for the production of therapeutic proteins. In 1997, Verma and coworkers [1] commented; 'The Achilles heel of gene therapy is gene delivery…'. This situation does not appear to have changed in 2002, despite the substantial progression of gene vector research in the past five years. Much research and development is being applied to obtaining better vectors of both viral and non-viral types. At present, this research is mainly directed to obtaining higher transfection efficiency of genes and increased safety of vector systems. By contrast, gene expression control is another important issue for gene therapies, particularly for those genes whose precise regulation is essential to obtain therapeutic effects. Here, methodologies for gene expression control will be discussed, and a temperature-responsive non-viral gene delivery system is introduced as a vector that can control gene expression by external physical stimuli. *In vitro* gene expression control using this temperature-responsive vector system in relation to *in vivo* and clinical use of the vector will also be described.

Selective gene expression for gene therapy

In most of the previously performed gene therapy protocols, gene expression in various tissues, organs and cells is not controlled precisely; neither are the timing and the period of expression. In other words, most gene therapies are limited to cases in which the precise control of gene expression is not required. If the control of gene expression can be done in a specific manner, particularly *in vivo*, this would contribute substantially to the development of gene therapy.

Specific gene expression – methodologies

To obtain site-, timing-, and duration-period-specific gene expression, the following three methodologies are necessary, as summarized in Box 1. First, specific promoters [2] can start the selective transcription of the encoded gene in specific environments. Physical signals (γ-ray [3,4] and temperature [5]) and chemical signals, such as lactose [6] and tetracycline [7,8], induce transcription of the genes placed downstream from the signal-inducible promoters. Alternatively, tissue- or organ-specific promoters have been used to obtain the corresponding gene expression even if signal species are not identified in some cases. Using these specific promoters, considerably high induction ratios (the expression ratio between the presence and the absence of the signals) of up to several-thousands-fold have been obtained. Promoter designs, however, are considerably limited because the specificity of these promoters is based on the specific binding properties of naturally occurring transcription factors. For example, the heat shock protein promoter only responds to a temperature (42°C) that is dangerous to biological cellular activity. Artificial protein engineering to alter this signal temperature is difficult using the

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Box 1. Methodologies to achieve selective gene expression

Specific promoters
- Signal-inducible (e.g. heat shock, lactose, tetracycline)
- Organ specific, tissue specific

Selective delivery to target with carriers

Stimuli-responsive gene carriers

current technology. Furthermore, the delivery of chemicals to therapeutic sites can be another problem for chemical signal-inducible promoters: in general, specific concentrations and duration periods of chemicals at specific sites are not easily achieved *in vivo*.

The second methodology for selective gene expression is delivery by carriers. As studied for the delivery of pharmaceutical drugs, several types of carriers, including antibodies, liposomes and synthetic polymers, have been used to achieve selective delivery of genes. Even without specific promoters, selective gene expression is possible by the selective delivery of genes. Furthermore, viral vectors themselves can be selective carriers by taking advantage of their infection tropism to specific cell types.

The third method of obtaining specific gene expression is the stimuli-responsive gene carrier. This synthetic vector induces transcription by the release of DNA from the DNA–carrier complex in specific environments. This is described in more detail in a later section.

Synthetic vectors

Viral vectors were most commonly used in gene therapy clinical trials because of their high transfection efficiency. However, viral vectors have several disadvantages, such as possible pathogenic and immunogenic problems. By 1 February 2001, a considerable proportion of patients (18%) in clinical trials received synthetic vector systems among all the gene therapy clinical trials (http://www.wiley.com/legacy/wileychi/genmed/clinical/). Non-viral (synthetic) gene carriers [9] have been studied in two types; lipids and polymers. Non-viral gene carriers in clinical gene therapy trails often used cationic lipids because of previous research developments [9,10], rather than polymeric gene carriers. However, polymeric gene carriers have been studied because of some advantages over the lipid systems: (1) relatively small size and narrow distribution of complex [11]; (2) high stability against nuclease; and (3) easy control of physical factors (e.g. hydrophilicity and charge) by copolymerization.

Classification of synthetic carriers

From another viewpoint, synthetic carriers can be classified into two types based on the way they enter the cytoplasm. Because anionic macromolecules, such as DNA, cannot pass through the cell membrane in high efficiency, synthetic vectors can aid this translocation. The first type of carrier transfers plasmid DNA from the outside of the cell to the cytoplasm directly through the cell membrane. This type of carrier uses special proteins; for example, the tat protein of HIV [12,13] or the fusion protein of the hemagglutinating virus of Japan (HVJ) [14–16]. This type of carrier has attracted much attention because of a shorter pass to cytoplasm than the second type, which is described later. At present, the number of studies for the first type is limited as a result of a shorter study history and more complicated preparation procedures than the second type of synthetic vectors, which include cationic lipids and cationic polymers. A complex is formed between anionic DNA and cationic lipid or polymer by ionic interactions. This complex is formed with a positive charge excess; therefore, it could interact with the anionic cell membrane to induce endocytosis of the complex in a highly efficient manner. By endocytosis, the complex goes into the endosome then moves into the cytoplasm in mechanisms that are described later. Because of the ease of preparation of both the complex and the synthetic materials, this second type of synthetic carrier is favorable for large-scale production.

Here, the intracellular processes of the cationic carrier–DNA complex are discussed. It is generally considered that the cationic carrier–DNA complex accomplishes transcription of the therapeutic gene by the following five steps: (1) the complex adheres to the cell surface by electrostatic interactions; (2) cellular endocytosis into endosomes occurs; (3) the complex translocates from the endosome to the cytoplasm by mechanisms such as lipid fusion [17] and the proton sponge effect [18,19]; (4) the complex, or the released DNA from the complex, moves into the nucleus; and (5) the transcription process is initiated. If complex formation and dissociation phenomena are summarized in relation to these processes, tight complex formation is preferable in processes (1) and (2) for efficient cellular uptake and evasion of DNA degradation. By contrast, complete dissociation of the complex is required for the final transcription process (5) for RNA polymerase reading DNA information.

Therefore, as shown in Fig. 1, cationic gene carrier systems have a common dilemma in that these systems must fulfill the following two opposite requirements simultaneously: (1) tight complex formation, favorable for cell uptake and evasion of DNA degradation, and (2) complex dissociation or loose complex formation, favorable for transcription by RNA polymerase. It is impossible to fulfill these two

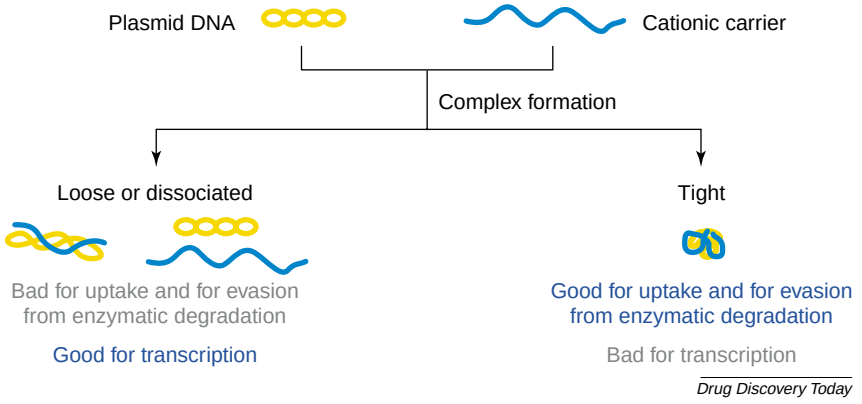

Figure 1. The dilemma of the DNA–polymer complex: just how tightly should DNA be complexed? In a cationic DNA–polymer complex the common dilemma is that these systems must fulfill two opposite requirements simultaneously: (1) tight complex formation, favorable for cell uptake and evasion of DNA degradation, and (2) complex dissociation or loose complex formation, favorable for transcription by RNA polymerase.

requirements effectively in a single gene carrier system because formation and dissociation are opposing phenomena. Tight complex formation is disadvantageous to the transcription process, and dissociation or loose formation of complex is disadvantageous to cell uptake and evasion of DNA degradation. This dilemma, which is observed in conventional cationic gene carrier systems, can be overcome by using the stimuli-responsive vectors described in the next section.

Stimuli-responsive synthetic vectors

To resolve the dilemma of cationic type synthetic carriers described previously, an intelligent system to control complex formation–dissociation by external stimuli is studied. By applying physical stimuli, such as light and temperature, complex formation–dissociation or tight–loose complex formation can be controlled to optimize the complex status for each intracellular process.

By using intelligent properties of the stimuli-responsive vector systems, two benefits can be obtained. The first benefit is high gene expression efficiency. As shown in Fig. 1, in the conventional cationic vector systems the complex must be formed with an intermediate tightness to fulfill the two opposite functions simultaneously; protection from enzymatic degradation and free access to RNA polymerase. This intermediate tightness of the complex formation can not afford the maximum efficiency for both functions. By contrast, for the stimuli-responsive vector systems, the function to evade DNA degradation can be optimized by forming a tight complex and, simultaneously, transcription efficiency can be maximized by complex dissociation or loose complex formation. The gene expression efficiency (transfection efficiency) might be a multiplication of efficiency of the five cellular processes described in the previous section and, therefore, is maximized in the stimuli-responsive gene carrier systems. In other words, the efficiency of each cellular step can be increased without decreasing the efficiencies of the other steps for the stimuli-responsive gene carriers.

The second benefit of the stimuli-responsive vectors is selective gene expression. Site-, timing- and duration-period-specific gene expression can be achieved with the stimuli-responsive synthetic vectors by releasing DNA from the DNA–carrier complex in a stimuli-responsive manner. As with the methodologies described previously, the stimuli-responsive synthetic vectors have several advantages. First, fine adjustments in stimuli applied to living bodies are possible, for example, applied temperature for the induction of gene expression is fixed for the heat shock protein promoter system, whereas in the stimuli-responsive synthetic systems the inducible temperature can be easily changed by designing the appropriate chemical structures. Second, site specificity is easily obtained by application of light and temperature. Light has the advantage over temperature with respect to site precision; however, temperature can also be applied to a specific site with considerable precision, such as within 5 mm using an ultrasound device [20,21]. Temperature stimuli is advantageous over light in terms of available depth from the surface. Such site-specificity can also be obtained in a uniform tissue (e.g. dermis and muscle) or an organ, whereas tissue- or organ-specific promoters can not achieve such site-specificity.

Nagasaki and coworkers [22] synthesized a new cationic L-lysine-modified polyazobenzene dendrimer as a synthetic vector for transfection of mammalian cells. The azo moiety of the dendrimer shows *trans* to *cis* isomerization upon irradiation by UV light. UV irradiation of the plasmid DNA complex into the cytoplasm caused a 50% increase in the transfection efficiency, compared with the negative control. A decrease of the cationic charge density on the dendrimer surface was considered to contribute to the transfection increase by promoting DNA release from the complex. This is the first demonstration of the control of transfection efficiency by light using a synthetic gene vector. A gene vector system with temperature expression control is described in the next section.

Temperature-responsive polymeric vector system

Yokoyama and Okano have developed a temperature-responsive polymeric vector system [23,24] based on poly(*N*-isopropylacrylamide) (PIPAAm), which is known to exhibit a temperature-dependent phase transition behavior with a lower critical solution temperature (LCST) at 32°C [25] and has been applied extensively to biomedical applications, such as hydrogels [26,27], bioconjugates [28] and polymeric micelles as drug carriers [29–31]. Below the LCST, PIPAAm is water-soluble and hydrophilic, existing in an extended chain form. Above the LCST, PIPAAm undergoes a reversible phase transition to an insoluble and hydrophobic aggregate. This phase transition temperature (the LCST) can be shifted by copolymerizing hydrophilic monomer units and hydrophobic monomer units to raise and lower the LCST, respectively. Using this method, a complex between the carrier polymer and DNA is tightly formed above the transition temperature by hydrophobic aggregation of the polymer, as shown in Fig. 2. Below the transition temperature this complex is dissociated or becomes loosely bound because of the decrease in hydrophobic nature of the polymer. A tightly formed complex is favorable for cellular uptake and evasion of enzymatic degradation of DNA, whereas free DNA released from the complex or loosely packed complex is favorable for transcription. Therefore, a change between the tight complex formation and its dissociation (or loose complex formation) can contribute to optimize efficiency of the five cellular processes described previously and, consequently, the total gene expression efficiency could be dramatically increased with the potential for selective gene expression.

Tumor hyperthermia local therapy

Temperature is one of the safest external signals that is applicable to a variety of sites of living bodies. Site-specific heating is now routinely performed in hyperthermia therapy against solid tumors [32]. This tumor hyperthermia local therapy is usually performed by microwave, which can selectively heat the target site with ~1 cm precision. Furthermore, because microwave heating can reach a considerable depth from the body surface, this therapy can treat most internal organs and tissues even though the microwave radiation is applied from outside the body. By contrast, lowering the temperature can be limited to the sites near the body surface if the cooling procedure is applied from outside the body. However, applicable sites can be expanded substantially using catheters. With current medical technology, most internal areas are accessible with catheters passing through blood vessels or via other sites, such as the urinary tract.

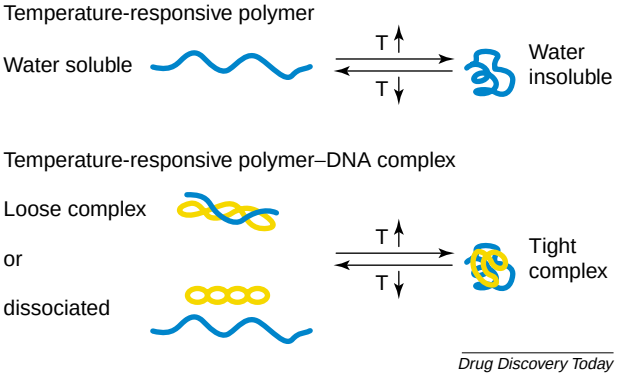

Figure 2. The concept of temperature-responsive gene carriers. A complex between the carrier polymer and the DNA is tightly formed above the transition temperature by hydrophobic aggregation of the polymer.

A temperature-responsive polymeric gene carrier system was designed as shown in Fig. 3. This polymeric carrier is composed of three components; a temperature-responsive unit, a cationic unit and a hydrophobic unit. The cationic unit interacts with anionic DNA to produce high DNA yields in the polymer–DNA complex formation. The hydrophobic unit is used to adjust the phase transition temperature of this carrier system. The PIPAAm homopolymer possesses an LCST of 32°C. The introduction of an amine unit to the PIPAAm chain raises the LCST. By contrast, the introduction of hydrophobic units lowers the LCST. In the first example of the temperature-responsive vector, the LCST of this copolymer was set below physiological body temperature for a proof-of-gene-expression

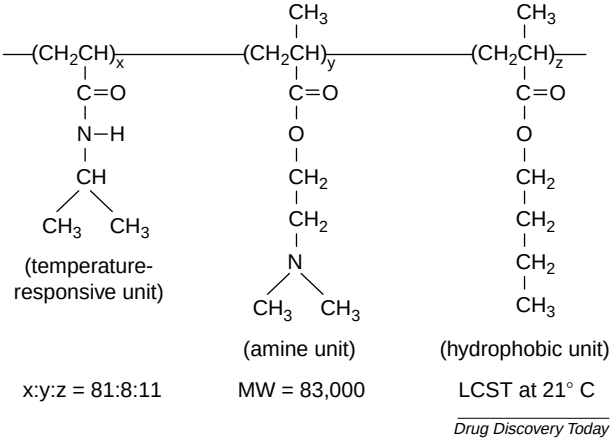

Figure 3. The chemical structure of a temperature-responsive gene carrier. This polymeric carrier is composed of three components; a temperature-responsive unit, a cationic amine unit and a hydrophobic unit. Abbreviations: LCST, lower critical solution temperature; MW, molecular weight.

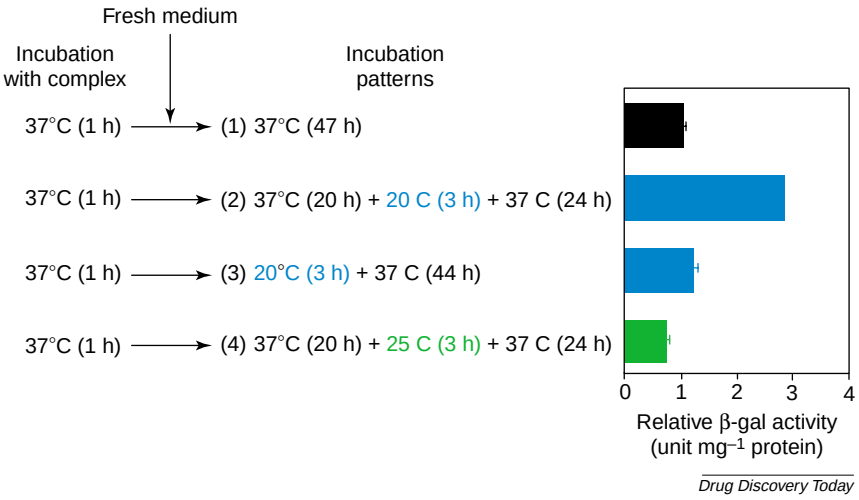

Figure 4. β-galactosidase activity can be increased by lowering the incubation temperature. A temperature-responsive copolymer was complexed with a plasmid (pCMV– β-gal) and this was added to COS1 cells in a 96-well plate. The cells were incubated for 1 h at 37°C. The cells were then cultured at various temperatures for various times and the activity of β-gal was assessed by the ONPG method.

enhancement by temperature. If the LCST is set above 37°C, gene expression enhancement by raising the temperature is thought to result from cell activation, as well as from the formation–dissociation control of the complex. The enhancement of gene expression obtained by lowering the temperature from 37°C demonstrates the designed function of this carrier system because cell activities, such as protein synthesis, are reduced at temperatures <37°C.

Polymer–DNA complex

A copolymer composed of the temperature-responsive unit, the amine unit and the hydrophobic unit shown in Fig. 3 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 found to be the same after complex formation with DNA. This complex [with a charge ratio (polymer:DNA) of 3:1] was added to COS1 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 plate and the cells were cultured at various times and temperatures as shown in Fig. 4. When the incubation patterns were compared, pattern (1), which lacked any cooling period at 20°C, had significantly lower β-galactosidase (β-gal) activity than pattern (2) (Fig. 4). The number of β-gal-expressing cells also increased in pattern (2) as shown in Fig. 5. Generally, temperatures below 37°C are disadvantageous for transfection. In fact, transfection efficiency of a homopolymer of the amine unit was found to decrease by a half upon lowering the incubation temperature [23]. Remarkably, gene expression of the temperature-responsive vector system was controlled by precise conditions of incubation patterns. When the cells were cooled immediately after the incubation period with the complex [pattern (3)], no increase in transfection was observed when compared with pattern (1). 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 [pattern (4)]. These results indicate that the enhanced transfection efficiency caused by lowering the temperature was probably provided by preferential DNA release in the later cellular processes in the cytoplasm and nucleus described as processes (4) and (5) in the *Classification of synthetic carriers* section.

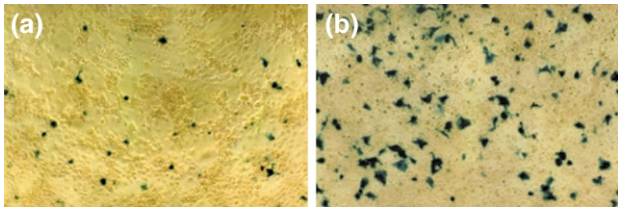

Figure 5. The amount of β-galactosidase expressing cells can be increased by decreasing the incubation temperature. **(a)** Cells incubated at 37°C for 47 h. **(b)** Cells incubated at 37°C for 20 h + 20°C for 3 h + 37°C for 24 h. The activity of β-galactosidase was monitored using the X-gal reagent.

To obtain an even greater enhancement of gene expression, the incubation period of the DNA–carrier complex with cells was varied [24]. The enhancement ratio (activity with the cooling procedure:activity without the cooling procedure) reached 8.6 times in the 3 h incubation period case. Such an enhancement could be useful in obtaining site- and timing-specific expression of foreign genes in biological and medical applications. Although these data were obtained *in vitro*, the *in vivo* developments are feasible because of the safe and easy application of temperature stimuli to living bodies.

Future perspectives of a temperature-responsive polymeric vector system

The aforementioned copolymer system describes the first example of gene expression control by temperature using a

synthetic vector. Because temperature is one of the easiest and safest signals applied to living bodies [32], this synthetic vector system could find applications for *in vivo* gene therapies.

In the previous section, gene expression control is described using the polymeric vector with a phase transition temperature below 37°C. In this case the target gene expression was enhanced by DNA release inside 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 can be obtained by localized heating at a specific site and time. After the selective heating is concluded, the complex in the cytoplasm or nucleus is dissociated at the body temperature, which is below this phase transition temperature, and gene expression occurs using DNA released from the complex. Consequently, gene expression is selectively obtained at the heated site. The feasibility of this scenario has recently been reported [24]. Consequently, the temperature-responsive gene carrier systems might possess wide applicability to *in vivo* gene therapies both by heating and cooling the therapeutic sites for gene expression.

An increase in transfection efficiency is needed for synthetic vectors [9]. At present, the efficiency of gene transfection of the temperature-responsive polymeric vector is approximately the same level as the commercially available transfection reagents, such as Lipofectin (Invitrogen, Paisley, Scotland) [23]. Improvement in the transfection efficiency might be required for *in vivo* applications. Higher transfection efficiency can be achieved in the following ways; optimization of the temperature response for DNA association and release, and an additional design to raise efficiencies of the initial cellular processes of adhesion, cell uptake and escape from the endosome.

Finally, the possible importance of DNA release outside the cells should be pointed out. This review has focused on intracellular delivery of DNA to the nucleus because the *in vivo* application (e.g. intravenous injection) of naked DNA was considered non-meaningful as a result of negligible transfection efficiency, except in the case of intramuscular injection. Recently, high *in vivo* transfection of naked DNA was reported by a hydrodynamics-based procedure [33,34]. [The hydrodynamic-based procedure is the injection of a large volume of DNA solution (1.5 ml to 3.0 ml in a mouse) for a short period of time (several seconds).] This indicates that naked DNA without any vector can transfect cells in high efficiency in certain physiological conditions. Gene expression efficiency obtained by the hydrodynamic method is lowered considerably by enzymatic DNA degradation according to reported rapid DNA degradation in the bloodstream [35,36]. Therefore, for the temperature-responsive vector systems, DNA release outside the target cells by temperature change is worth being examined, as well as DNA release inside the target cells.

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