Expert Opinion

Stimulus-controlled delivery of drugs and genes

David Oupicky†, Harender S Bisht, Devika Soundara Manickam & Qing-hui Zhou
† *Wayne State University, Department of Pharmaceutical Sciences, Detroit, MI 48202, USA*

Macromolecular and colloidal systems used for the systemic delivery of drugs and genes promise to improve the way we treat and prevent numerous diseases. New generations of drug and gene delivery systems (DGDS) are being designed to enhance further efficiency by using a range of endogenous and external stimuli. This review focuses on three qualitatively distinct ways a stimulus can improve the efficiency of DGDS; namely, by selectively triggering release of the therapeutic agent from the DGDS, by modulating physical properties of DGDS and by favourably altering physiological properties of tissues to enhance DGDS transport. Recent developments in these areas are discussed to illustrate the potential of stimulus-controlled DGDS in the development of new generations of therapeutics.

Keywords: drug delivery, gene delivery, heat shock, hyperthermia, redox potential gradient, stimulus, ultrasound

Expert Opin. Drug Deliv. (2005) 2(4):653-665

1. Introduction

Macromolecular and colloidal systems used for the systemic delivery of traditional small-molecule drugs and modern biotech macromolecular drugs evolved immensely in the last decade, resulting in a number of successful clinical applications, with more promising clinical results arising from trials [1]. Such drug and gene delivery systems (DGDS) use water-soluble polymers that allow flexible optimisation of their structure and properties, subsequent to the known pathophysiology of the target disease. The use of DGDS, however, often creates a new set of challenges that are usually related to their relatively large size. Irrespective of the size, a drug must safely reach its target cell and the appropriate location within the target cell to achieve the desired therapeutic effect. Small-molecule drugs are transported freely and enter cells by diffusion; therefore, reaching the desired subcellular location is often straightforward. Unlike small-molecule drugs, the transport of DGDS is severely restricted due to their large size, which can range from several nanometers up to several micrometers. Distribution of intravenously injected DGDS is, therefore, usually restricted to the intravascular space, due to the low capillary permeability in most organs with continuous capillary beds. The physiology of liver and many solid tumours characterised by discontinuous endothelial capillaries provides a reliable rationale for the advantageous use of DGDS in tumour and hepatic targeting. Similarly, DGDS cannot usually cross cellular membranes and are restricted to fewer subcellular compartments compared with small-molecule drugs. Although the limited ability of DGDS to enter cells is widely exploited to their advantage by attaching ligands that can target them to specific cells, the overall difficulties in crossing biological membranes are the major problems compromising the therapeutic efficiency of many DGDS. Therefore, the efficient delivery of therapeutic macromolecules to designated cells and subcellular locations greatly relies on the successful passage across biological membranes. Properties of DGDS can often be modified to improve the efficiency of their transport across a selected barrier, but it has become apparent that different biophysical and biological properties are required for the

Ashley Publications
www.ashley-pub.com

DGDS to successfully overcome each of the barriers faced by intravenously administered delivery systems. Growing expertise in designing more complex and sophisticated macromolecules and nanostructures led to the development of second-generation delivery technologies, containing a variety of specific responsive elements. One of the most promising approaches to overcome the multiple barriers faced by DGDS relies on the use of a specific stimulus to improve the efficiency of DGDS-mediated delivery by temporal and spatial control of drug release from DGDS, properties of DGDS, or physiological properties of target tissues. This review focuses on recent developments in the three selected areas of stimuli-controlled delivery of drugs and genes. To document the opportunities available, examples of approaches that use redox potential gradients and hyperthermia as the specific endogenous and external stimuli have been selected.

2. Selected stimuli for improvement of drug and gene delivery systems efficiency

A variety of external and endogenous stimuli can be used to improve the overall efficiency of DGDS. Although the external stimuli are by nature physical (ultrasound, heat, magnetic field, light), the endogenous ones offer a wider variety, ranging from simple chemical to complex biochemical stimuli. Irrespective of its nature, a given stimulus can improve DGDS efficiency by one of three distinct mechanisms; first, a stimulus can be used to facilitate spatial and temporal control of the release of a therapeutic agent from DGDS. Stimuli-triggered release of drugs and genes from DGDS is probably the most widely used and investigated approach among those discussed. The mechanism of action is usually based on chemical or biochemical gradients existing within body. Examples of successfully used stimuli to release small-molecule drugs from macromolecular DGDS include pH gradient and redox potential gradient. In addition, site-specific enzymatic release of drugs is another widely used approach [2-4]. Second, a stimulus can be used to alter properties of DGDS, enabling them to overcome selected barriers. This approach has not been as extensively investigated as the first one and can be based on both external and endogenous stimuli (heat, ultrasound, pH gradient, redox potential gradient). Third, a stimulus can be used to favourably alter the physiological properties of target tissues to increase the efficiency of DGDS. This approach creates potential risks, as well as benefits, and is limited to external stimuli such as local hyperthermia or acoustic cavitation. Three stimuli for discussion have been selected in this review to illustrate the potential of all three mechanisms of action for improving DGDS efficiency.

2.1 Redox potential gradient

One of the several microenvironmental features that can be exploited for improving the efficiency of DGDS is the redox potential gradient existing between the extracellular environment and various subcellular organelles in normal, as well as pathological, states. The existence of a high redox potential gradient between the oxidising extracellular space and the reducing environment of subcellular organelles has been exploited, mainly by incorporating a disulfide bond(s) into the structure of the DGDS to provide them with the capability to release the therapeutic agent (drug or gene) selectively in the subcellular reducing space [5]. Two features make the disulfide bonds especially attractive; easy intracellular reversibility and relative extracellular stability. The intracellular reduction of disulfide bonds is mediated by small redox molecules such as glutathione (GSH) and thioredoxin, either alone or with the help of redox enzymes [6]. The concentrations of GSH, the most abundant cellular reducing component, are in a mM range. In contrast, GSH concentrations in blood plasma are typically ~ 10 μM [7,8]. GSH plays a role in various cellular processes including DNA and protein synthesis, the maintenance of cell membrane integrity, drug and chemical metabolism and protection from oxidative stress. The lowest redox potential in the cell can be found within the nucleus, where it is required for DNA synthesis and repair as well as to maintain a number of transcription factors in a reduced state [8-11]. The concentration of GSH has been reported to be ~ 4 mM in the cytoplasm and ~ 20 mM in the nucleus [12-14]. The subcellular distribution of GSH suggests that the intracellular reduction of disulfide bonds in the DGDS will proceed mainly in the cytoplasm and nucleus [15-17]. Available evidence also suggests the possibility of plasma membrane, endosomal and lysosomal reduction [18-20].

In addition, altered GSH concentration and compartmentalisation associated with a variety of pathological states represent an exciting opportunity for enhanced selectivity of DGDS delivery in specific diseased cells [10,21-26]; for example, overexpression of the antiapoptotic protein Bcl-2, which is a critical regulator of susceptibility towards cell death, is known to be associated with increased GSH levels and altered subcellular distribution of GSH [27,28]. Using HeLa cells transfected with Bcl-2 under the control of a tetracycline-repressible promoter, Voehringer *et al.* demonstrated that Bcl-2 overexpression leads to GSH redistribution from the cytoplasm to the cell nucleus. This was documented by the GSH nuclear concentration increasing from 6 mM in cells with Bcl-2 expression switched off, to 16 mM in cells with the Bcl-2 expression switched on [22]. At the same time, the overall cellular GSH content underwent no statistically significant change, suggesting its redistribution to the nucleus. Bcl-2 overexpression is often implicated in the resistance of cancer cells to apoptosis through antioxidant pathways, which involve cellular sulfhydryls and, as such, can potentially be used for enhanced therapeutic selectivity in Bcl-2-overexpressing tumours [29,30].

2.2 Local hyperthermia

Localised increase of temperature (hyperthermia) is a relatively easily applicable external stimulus that can be used to control

the delivery of drugs and genes. Part of the attractiveness of using locally elevated temperatures as a specific stimulus for improving DGDS delivery is the wide availability of the necessary instrumentation to many radiology departments. In general, hyperthermia is a clinical procedure based on raising the temperature of a tumour region of the body to treat cancer. Hyperthermia itself has been successfully used as an adjunct therapy in multimodal oncological strategies with other established cancer treatments, such as radiotherapy and chemotherapy. Several clinical trials have shown a beneficial effect of hyperthermia for the local control or management of various types of tumours, including recurrent breast cancer, melanoma, or head and neck cancers [31-37]. Novel oncological therapies, such as gene therapy, could be triggered by a temperature increase or could be improved with hyperthermia; for example, a novel and very promising approach to targeting therapeutic gene expression to tumour cells has been developed and relies on engineering a hyperthermia-activated heat-shock promoter into gene delivery vectors. The heat-shock protein promoter 70 has been studied most extensively. The promoter is induced by up to three orders of magnitude following the application of hyperthermia.

The strong transcriptional response exhibited by heat-shock genes, in addition to the beneficial therapeutic effects of hyperthermia, has led to the development of heat-directed gene-targeting strategies for cancer treatment [38,39]. The major advantage of this therapeutic strategy is the potential to control and limit transgene expression within the body, even when the vectors are administered systemically. Hyperthermia itself has a marked effect at every biological level; for example, it decreases DNA synthesis, alters protein synthesis (including the induction of heat-shock proteins), disrupts the microtubule organising centre, alters the expression of receptors and the binding of growth factors and changes cell morphology and attachments. Importantly for DGDS anticancer therapies, hyperthermia, at temperatures between 40 and 45°C, is also known to increase tumour blood flow and vascular permeability compared with normal vasculature; indeed, the increased thermal sensitivity of tumour vasculature compared with normal vasculature has led to the use of local hyperthermia as a therapeutic modality for solid tumours in combination with thermally insensitive drugs. To reach and maintain temperatures clearly above the systemic temperature of 37°C in a defined target volume is a technical challenge and still under development. The major problem, especially with maintaining the increased local temperature, is that the physiological response of the body is to increase perfusion rates in the area to counteract the temperature rise. Perfusion rates in human tumours are around 5 – 15 ml/100 g/min, and vary widely. To reach a therapeutic temperature of ~ 42°C, at least in some parts of such tumours, necessitates power density of about 20 – 40 W/kg at the target region [40]. In comparison, human basal metabolic rate is ~ 1 W/kg. The potential to control power distributions *in vivo* has been significantly improved lately by the development of techniques that offer better spatial control of the hyperthermia. The typical temperature increase of tumour tissues to 40 – 43°C can be achieved by various methods, which are, in many cases, already in clinical use [40,41].

2.3 Non-thermal ultrasound effects

Ultrasound is the primary example of a stimulus that can improve DGDS delivery by altering the physiological properties of treated tissues [42]. The biological effects of ultrasound, which can potentially cause physical perturbation of cells and tissues, may generally be categorised as thermal (heating) and non-thermal (cavitation). The thermal bioeffects are manifested by heating, which occurs as the ultrasonic energy is absorbed in a medium; for example, the intensity of ultrasound at a frequency of 1 MHz can decrease to about a half for each 10 cm of propagation, most of which appears as heat.

Any process that can produce a biological effect without a significant degree of heating (< ~ 1°C) is a non-thermal mechanism. Acoustic cavitation is a non-thermal interaction between a propagating ultrasound pressure wave and a gaseous inclusion in aqueous media. A gas bubble pulsates at ultrasound amplitudes that depend on its size and are maximised at the resonance frequency of the bubble; for example, a resonance size of a free bubble in water at 1 MHz is ~ 7 µm in diameter. As a part of cavitation activity, gas bubbles implode, leading to the generation of intense heat and light (sonoluminescence), free radical production and the generation of wave, which can introduce secondary bioeffects whenever bubbles or cavitation nuclei are present in a biological medium exposed to ultrasound. Cavitation causes mechanical perturbation in the vicinity of active bubbles, which can lead to membrane effects on individual cells and to capillary rupture *in vivo* [43,44]. The membrane effects range from sonoporation (the transient opening of holes in the membrane) to cell lysis or even fragmentation. During sonoporation, sublethal membrane damage occurs, allowing cells to freely exchange large molecules with the surrounding medium through the transient holes, followed by membrane sealing and cell survival. This allows foreign macromolecules and particles to be trapped inside the cell. It comes as no surprise that tissues naturally containing gas–liquid interfaces are more susceptible to the mechanical effects of ultrasound because cavitation depends on the presence of gas bodies. Microscopic haemorrhage in the lungs and intestine was repeatedly demonstrated after exposure to high acoustic pressures. Among other reported non-thermal bioeffects of ultrasound are increased vasoconstriction, vessel rupture and enhanced thrombolysis. The development of ultrasound contrast agents (suspensions of stabilised gas bodies of diameters suitable to passage through the circulation and also for return of strong echoes) for diagnostic purposes, introduced cavitation phenomena into an otherwise high threshold *in vivo* environment, with the potential for new therapeutic (or deleterious) biological effects. Promising applications mediated by ultrasound contrast agents include the reduction of

established blood clots [45], delivery of drugs from the blood to the interstitium and across the blood–brain barrier [46-48], and gene transfer [49]. The drug delivery application is particularly robust; for example, diagnostic contrast ultrasound (as for echocardiography) readily yields microvascular permeabilisation above pressure amplitudes of 0.4 MPa, with a substantial and reliable delivery of large molecules and particles up to the size of erythrocytes to the interstitium [50,51].

3. Selected examples of stimuli-controlled drug and gene delivery systems

Two examples have been selected that illustrate the potential of stimulus-controlled DGDS in the development of new generations of the delivery systems. The possibility to use redox potential gradients to control the release of therapeutic nucleic acids from DGDS specifically in selected intracellular compartments as well as to control the toxicity of DGDS is discussed first. The utility of local hyperthermia for altering properties of temperature-responsive DGDS in order to increase their accumulation in tumours and/or control their activity is described in the following section. The potential of the third discussed stimulus, ultrasound treatment for improving vascular extravasation and transmembrane transport of DGDS by altering physiological properties of treated tissues, has been extensively discussed in a recent issue of this journal [52].

3.1 Gene delivery controlled by redox potential gradients

The original interest in gene delivery systems controlled by redox potential gradients was guided by the need to transiently enhance physical stability of polycation-based vectors (polyplexes) during systemic *in vivo* transport. The tendency to undergo polyelectrolyte exchange reactions in the bloodstream seems to be one of the most challenging aspects of the instability of polyplexes. Many cells, surfaces and macromolecules *in vivo* have polyionic characteristics and can compete with polyions in the polyplexes for binding, leading to destabilisation of the polyplexes and rapid clearance from the plasma. It was hypothesised that, not only careful control of surface properties and steric stability, but also significantly increased resistance against polyelectrolyte exchange reactions are required to permit increased circulation times of polyplexes. The adopted approach relies on decreasing or fully suppressing the disassembly of polyplexes in the oxidative plasma environment by introducing disulfide bonds into the structure of linear polycations, or by crosslinking the polyplex nanoparticles. In both cases, the disassembly rates of polyplexes are controlled by modifying the molecular weight of the polycations. This approach was successfully applied to improving systemic intravenous delivery of various peptide-based polyplexes; for example, Park *et al.* synthesised disulfide crosslinked polyplexes further equipped with a protective polyethylene glycol layer and carbohydrate targeting ligands. The authors demonstrated that these polyplexes can efficiently target the transgene expression selectively into hepatocytes and provide expression of secreted alkaline phosphatase in mouse serum for an extended period of 12 days [53].

An alternative approach to disulfide crosslinking aimed at achieving a reversible stabilisation of intravenously injected polyplexes was proposed by Oupicky *et al.* [54,55]. Multivalent *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers were used to bind around the surface of polyplexes, linking surface amino groups and providing the polyplexes with a combination of steric and lateral stability (**Figure 1**). The lateral stabilisation was distinctly demonstrated by reduced disassembly rates and increased resistance to polyelectrolyte exchange reactions. Whereas typical polyplexes release DNA following incubation with polyanions, the polyplexes coated with multivalent HPMA copolymers were laterally stabilised and resistant to the exchange reactions. The polyplexes coated with multivalent HPMA copolymers exhibited significantly extended plasma circulation compared with the parent polyplexes. The authors identified several structural factors affecting the pharmacokinetics of these polyplexes, including molecular weight of the polycation, molecular weight of the HPMA copolymer and the amount of HPMA attached to the polyplexes. It was shown that efficient lateral stabilisation and subsequent prolonged circulation of the HPMA-coated polyplexes demands the use of HPMA copolymers with a minimal molecular weight of 30,000 and that the concentration of HPMA used plays a vital role in enabling prolonged circulation. Using 2 mg/ml of HPMA copolymers in the coating reaction led, not only to significantly increased circulatory half-life compared with 0.2 mg/ml, but, most importantly, to these polyplexes exhibiting long circulating properties, even at low administered doses. The increased amount of HPMA bound to the polyplexes at 2 mg/ml, together with the possibly of further improved lateral stability, are most likely to be important reasons. Overall, increasing the concentration of HPMA resulted in increased lateral stability, reduced rate of hepatic clearance, reduced dose-dependence of the pharmacokinetics, and increased circulation times. Using the optimised formulation of HPMA-coated polyethylenimine (PEI)/DNA polyplexes resulted in circulation half-lifes of almost 90 min in mice after intravenous injection, a significant improvement over the typical 3 – 5 min observed for typical polycation/DNA polyplexes. The prolonged plasma circulation also enabled passive accumulation of the HPMA-coated polyplexes in solid subcutaneous tumours at levels comparable to sterically-stabilised liposomes. Unfortunately, the irreversibility of HPMA-crosslinking in the originally developed polyplexes resulted in significantly reduced transfection activity due to the low disassembly rates that had adverse effects on the transcriptional availability of DNA. A dependence of the HPMA-induced stabilisation on the molecular weight of the polycations was, therefore, utilised for intracellular-specific activation of the vectors following cellular uptake (**Figure 1**). A high molecular weight reducible poly-L-lysine (rPLL), containing disulfide bonds in the backbone, was synthesised by oxidative polycondensation of Cys-$(Lys)_{10}$-Cys peptide, and used for the preparation of the

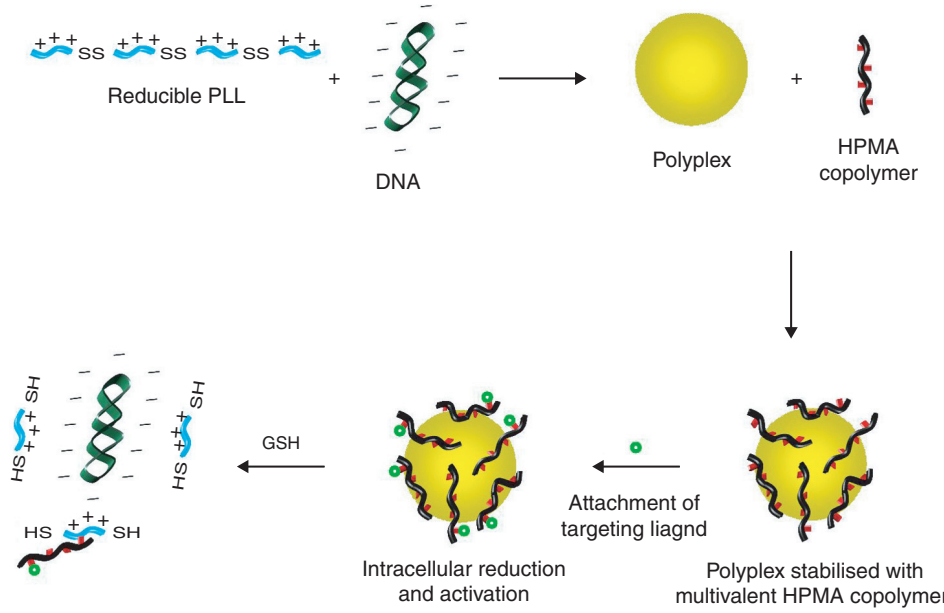

Figure 1. Schematic representation of the approach leading to targeted polyplexes with combined lateral and steric stability achieved by surface modification with multivalent HPMA copolymers, and the principle of intracellular-specific activation of reducible PLL-containing polyplexes by reduction with GSH. Adapted from OUPICKY D, PARKER AL, SEYMOUR LW: Laterally stabilized complexes of DNA with linear reducible polycations: strategy for triggered intracellular activation of DNA delivery vectors. *J. Am. Chem. Soc.* (2002) **124**:8-9.
GSH: Glutathione; HPMA: *N*-(2-Hydroxypropyl)methacrylamide; PLL: Poly-L-lysine; SH: Sulfhydryl group; SS: Disulfide bond.

polyplexes. These polycations were capable of reductive degradation into low molecular weight species in the reducing intracellular environment, and permitted to reverse the lateral stabilisation and facilitate the release of the DNA (**Figure 1**). Efficient intracellular activation of rPLL-based polyplexes by reductive degradation of rPLL was implied by > 60-fold increase of transfection activity of the polyplexes compared with control polyplexes based on PLL. In addition, the circulation half-life of rPLL polyplexes coated with HPMA copolymers was shorter but comparable to that of parent PLL-based polyplexes (Unpublished observation, Oupicky [2002]). This observation confirmed that the reduction-mediated destabilisation of the vectors in blood plasma is not significant and that rPLL-based vectors are suitable for systemic application.

Using multivalent HPMA copolymers for coating of the polyplexes permits simultaneous easy linkage of protein-targeting ligands to the surface of the polyplexes without the need for a complex chemistry (**Figure 1**). Such polyplexes then offer an open platform, where a variety of biologically active compounds can be incorporated, allowing an easy control of the tropism of the polyplexes. Vascular endothelial growth factor, transferrin and basic fibroblast growth factor have each been linked to HPMA-stabilised polyplexes [55-57]. Such ligand-targeted polyplexes demonstrated an increased uptake into receptor-positive cells that could be antagonised with excess free ligand. Targeted polyplexes also exhibited increased transfection activity and resistance to inhibition by serum when compared with non-targeted polyplexes. In a recently reported alternative approach, reducible disulfide bonds were introduced into the backbone of the coating of HPMA copolymers, instead of the polycation, and used to reversibly stabilise PEI/DNA polyplexes, while successfully enhancing the transfection efficiency [58].

Polyplexes capable of responding to environmental changes by altering their properties and behaviour could significantly improve qualitative and quantitative understanding of the gene delivery process. The redox potential gradient represents a highly specific stimulus that can be exploited for such purposes. Based on the available evidence, the subcellular trafficking of polyplexes based on reducible polycations can be schematically described as shown in **Figure 2**. The overall transfection activity of DNA polyplexes in such a scheme is determined by the relative rates of the steps leading to nuclear delivery and expression compared with the steps leading to DNA degradation. One can hypothesise that the intracellular reduction of reducible polycations within polyplexes leads to increased rates of polyplex disassembly, and that this ultimately promotes better transcription/translation of the delivered DNA/mRNA. One can also expect that, not only the total rates and extent of the reduction, but also the location, and, therefore, the subcellular compartmentalisation of GSH, will modulate the overall levels and kinetics of the transgene expression. Indeed, available evidence clearly suggests that altering the overall GSH levels affects the binding affinity of nucleic acids to the reducible polycations and, subsequently, transfection efficiency [58-60]. Using different means of

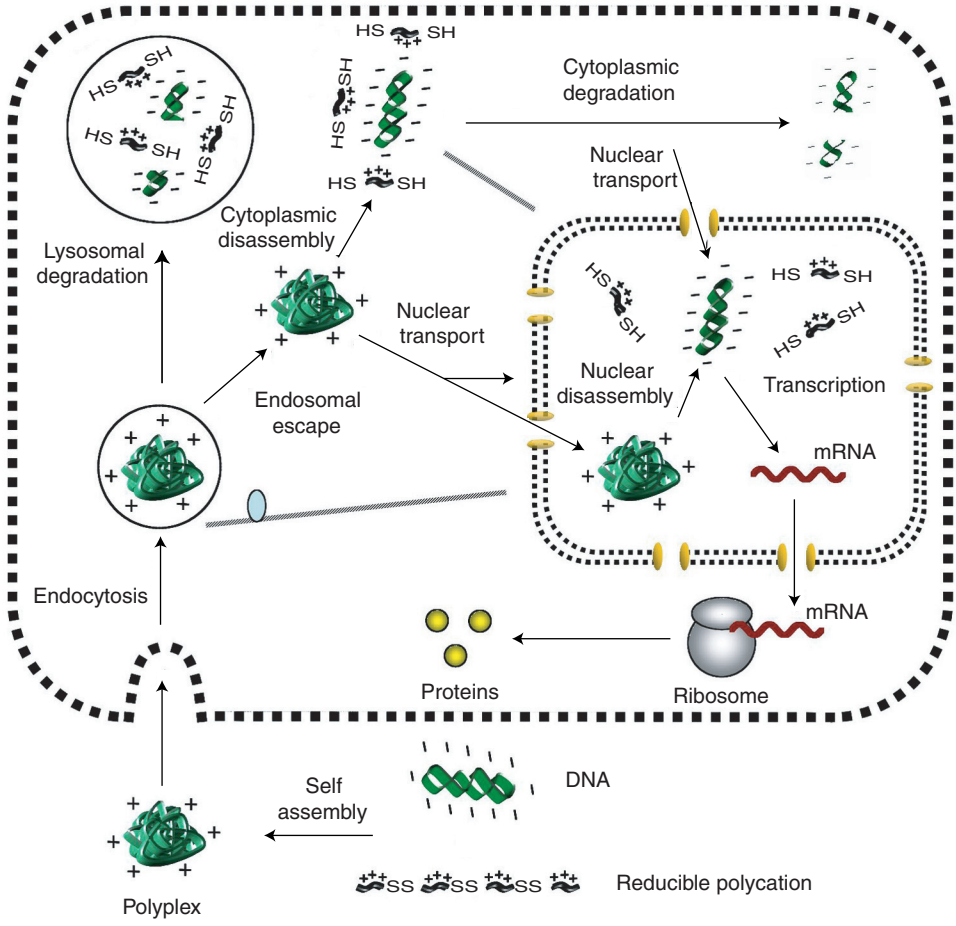

Figure 2. Schematic representation of the subcellular trafficking of DNA polyplexes based on reducible polycations. The polyplexes are formed by the self-assembly of plasmid DNA and reducible polycations, and internalised in the cell by endocytosis. Some polyplexes escape from the endosomes into the cytoplasm, whereas the rest are routed for degradation into lysosomes. Polyplexes located in the cytoplasm undergo full or partial reduction by the cytosolic GSH, leading to their partial or full disassembly. DNA from the polyplexes that undergo cytoplasmic disassembly can be degraded by cytosolic nucleases or transported to the nucleus. Polyplexes that do not disassemble in the cytoplasm are transported into the nucleus either passively by association with nuclear material during breakdown of the nuclear envelope during cell division, or actively through nuclear pore complexes. GSH present in the nucleus causes degradation of the reducible polycations within polyplexes into non-toxic small cationic fragments, and release of DNA from the polyplex due to enhanced disassembly rates. The polyplex disassembly then allows the transcription apparatus access to the DNA.
GSH: Glutathione; SH: Sulfhydryl group; SS: Disulfide bond.

inhibition of GSH, it is even possible to alter GSH concentrations within selected subcellular compartments; for example, buthionine sulfoximine (BSO) inhibits γ-glutamylcysteine synthetase, which is necessary for cytosolic GSH biosynthesis [61]. On the other hand, diethyl maleate permits the depletion of GSH within all cellular pools by directly conjugating with thiols. Membrane permeable ethyl ester of GSH (GSH-Et) can be used to increase the intracellular GSH concentration [59,62]. It was shown, for example, that depleting intracellular GSH by inhibiting its *de novo* synthesis with BSO decreased the efficiency of early gene expression of DGDS sensitive to the redox potential gradients, whereas augmentation of intracellular GSH by means of membrane-permeable GSH-Et, had the opposite effect. Similar effects of increasing GSH concentrations on enhanced transfection efficiency were reported by Read *et al.* [60]. Because of the higher GSH concentration present in the cell nucleus compared with the cytoplasm, polyplex disassembly is likely to occur more efficiently in the nucleus than in the cytoplasm. Improved subcellular disassembly of polyplexes based on reducible polycations proved to be highly beneficial for enzyme prodrug gene therapy, demonstrating efficiency comparable with that previously attained with adenoviral vectors [60].

The benefits of controlled subcellular disassembly of polyplexes for efficient delivery of nucleic acids have also been demonstrated for mRNA. The use of mRNA in gene therapy has been limited due to its poor stability and short duration of expression [63-66]. Unlike DNA, only cationic lipids can be

used to efficiently deliver mRNA. It was suggested that the high intracellular stability (low disassembly rates) of complexes of high molecular weight polycations is responsible for extremely low mRNA expression [67]. It has been shown that polyplexes based on high molecular weight polycations such as PEI (25 kDa) and PLL (54 kDa) cannot mediate efficient translation of mRNA due to their high stability against disassembly in the cytoplasm [67]. In comparison, polyplexes formed using smaller PEI (2 kDa) and PLL (3 kDa) mediated relatively high levels of gene expression. It was demonstrated that the use of polyplexes based on intracellularly degradable polycations can mediate high levels of transfection with mRNA, even when high molecular weight polycations are used [60]. The results proved that cytosolic reduction of the disulfide bonds in reducible polycations causes increased cytosolic availability of mRNA. The results obtained from mRNA transfection experiments serve to demonstrate the feasibility of using redox-potential gradient responsive polyplexes for efficient cytosolic delivery of mRNA, and confirm the contribution of GSH-mediated degradation to the intracellular disassembly of such vectors.

The toxicity of polycations such as PEI is a well-known phenomenon [68-70]. A successful gene delivery carrier, however, should be able to deliver transcriptionally active genes to the cell without negatively affecting normal functions of the host cell. Although the precise basis of the cytotoxic action of polycations remains unknown, based on the published evidence, three mechanisms can be postulated by which they exert cytotoxic activity [70-76]: direct destabilisation of plasma membranes by free polycations; destabilisation of intracellular membranes (e.g., lysosomal, nuclear) by free polycations and polycations released from the polyplexes; and interference of free polycations and polycations released from the polyplexes with vital cellular processes (e.g., interaction with nucleic acids, proteins and activation of intracellular signal transduction pathways). Structural features believed to most affect the way in which polycations interact with cell membranes include charge density, molecular weight, type of charged centre (type of amino group), and molecular flexibility [77-80]. Rigid molecules have more difficulties attaching to the membranes than flexible molecules [73]; therefore, high cationic charge densities and highly flexible polymers, such as PEI, are expected to cause greater cytotoxic effects than those with low cationic charge density and more rigid chains. Available data on cytotoxicity of the reducible polycations and their DNA polyplexes show that all the reducible polycations exhibit only a minimal toxicity when compared with control non-reducible polycations [60,81]. In fact, the observed cytotoxicity is often similar to that obtained for low molecular weight degradation products of the reducible polycations. It can be hypothesised that the observed low cytotoxicity of the disulfide-containing polycations is a direct consequence of the reduced binding affinity for cell membranes and vital proteins and nucleic acids after rapid intracellular reduction, and subsequent decrease of molecular weight of these polycations. The available results also suggest that a significant contribution to the cytotoxicity of polycations originates from their intracellular action, and that direct destabilisation of the plasma membrane, may not be the major contributor to the polycation toxicity.

3.2. Hyperthermia-controlled delivery and activity of drugs and genes

Existing local hyperthermia treatment protocols are well suited as the stimulus for DGDS-containing polymers, which exhibit lower critical solution temperature (LCST) behaviour. The possibility to control properties of soluble DGDS by local hyperthermia has so far been utilised in several different types of applications. This is an emerging group of stimulus-controlled delivery systems that relies on temperature-induced phase transition of the polymer carrier. It is the rapid, highly nonlinear and reversible nature of such phase transitions in response to small changes of temperature that makes these vectors very promising in the design of delivery vectors where rapid changes of properties are required. Temperature-responsive vectors described thus far use synthetic acryl-based copolymers or genetically engineered elastin-like polypeptides as the temperature responsive component. One of the most widely used polymers, poly-*N*-isopropylacrylamide (PNIPA), is a water-soluble polymer that undergoes a coil-to-globule phase transition at 32°C. This transition is caused by breaking hydrogen bonds between the polymer and water when moving above the phase transition temperature; the polymer expels water molecules and effectively precipitates. The transition temperature can be easily engineered by copolymerisation with a suitable comonomer to levels fully compatible with the protocols of clinical hyperthermia.

The possibility to control the solubility of DGDS by local hyperthermia has been utilised as a simple way to enhance their tumour accumulation; for example, hyperthermia (40 – 42°C) induced precipitation of conjugates of genetically engineered elastin-like polypeptides and NIPA copolymers was used to selectively increase accumulation in solid tumours [82]. It was found that local tumour hyperthermia increased the total accumulation of the thermally responsive DGDS twofold. Intravital fluorescence videomicroscopy further suggested that the observed increase in total accumulation in heated tumours of the thermally responsive carriers was due, both to an increased extravasation of the thermally responsive polymer, and to the accumulation of aggregated carrier in the vasculature.

When temperature-responsive statistical copolymers of NIPA with 2-(dimethylamino)ethyl methacrylate and butyl methacrylate were used as part of gene delivery vectors, increased affinity of the copolymers to DNA above the phase transition temperature was observed, and the ability to control transfection activity by temperature was also demonstrated [83]. The use of a copolymer with the phase transition of 21°C (i.e., the copolymer is insoluble above and soluble below 21°C), enabled the affinity of the copolymer to plasmid DNA to be controlled. Although the DNA was fully retained

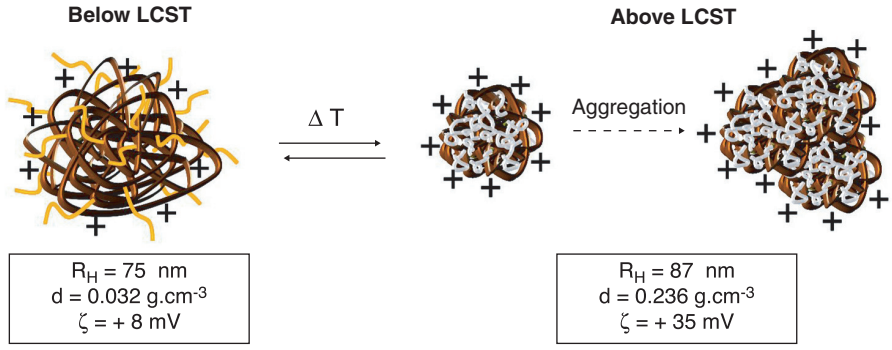

Figure 3. Controlling properties of DNA polyplexes by phase state of PNIPA. Below the phase transition temperature (LCST), the hydrated PNIPA chains (light colour) mask the positive surface charge, solubilise and cause internal swelling of polyplexes formed between DNA, and graft copolymers between poly-L-lysine and PNIPA. As a result, polyplexes with low structural density (d) and zeta potential (ζ) are formed. Above LCST, the collapsed PNIPA chains show reduced ability to mask the positive surface charge, causing a significant increase in the structural density and secondary aggregation of polyplexes, documented by increased hydrodynamic radius, R_H [84].
LCST: Lower critical solution temperature; PNIPA: Poly-*N*-isopropylacrylamide.

in the origin of the gel during electrophoresis at temperatures above the phase transition, at temperatures below the phase transition, the plasmid became partially dissociated from the copolymer. In addition, the authors established that a temperature regimen can be identified that enables the disassembly of the vector inside the cells to be controlled and transfection levels to be elevated.

A recent study suggested that hyperthermia can be used to control the biophysical properties of DNA polyplexes. In that study, biophysical properties of complexes of DNA with graft copolymers of PLL and PNIPA were investigated [84]. The objective of the study was to demonstrate that physicochemical properties of DNA polyplexes are controlled by the phase state of NIPA present in their structure. A graft copolymer of PLL and PNIPA was synthesised by randomly grafting semitelechelic PNIPA onto PLL [85]. It was shown that the phase transition temperature (LCST) of PNIPA grafts was not significantly affected by grafting onto PLL chains, and it remained at ~ 31 – 32°C. The effect of the phase transition of PNIPA on the properties of polyplexes was studied at two selected temperatures; 25 and 38°C, representing 6°C above and 6°C below the observed LCST of the PNIPA. Although increasing the temperature from 25 to 38°C only had a negligible effect on the physicochemical properties of polyplexes not containing PNIPA, it caused marked changes in the properties of NIPA-containing polyplexes. The PNIPA transition is triggered by the loss of hydrating water, and is manifested by a collapse of the coil conformation into the more compact globule, and by increased polymer hydrophobicity. The authors demonstrated that the phase transition of PNIPA chains caused an increased surface charge and structural density of polyplexes; observations that are compatible with the coil-to-globule collapse. The increase of the structural density of polyplexes implied a reduction in their size. Concomitant, hydrophobically-driven aggregation of the polyplexes, however, prevented in most cases, the clear demonstration of the size reduction of individual polyplexes. **Figure 3** schematically summarises the main physicochemical effects induced by PNIPA-phase transition on properties of DNA polyplexes and also shows a specific example of the reported changes of physical properties induced by the PNIPA-phase transition. For example, the surface charge of some of the studied DNA complexes was increased from 8 to 32 mV by increasing the temperature above the LCST of the PNIPA. At the same time, the internal structure of the complexes was also altered, manifesting an almost 10-fold increase in the structural density of the polyplex nanoparticles. The authors hypothesised that the possibility to alter surface properties of these complexes by small changes of temperature can be translated into controlling the tropism of the polyplexes. Similar control of biophysical properties by temperature was demonstrated for polyplexes based on graft copolymers of PEI and NIPA copolymers [86]. The major difference between the vectors discussed in [84,86,87] and those developed by the group of Okano [83,88], is the direct involvement of the temperature-responsive component in DNA binding in [83,88]. The limited amount of data that is currently available does not allow a clear conclusion to be made as to which of the copolymer architectures will prove to be more beneficial for potential hyperthermia-controlled gene delivery. More importantly, despite the promising properties of these vectors, they have yet to demonstrate a convincing performance under more realistic physiological conditions.

Conjugation of temperature sensitive copolymers to protein molecules has been exploited in the design of conjugates with temperature controlled activity. These systems offer the possibility to design prodrug types of delivery system, with their activity reversibly switched on and off in the

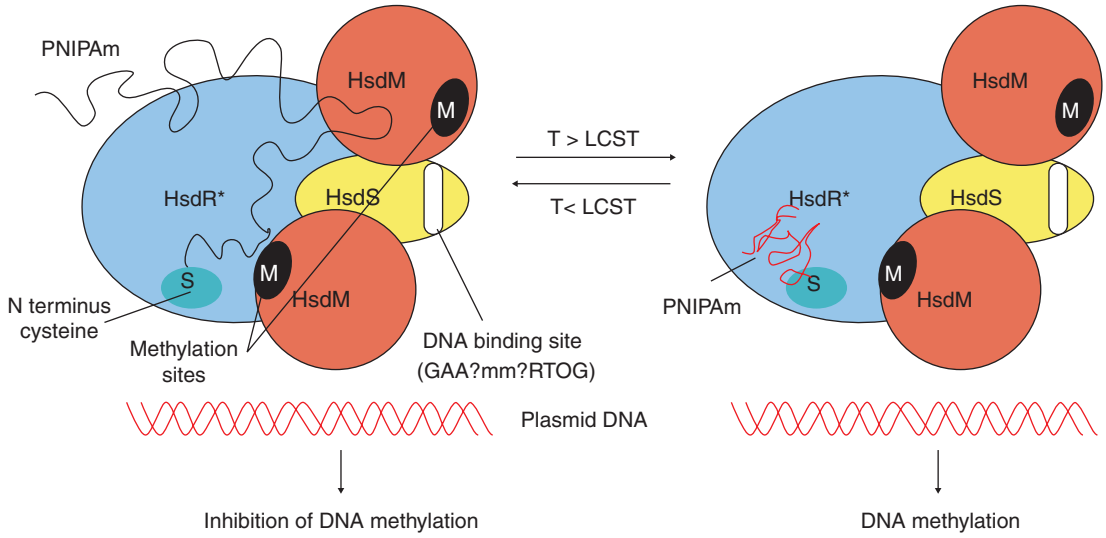

Figure 4. Schematic representation of the *N*-isopropylacrylamide switch. Reprinted with permission from PENNADAM SS, LAVIGNE MD, DUTTA CF *et al.*: Control of a multisubunit DNA motor by a thermoresponsive polymer switch. *J. Am. Chem. Soc.* (2004) **126**:13208-13209. Copyright 2004 American Chemical Society.
HsdM, HsdR, HsdS: Subunits of the enzyme EcoR124I; LCST: Lower critical solution temperature; PNIPAm: Poly-(*N*-isopropylacrylamide); T: Temperature.

organism by the application of a local hyperthermia. Many of the initial studies focused on model systems, such as streptavidin/biotin, to gain a basic understanding of the factors underlying the activity control by covalent attachment of temperature-sensitive polymers [89-92].

Depending on the selected protein and the site of attachment, the systems can be engineered to have their activity switched on by the hyperthermia or switched off, providing a desired flexibility for potential therapeutic use; for example, a site-specific conjugation of *N*,*N*-dimethylacrylamide/*N*-4-phenylazophenylacrylamide copolymers to a unique cysteine residue positioned near the active site of an enzyme endoglucanase 12A, led to a conjugate where the copolymer acted as a molecular switch of the enzymatic activity triggered by temperature change (increasing the temperature acting as the switch-off trigger) [93]. Depending on the conjugation site, the conjugate displayed different shut-off efficiency in the collapsed polymer state. Increasing the molecular weight of the polymer was also shown to increase the shut-off efficiency of the switch. In addition, the molecular switch efficiency was dependent on the size of the substrate molecules.

In a recent example of an opposite molecular switch, where the protein activity is switched on by a temperature increase, Pennadam *et al.* conjugated NIPA copolymers to a subunit of a hybrid EcoR124I restriction-modification enzyme (**Figure 4**) [94,95]. The NIPA polymer switch was covalently attached, specifically at a subunit distal to the DNA recognition and restriction sites. The authors reported that only 10% methylation was observed below the phase transition temperature of the NIPA copolymer. Above the transition temperature, 90 – 100% methylation was observed, clearly indicating an activity switch due to the coil-to-globule transition of the NIPA chains. Furthermore, the activity of the conjugate was fully reversible and could be repeated over multiple cycles.

4. Expert opinion

DGDS sensitive to redox potential gradients are likely to receive increased attention in the future. In particular, their use in gene delivery has the potential to contribute to overcoming some of the obstacles currently faced by researchers developing polyplex-based gene therapies. The redox potential gradient represents a highly reliable stimulus for the intracellular release of drugs and genes; however, there is a potential for premature release of the therapeutic cargo in the blood plasma. Although plasma environment is predominantly oxidising, reduction of disulfide bonds does occur due to the presence of low concentrations of cysteine and GSH. This has to be taken into consideration, especially in the design of DGDS for intravenous systemic administration, where extended plasma circulation times are expected. Local hyperthermia, the second stimulus discussed in this review, has so far shown great promise in combination with adenoviral gene delivery vectors containing the heat-shock protein promoter. The use of hyperthermia to control the behaviour of DGDS based on phase-transition polymers has so far been less successful until now. The current limitations in the use of DGDS responsive to hyperthermia are, at least partly, caused by the difficulties in controlling the phase transition behaviour in complex biological fluids such as blood plasma. The LCST of these systems is affected by a variety of solutes and, therefore, the optimisation of the DGDS properties and their reproducibility is often difficult. In addition, the synthetic methodology used in the preparation of the

temperature-responsive polymers, is usually not compatible with producing uniform polymers both in terms of molecular weight and composition. Response uniformity is potentially another major problem in using soluble DGDS based on temperature-responsive polymers. Although this approach has yet to clearly demonstrate utility under real physiological conditions, modern approaches based on genetic engineering or novel synthetic methodologies, such as living radical polymerisation, show promise in overcoming many of the limitations. Enhancing the microvascular and cell membrane permeability by a local ultrasound exposure seems to represent an attractive approach to overcoming the limited ability of blood-borne DGDS to extravasate from the vasculature and cross cellular membranes effectively. This approach can potentially be applied for improving the efficiency of DGDS delivery to a wide range of tissues and organs, including brain and solid tumours. Potential risks of using ultrasound exposure to control properties of tissues include microvascular damage, haemorrhaging, and the possibility of facilitating metastatic spread from the treated tumours.

This review discussed recent examples of polymer-based drug and gene delivery vectors that are capable of responding to specific endogenous or exogenous stimuli by altering their properties or behaviour. One of the key motivations for the development of such vectors is the requirement for diverse properties necessary to overcome the variety of physiological barriers encountered by the vectors during the systemic intravenous delivery. Further developments of the stimuli-responsive DGDS will critically depend on the enhanced knowledge of the correlations between specific physical properties of the vectors and their biological behaviour. In particular, detailed quantitative description of the extracellular and intracellular phases of the delivery process will be of importance for optimisation of the design of such vectors.

Bibliography

Papers of special interest have been highlighted of interest (•) or of considerable interest (••) to readers.

1. DUNCAN R: The dawning era of polymer therapeutics. *Nat. Rev. Drug Discov.* (2003) **2:**347-360.
2. GOPIN A, PESSAH N, SHAMIS M, RADER C, SHABAT D: A chemical adaptor system designed to link a tumor-targeting device with a prodrug and an enzymatic trigger. *Angew. Chem. Int. Ed.* (2003) **42:**327-332.
3. SHABAT D, RADER C, LIST B, LERNER RA, BARBAS CF 3rd: Multiple event activation of a generic prodrug trigger by antibody catalysis. *Proc. Natl. Acad. Sci. USA* (1999) **96:**6925-6930.
4. GIANASI E, WASIL M, EVAGOROU EG, KEDDLE A, WILSON G, DUNCAN R: HPMA copolymer platinates as novel antitumour agents: *in vitro* properties, pharmacokinetics and antitumour activity *in vivo*. *Eur. J. Cancer* (1999) **35:**994-1002.
5. SAITO G, SWANSON JA, LEE KD: Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities. *Adv. Drug Deliv. Rev.* (2003) **55:**199-215.

• **A comprehensive review of drug delivery systems sensitive to redox potential gradients.**

6. KRETZ-REMY C, ARRIGO AP: Gene expression and thiol redox state. *Methods Enzymol.* (2002) **348**:200-215.
7. MEISTER A, ANDERSON ME: Glutathione. *Ann. Rev. Biochem.* (1983) **52**:711-760.
8. BELLOMO G, VAIRETTI M, STIVALA L, MIRABELLI F, RICHELMI P, ORRENIUS S: Demonstration of nuclear compartmentalization of glutathione in hepatocytes. *Proc. Natl. Acad. Sci. USA* (1992) **89:**4412-4416.
9. ARRIGO AP: Gene expression and the thiol redox state. *Free Radic. Biol. Med.* (1999) **27:**936-944.
10. AULT JG, LAWRENCE DA: Glutathione distribution in normal and oxidatively stressed cells. *Exp. Cell Res.* (2003) **285:**9-14.
11. ARRICK BA, NATHAN CF: Glutathione metabolism as a determinant of therapeutic efficacy. *Cancer Res.* (1984) **44:**4224-4232.
12. WU X, BISHOPRIC NH, DISCHER DJ, MURPHY BJ, WEBSTER KA: Physical and functional sensitivity of zinc finger transcription factors to redox change. *Mol. Cell Biol.* (1996) **16:**1035-1046.
13. HWANG C, LODISH HF, SINSKEY AJ: Measurement of glutathione redox state in cytosol and secretory pathway of cultured cells. *Methods Enzymol.* (1995) **251**:212-221.
14. HWANG C, SINSKEY AJ, LODISH HF: Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* (1992) **257:**1496-1502.
15. LASH LH, PUTT DA, HUENI SE *et al.*: Cellular energetics and glutathione status in NRK-52E cells: toxicological implications. *Biochem. Pharmacol.* (2002) **64:**1533-1546.
16. STAAL FJT, ROEDERER M, HERZENBERG LA: Intracellular thiols regulate activation of nuclear factor kappa-B and transcription of human-immunodeficiency-virus. *Proc. Natl. Acad. Sci. USA* (1990) **87:**9943-9947.
17. TOWNSEND DM, TEW KD, TAPIERO H: The importance of glutathione in human disease. *Biomed. Pharmacother.* (2003) **57:**145-155.
18. COLLINS DS, UNANUE ER, HARDING CV: Reduction of disulfide bonds within lysosomes is a key step in antigen processing. *J. Immunol.* (1991) **147:**4054-4059.
19. DONOGHUE N, YAM PTW, JIANG XM, HOGG PJ: Presence of closely spaced protein thiols on the surface of mammalian cells. *Protein Sci.* (2000) **9:**2436-2445.
20. PHAN UT, ARUNACHALAM B, CRESSWELL P: Gamma-interferon-inducible lysosomal thiol reductase (GILT) – maturation, activity, and mechanism of action. *J. Biol. Chem.* (2000) **275:**25907-25914.
21. VOEHRINGER DW: BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. *Free Radic. Biol. Med.* (1999) **27:**945-950.
22. VOEHRINGER DW, MCCONKEY DJ, MCDONNELL TJ, BRISBAY S, MEYN RE: Bcl-2 expression causes redistribution of glutathione to the nucleus.

Proc. Natl. Acad. Sci. USA (1998) **95:**2956-2960.

23. VOEHRINGER DW, MEYN RE: Redox aspects of Bcl-2 function. *Antioxid. Redox Signal.* (2000) **2:**537-550.
24. SIES H: Glutathione and its role in cellular functions. *Free Radic. Biol. Med.* (1999) **27:**916-921.
25. FITZGERALD UF, GILBEY T, BRODIE S, BARNETT SC: Transcription factor expression and cellular redox in immature oligodendrocyte cell death: effect of Bcl-2. *Mol. Cell. Neurosci.* (2003) **22:**516-529.
26. ORTEGA A, FERRER P, CARRETERO J *et al.*: Down-regulation of glutathione and Bcl-2 synthesis in mouse B16 melanoma cells avoids their survival during interaction with the vascular endothelium. *J. Biol. Chem.* (2003) **278:**39591-39599.
27. HOETELMANS RW, VAHRMEIJER AL, MULDER GJ, VAN DE VELDE CJ, NAGELKERKE JF, VAN DIERENDONCK JH: Bcl-2 overexpression does not prevent but retards adriamycin toxicity in CC531 colon carcinoma cells. *Chemotherapy* (2003) **49:**309-315.
28. HOETELMANS RW, VAHRMEIJER AL, VAN VLIERBERGHE RL *et al.*: The role of various Bcl-2 domains in the anti-proliferative effect and modulation of cellular glutathione levels: a prominent role for the BH4 domain. *Cell Prolif.* (2003) **36:**35-44.
29. STORY MD, MEYN RE: Modulation of apoptosis and enhancement of chemosensitivity by decreasing cellular thiols in a mouse B-cell lymphoma cell line that overexpresses bcl-2. *Cancer Chemother. Pharmacol.* (1999) **44:**362-366.
30. MIRKOVIC N, VOEHRINGER DW, STORY MD, MCCONKEY DJ, MCDONNELL TJ, MEYN RE: Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols. *Oncogene* (1997) **15:**1461-1470.
31. AMICHETTI M, ROMANO M, CRISTOFORETTI L, VALDAGNI R: Hyperthermia and radiotherapy for inoperable squamous cell carcinoma metastatic to cervical lymph nodes from an unknown primary site. *Int. J. Hyperthermia* (2000) **16:**85-93.
32. VALDAGNI R, AMICHETTI M: Report of long-term follow-up in a randomized trial comparing radiation therapy and radiation therapy plus hyperthermia to metastatic lymph nodes in stage IV head and neck patients. *Int. J. Radiat. Oncol. Biol. Phys.* (1994) **28:**163-169.
33. OVERGAARD J, GONZALEZ D, HULSHOF MC *et al.*: Randomised trial of hyperthermia as adjuvant to radiotherapy for recurrent or metastatic malignant melanoma. European Society for Hyperthermic Oncology. *Lancet* (1995) **345:**540-543.
34. LI G, MITSUMORI M, OGURA M *et al.*: Local hyperthermia combined with external irradiation for regional recurrent breast carcinoma. *Int. J. Clin. Oncol.* (2004) **9:**179-183.
35. ROTHBARTH J, TOLLENAAR RA, SCHELLENS JH *et al.*: Isolated hepatic perfusion for the treatment of colorectal metastases confined to the liver: recent trends and perspectives. *Eur. J. Cancer* (2004) **40:**1812-1824.
36. FELDMAN ED, WU PC, BERESNEVA T *et al.*: Treatment of patients with unresectable primary hepatic malignancies using hyperthermic isolated hepatic perfusion. *J. Gastrointest. Surg.* (2004) **8:**200-207.
37. GLEHEN O, MOHAMED F, GILLY FN: Peritoneal carcinomatosis from digestive tract cancer: new management by cytoreductive surgery and intraperitoneal chemohyperthermia. *Lancet Oncol.* (2004) **5:**219-228.
38. BORRELLI MJ, SCHOENHERR DM, WONG A, BERNOCK LJ, CORRY PM: Heat-activated transgene expression from adenovirus vectors infected into human prostate cancer cells. *Cancer Res.* (2001) **61:**1113-1121.
39. BRADE AM, SZMITKO P, NGO D, LIU FF, KLAMUT HJ: Heat-directed suicide gene therapy for breast cancer. *Cancer Gene Ther.* (2003) **10:**294-301.
40. WUST P, HILDEBRANDT B, SREENIVASA G *et al.*: Hyperthermia in combined treatment of cancer. *Lancet Oncol.* (2002) **3:**487-497.
41. HYNYNEN K, CLEMENT GT, MCDANNOLD N *et al.*: 500-Element ultrasound phased array system for noninvasive focal surgery of the brain: a preliminary rabbit study with *ex vivo* human skulls. *Magn. Reson. Med.* (2004) **52:**100-107.
42. MILLER DL, PISLARU SV, GREENLEAF JE: Sonoporation: mechanical DNA delivery by ultrasonic cavitation. *Somat. Cell Mol. Genet.* (2002) **27:**115-134.
43. TANIYAMA Y, TACHIBANA K, HIRAOKA K *et al.*: Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. *Gene Ther.* (2002) **9:**372-380.
44. SKYBA DM, PRICE RJ, LINKA AZ, SKALAK TC, KAUL S: Direct *in vivo* visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue. *Circulation* (1998) **98:**290-293.
45. UNGER EC, MATSUNAGA TO, MCCREERY T, SCHUMANN P, SWEITZER R, QUIGLEY R: Therapeutic applications of microbubbles. *Eur. J. Radiol.* (2002) **42:**160-168.
46. PRICE RJ, SKYBA DM, KAUL S, SKALAK TC: Delivery of colloidal, particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. *Circulation* (1998) **98:**1264-1267.

•• **Demonstration of efficient enhancement of vascular extravasation of synthetic nanoparticles by localised ultrasound treatment.**

47. HYNYNEN K, MCDANNOLD N, VYKHODTSEVA N, JOLESZ FA: Non-invasive opening of BBB by focused ultrasound. *Acta Neurochir. Suppl.* (2003) **86:**555-558.
48. LINDNER JR, KAUL S: Delivery of drugs with ultrasound. *Echocardiography* (2001) **18:**329-337.
49. MILLER DL, SONG J: Lithotripter shock waves with cavitation nucleation agents produce tumor growth reduction and gene transfer *in vivo*. *Ultrasound Med. Biol.* (2002) **28:**1343-1348.
50. SONG J, CHAPPELL JC, QI M, VANGIESON EJ, KAUL S, PRICE RJ: Influence of injection site, microvascular pressure and ultrasound variables on microbubble-mediated delivery of microspheres to muscle. *J. Am. Coll. Cardiol.* (2002) **39:**726-731.
51. LI P, ARMSTRONG WF, MILLER DL: Impact of myocardial contrast echocardiography on vascular permeability: comparison of three different contrast

agents. *Ultrasound Med. Biol.* (2004) **30:**83-91.

52. PITT WG, HUSSEINI GA, STAPLES BJ: Ultrasonic drug delivery – a general review. *Expert Opin. Drug Deliv.* (2004) **1:**37-56.
53. KWOK KY, PARK Y, YANG Y, MCKENZIE DL, LIU Y, RICE KG: *In vivo* gene transfer using sulfhydryl cross-linked PEG-peptide/glycopeptide DNA co-condensates. *J. Pharm. Sci.* (2003) **92:**1174-1185.
54. OUPICKY D, OGRIS M, HOWARD KA, DASH PR, ULBRICH K, SEYMOUR LW: Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol. Ther.* (2002) **5:**463-472.
55. OUPICKY D, PARKER AL, SEYMOUR LW: Laterally stabilized complexes of DNA with linear reducible polycations: Strategy for triggered intracellular activation of DNA delivery vectors. *J. Am. Chem. Soc.* (2002) **124:**8-9.

• **Strategy for specific intracellular activation of DNA polyplexes stabilised by surface crosslinking with multivalent copolymers.**

56. FISHER KD, ULBRICH K, SUBR V *et al.*: A versatile system for receptor-mediated gene delivery permits increased entry of DNA into target cells, enhanced delivery to the nucleus and elevated rates of transgene expression. *Gene Ther.* (2000) 7:1337-1343.
57. DASH PR, READ ML, FISHER K *et al.*: Decreased binding to proteins and cells of polymeric gene delivery vectors surface modified with a multivalent hydrophilic polymer and retargeting through attachment of transferrin. *J. Biol. Chem.* (2000) **275:**3793-3802.
58. CARLISLE RC, ETRYCH T, BRIGGS SS, PREECE JA, ULBRICH K, SEYMOUR LW: Polymer-coated polyethylenimine/DNA complexes designed for triggered activation by intracellular reduction. *J. Gene. Med.* (2004) **6:**337-344.
59. BALAKIREV M, SCHOEHN G, CHROBOCZEK J: Lipoic acid-derived amphiphiles for redox-controlled DNA delivery. *Chem. Biol.* (2000) 7:813-819.
60. READ ML, BREMNER KH, OUPICKY D, GREEN NK, SEARLE PF, SEYMOUR LW: Vectors based on reducible polycations facilitate intracellular release of nucleic acids. *J. Gene. Med.* (2003) **5:**232-245.
61. JARUGA E, BIELAK-ZMIJEWSKA A, SIKORA E *et al.*: Glutathione-independent mechanism of apoptosis inhibition by curcumin in rat thymocytes. *Biochem. Pharmacol.* (1998) **56:**961-965.
62. LEVY EJ, ANDERSON ME, MEISTER A: Transport of glutathione diethyl ester into human cells. *Proc. Natl. Acad. Sci. USA* (1993) **90:**9171-9175.
63. ANDERSON DM, HALL LL, AYYALAPU AR, IRION VR, NANTZ MH, HECKER JG: Stability of mRNA/cationic lipid lipoplexes in human and rat cerebrospinal fluid: Methods and evidence for nonviral mRNA gene delivery to the central nervous system. *Hum. Gene Ther.* (2003) **14:**191-202.
64. MITCHELL DA, NAIR SK: RNA-transfected dendritic cells in cancer immunotherapy. *J. Clin. Invest.* (2000) **106:**1065-1069.
65. HEISER A, DAHM P, YANCEY DR *et al.*: Human dendritic cells transfected with RNA encoding prostate-specific antigen stimulate prostate-specific CTL responses *in vitro*. *J. Immunol.* (2000) **164:**5508-5514.
66. MALONE RW, FELGNER PL, VERMA IM: Cationic Liposome-Mediated RNA Transfection. *Proc. Natl. Acad. Sci. USA* (1989) **86:**6077-6081.
67. BETTINGER T, CARLISLE RC, READ ML, OGRIS M, SEYMOUR LW: Peptide-mediated RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic cells. *Nucleic Acids Res.* (2001) **29:**3882-3891.

• **High molecular weight polycations are not compatible with mRNA transfection.**

68. ZOU SM, ERBACHER P, REMY JS, BEHR JP: Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J. Gene. Med.* (2000) **2:**128-134.
69. CHOLLET P, FAVROT MC, HURBIN A, COLL JL: Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. *J. Gene. Med.* (2002) **4:**84-91.
70. FISCHER D, LI Y, AHLEMEYER B, KRIEGLSTEIN J, KISSEL T: *In vitro* cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* (2003) **24:**1121-1131.
71. LEROY D, SCHMID N, BEHR JP *et al.*: Direct identification of a polyamine binding domain on the regulatory subunit of the protein-kinase casein kinase-2 by photoaffinity-labeling. *J. Biol. Chem.* (1995) **270:**17400-17406.
72. CHOKSAKULNIMITR S, MASUDA S, TOKUDA H, TAKAKURA Y, HASHIDA M: *In-vitro* cytotoxicity of macromolecules in different cell-culture systems. *J. Control. Release* (1995) **34:**233-241.
73. SINGH AK, KASINATH BS, LEWIS EJ: Interaction of polycations with cell-surface negative charges of epithelial-cells. *Biochim. Biophys. Acta* (1992) **1120:**337-342.
74. MORGAN DML, LARVIN VL, PEARSON JD: Biochemical-characterization of polycation-induced cytotoxicity to human vascular endothelial-cells. *J. Cell Sci.* (1989) **94:**553-559.
75. BIEBER T, MEISSNER W, KOSTIN S, NIEMANN A, ELSASSER HP: Intracellular route and transcriptional competence of polyethylenimine-DNA complexes. *J. Control. Release* (2002) **82:**441-454.
76. GODBEY WT, WU KK, MIKOS AG: Poly(ethylenimine)-mediated gene delivery affects endothelial cell function and viability. *Biomaterials* (2001) **22:**471-480.
77. GODBEY WT, WU KK, MIKOS AG: Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. *J. Biomed. Mater. Res.* (1999) **45:**268-275.
78. FISCHER D, BIEBER T, LI YX, ELSASSER HP, KISSEL T: A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm. Res.* (1999) **16:**1273-1279.
79. BRISSAULT B, KICHLER A, GUIS C, LEBORGNE C, DANOS O, CHERADAME H: Synthesis of linear polyethylenimine derivatives for DNA transfection. *Bioconjugate Chem.* (2003) **14:**581-587.
80. MORGAN DML, CLOVER J, PEARSON JD: Effects of synthetic polycations on leucine incorporation, lactate-dehydrogenase release, and morphology of human umbilical vein endothelial-cells. *J. Cell Sci.* (1988) **91:**231-238.
81. SOUNDARA MANICKAM D, BISHT HS, WAN L, MAO G, OUPICKY D: Influence of TAT-peptide polymerization on properties and transfection activity of TAT/DNA polyplexes. *J. Control. Release* (2005) **102:**293-306.

82. MEYER DE, SHIN BC, KONG GA, DEWHIRST MW, CHILKOTI A: Drug targeting using thermally responsive polymers and local hyperthermia. *J. Control. Release* (2001) **74:**213-224.

83. KURISAWA M, YOKOYAMA M, OKANO T: Gene expression control by temperature with thermo-responsive polymeric gene carriers. *J. Control. Release* (2000) **69:**127-137.

• **First published evidence of temperature-controlled transfection activity of non-viral vectors using temperature-responsive copolymers.**

84. OUPICKY D, RESCHEL T, KONAK C, OUPICKA L: Temperature controlled behavior of self-assembly gene delivery vectors based on complexes of DNA with poly-L-lysine-graft-poly-*N*-isopropylacrylamide). *Macromolecules* (2003) **36:**6883-6872.

• **Demonstration of the possibility to control biophysical properties of DNA polyplexes by temperature-responsive copolymers.**

85. KONAK C, RESCHEL T, OUPICKY D, ULBRICH K: Thermally controlled association in aqueous solutions of poly(L-lysine) grafted with poly (*N*-isopropylacrylamide). *Langmuir* (2002) **18:**8217-8222.

86. TWAITES BR, DE LAS HERAS ALARCON C, CUNLIFFE D *et al.*: Thermo and pH responsive polymers as gene delivery vectors: effect of polymer architecture on DNA complexation *in vitro*. *J. Control. Release* (2004) **97:**551-566.

87. TURK M, DINCER S, YULUG IG, PISKIN E: *In vitro* transfection of HeLa cells with temperature sensitive polycationic copolymers. *J. Control. Release* (2004) **96:**325-340.

88. KURISAWA M, YOKOYAMA M, OKANO T: Transfection efficiency increases by incorporating hydrophobic monomer units into polymeric gene carriers. *J. Control. Release* (2000) **68:**1-8.

89. BULMUS V, DING ZL, LONG CJ, STAYTON PS, HOFFMAN AS: Site-specific polymer-streptavidin bioconjugate for pH-controlled binding and triggered release of biotin. *Bioconjugate Chem.* (2000) **11:**78-83.

90. DING Z, LONG CJ, HAYASHI Y, BULMUS EV, HOFFMAN AS, STAYTON PS: Temperature control of biotin binding and release with a streptavidin-poly(*N*-isopropylacrylamide) site-specific conjugate. *Bioconjugate Chem.* (1999) **10:**395-400.

91. DING ZL, FONG RB, LONG CJ, STAYTON PS, HOFFMAN AS: Size-dependent control of the binding of biotinylated proteins to streptavidin using a polymer shield. *Nature* (2001) **411:**59-62.

•• **Temperature-responsive copolymers control binding of protein substrates in a size-dependent manner.**

92. STAYTON PS, SHIMOBOJI T, LONG C *et al.*: Control of protein-ligand recognition using a stimuli-responsive polymer. *Nature* (1995) **378:**472-474.

93. SHIMOBOJI T, LARENAS E, FOWLER T, HOFFMAN AS, STAYTON PS: Temperature-induced switching of enzyme activity with smart polymer-enzyme conjugates. *Bioconjugate Chem.* (2003) **14:**517-525.

94. PENNADAM SS, FIRMAN K, ALEXANDER C, GORECKI DC: Protein-polymer nano-machines. Towards synthetic control of biological processes. *J. Nanobiotech.* (2004) **2:**8.

95. PENNADAM SS, LAVIGNE MD, DUTTA CF *et al.*: Control of a multisubunit DNA motor by a thermoresponsive polymer switch. *J. Am. Chem. Soc.* (2004) **126:**13208-13209.

Affiliation

David Oupicky†, Harender S Bisht, Devika Soundara Manickam & Qing-hui Zhou
†Author for correspondence
Wayne State University, Department of Pharmaceutical Sciences, Detroit, MI 48202, USA
Tel: +1 313 993 7669; Fax: +1 313 577 2033;
E-mail: oupicky@wayne.edu