Expert Opinion

Temperature- and pH-responsive smart polymers for gene delivery

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Biopolymer therapeutics are likely to be the next generation of medicines, and nucleic acids are among the most important of these potential drugs. The challenges of delivering genes are formidable and advanced biomimetic materials are expected to be required. Polymers that can respond to changes in temperature and pH are good candidates for gene delivery vehicles, as the stimulus response can be used to alter their interactions with the drug payload. In this review, the chemistries underlying these responsive polymers are considered, and the possible mechanisms by which nucleic acids, primarily DNA, can be protected during transit and released at target sites are outlined. Sophisticated multicomponent polymers are being developed, with functionalities designed to overcome the barriers to gene delivery at both the systemic and local level; key examples are highlighted. The extension of these materials to yet more advanced therapies, such as cell delivery and regenerative medicine, is outlined as an emerging technology for the future.

Keywords: gene delivery, nanoparticles, plasmid DNA, polyelectrolyte complexes, polyethyleneimine, polymer therapeutics, poly(*N*-isopropylacrylamide), responsive polymers, transfection

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1. Introduction

An ideal therapeutic delivery system is one that can carry a drug to where it is needed the most in the body and then to be able to release it at exactly the right time and in the right dose. If this system can deliver the drug through external control, or via a response to a biological stimulus, its behaviour may then be termed 'smart', and many studies in drug delivery research are accordingly directed towards the development of these materials. Polymers that respond reversibly to relatively small variations in their environment through a phase transition or a discontinuous conformation change are perhaps the most actively researched smart materials. These can be synthesised to respond to changes in environmental temperature [1], pH [2], ionic strength [3], light [4,5], electric or magnetic fields [6,7], through to specific chemical actuation via solvent, ligands (e.g., glucose [8]) or enzymes [9]. Smart polymers have many potential applications in conventional drug delivery (e.g., as pulsatile drug release systems) as well as site-specific and controllable release carriers, but also have a role in newer biomedical applications, such as cell delivery, tissue engineering and regenerative medicine. This review considers primarily temperature- and pH-responsive synthetic polymers for delivery of genes, but also highlights some exciting recent developments in cell delivery that have the potential to revolutionise many aspects of medicine.

2. Gene delivery: biological barriers to nucleic acid therapeutics

Biopolymers such as nucleic acids offer enormous therapeutic potential, but are rapidly degraded when directly administered, as the barriers against exogenous DNA are formidable [10,11]. In order to express a therapeutic gene, the DNA must be protected

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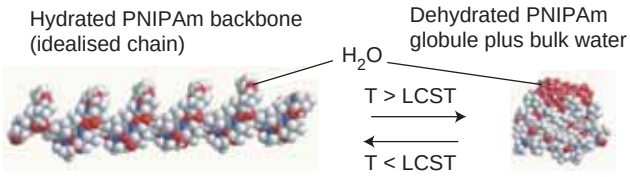

Figure 1. Schematic of PNIPAm coil–globule phase transition.
LCST: Lower critical solution temperature; PNIPAm: Poly(*N*-isopropylacrylamide); T: Temperature.

in the extracellular environment and carried to the desired tissue where it needs to cross the cellular membranes of the targeted cell, gain entry into the cytoplasm, and ultimately be taken to the nucleus where it must be released intact for transcription and subsequent translation. Carriers (vectors) are thus required that meet two basic, but conflicting, needs: strong binding to protect the genetic material during transit; and weak binding at the target site (cytoplasm for RNA, nucleus for DNA) to enable release. Barriers to delivering biopharmaceuticals are also of a technological nature, in that stable and industrially practicable (gentle) formulation methodologies of these delicate macromolecules are not always attainable [12,13]. Natural DNA and RNA transfection systems (i.e., viruses) are very effective in gently packaging nucleic acids until required and then releasing the genetic material with very high efficiency, but there are concerns with the safety of viral vectors and, hence, there are issues of patient confidence. Synthetic polymers can be envisaged as replacements for viruses in gene transfer, and have much to offer regarding protection of nucleic acids and their formulation into medicines. Responsive polymers in particular are desirable, as the switching of a polymer between two states may be the key to meeting the conflicting requirements of transport and release.

3. Responsive polymers for gene delivery

The most conceptually simple stimulus response of a polymer for gene therapy is one that changes the affinity of the polymer with the nucleic acid at the target site. Thus, a polymer can be designed to form highly stable complexes with DNA during transport to target cells and intracellular trafficking, and then application of a stimulus causes a polymer response, which, in turn, reduces the stability of the complex with DNA, thus allowing release. A second strategy may be to employ a polymer response to alter the site, timing and duration of gene expression (e.g., via a locally applied or temporally controlled stimulus that affects a cellular process either upstream or downstream of DNA transcription). An illustrative concept for this is a carrier vehicle in the cytoplasm that is activated by a stimulus to display a ligand, enabling transfer of DNA across the nuclear membrane. Until this signal is received by the polymer, it could release DNA into the cytosol without causing significant transgene expression, but when the nuclear localisation sequence is expressed, the efficiency of expression would dramatically increase. However, in all cases, it is advantageous if the polymer system is able to undergo sharp on/off switching: ideally a highly non-linear response.

3.1 Temperature-sensitive polymers

Temperature-responsive polymers are the most widely investigated smart systems in gene delivery. This is primarily because a number of different polymers are available, or are easy to synthesise, that exhibit non-linear temperature-dependent solution behaviour. Polymers that undergo coil-to-globule phase transitions in water at their lower critical solution temperature (LCST) have attracted the most attention, as the change from a chain-extended system to a chain collapsed state/aggregate results in a macroscopic hydrophilic/hydrophobic polymer switch (Figure 1). The '*Drosophila*' of the responsive polymer field is poly(*N*-isopropylacrylamide [PNIPAm]), which exhibits an LCST of 32°C in pure water. Below the LCST, the polymer is water soluble and hydrophilic, whereas above the LCST, the polymer undergoes a reversible entropy-driven phase transition to an insoluble, hydrophobic aggregate. The LCST of PNIPAm copolymers can be tailored very easily by incorporating either more hydrophilic or more hydrophobic comonomers in the polymerisation mixture; thus, it is possible to design polymers with phase transitions above, at, and below body temperature.

3.2 Selected examples of temperature-responsive copolymers for gene delivery

The first temperature-responsive polymer system used in gene delivery was reported by Hinrichs *et al.*, who prepared copolymers from *N*-isopropylacrylamide and *N*,*N*-dimethylaminoethylmethacrylate (PNIPAm-co-PDMAEMA) [14]. The DMAEMA groups were protonated at cytosolic pH and, thus, bound electrostatically to the DNA polyanion backbone, while also raising the temperature of the PNIPAm phase transition. copolymers containing as little as 15 mol% DMAEMA were able to bind DNA through a combination of charge–charge and hydrophobic (solvophobic) interactions. PNIAm-co-PDMAEMA polymers of varying molecular weights and monomer contents complexed to the pCMV-LacZ plasmid successfully transfected human ovarian cancer (NIH-OVCAR-3) cells, but the efficiency varied with a number of experimental parameters. Complexes with plasmid DNA (pDNA) and copolymers formed at 25°C tended to precipitate as the overall LCST of the polymer conjugate decreased, and particles of ~ 200 nm diameter were formed at higher copolymer:plasmid ratios, indicating condensation of the plasmid within the complex. For low molecular weight copolymers bound to DNA, aggregation to larger particles took place at 37°C (i.e., above the LCST of PNIPAm), whereas the higher molecular weight copolymers or those with greater NIPAm content were relatively stable at the higher temperature, and these more stable 200-nm complexes proved to be the most effective at gene transfection. Increasing the NIPAm

content of the copolymers decreased the zeta potential and the cytotoxicity of the copolymers. However, the less-charged polymers were also less effective in transfection. The data from Hennink's group supported the conclusions in other studies of (non-responsive) cationic DNA-delivery vehicles in that there was a trade-off between efficient transfection and increased cytotoxicity through factors such as membrane disruption caused by highly charged polycations [14].

The first reports of temperature-dependent transfection using thermosensitive polymers were from Yokoyama *et al.* who used NIPAm and DMAEMA in combination with a third comonomer, butylmethacrylate, in order to control overall polymer LCST and potentially enhance hydrophobic interactions with DNA [15-18]. Enhanced pCMV-LacZ expression was achieved in COS-1 cells (SV-40-transformed African green monkey) using an experimental protocol, in which the temperature of the cell suspension was reduced to below the LCST of the copolymer during transfection assays. The increase in transgene expression was attributed partially to extra hydrophobic stabilisation of the copolymer–DNA complex, and possible enhancement of cell-surface binding prior to subsequent endocytosis, but also to a reduced stability of the polymer–DNA complexes at temperatures below the LCST. Temperature-dependent DNA association–dissociation was shown in agarose gel electrophoretic mobility studies, suggesting a strong correlation between the changing physicochemical properties and transfection. The proposed mechanism was that above the LCST, a tight complex was formed between the polymer and DNA by hydrophobic aggregation of the polymer, whereas below the LCST, the complex dissociated or became loosely bound because of the increase in polymer hydrophilicity.

The complexity of intracellular trafficking has made complete verification of this hypothesis difficult, but nevertheless the results were of considerable significance, as they implied that appropriate choice of comonomers and control of polymer composition and structure could be used to regulate the release of DNA for gene transfection. Following on from these reports, Oupicky *et al.* developed an approach using PNIPAm grafted to linear poly(L-lysine) (PLL) with the intention of variable affinity DNA complexation [19]. In a comprehensive physicochemical study, this study group showed that PLL–graft–PNIPAm polymers were able to form small complexes (hydrodynamic radius [R_H] ~ 75 nm) with pDNA, and that these compacted when above the PNIPAm LCST. Increasing the time above the LCST, or the proportion of PNIPAm attached to the PLL backbone, increased the tendency for the complexes to aggregate. Changes in charge density also occurred, as may be expected, during LCST transitions, and, importantly, the resistance of the complexes to heparin-induced dissociation increased after longer incubation times above LCST. These data strongly supported the mechanistic interpretation of enhanced DNA binding and increased compaction of complexes above LCST, although transfection data were not reported for these polymers.

Marked differences in polymer–plasmid (pX 61 [20]) DNA complexes were observed for PNIPAm–graft–bPEI (PEI–PNIPAm-1) with the LCST 30 – 33°C) and linear PNIPAm-co-DMAEMA polymers (PNDHA-1; LCST 22 – 30°C) when temperatures were increased above the phase transition temperatures of the polymers. Compaction of complexes from an average of ~ 140 nm atomic force microscopy diameter to ~ 100 nm took place, along with changes in gross morphology; this was in agreement with similar data obtained in the Oupicky group study for PLL–PNIPAm systems. Dynamic light scattering further demonstrated the differences in polymer–DNA complex structure with temperature and that these occurred reversibly. Compaction of polymer–DNA complexes over temperature ranges correlated with vector phase transitions, suggesting polymer response to temperature was a controlling factor in particle size (i.e., compaction) characteristics [21].

Confocal micrographs (Figure 2) and transfection studies showed that although PNDHA-1 polymers rapidly delivered pDNA to mouse muscle (C2C12) cell nuclei when initial complex formation was carried out below LCST, transgene expression was low at both temperatures. However, for the PEI–PNIPAm-1 polymer, an increased number of cells expressed green fluorescent protein when complex formation was carried out at 25°C compared with at 37°C, demonstrating that differences in polymer complex formation via LCST were also manifest in transgene expression. In addition, a related PEI–PNIPAm polymer with 46-kDa PNIPAm grafts was more effective in transfecting C2C12 cells than commercial JetPEI™ (PolyPlus Transfection) using standard transfection protocols. The polymers were not found to be toxic to these cells at these concentrations in standard cell viability (MTT) assays [22], and transfection efficiencies of up to 10% in other cell lines (COS-7) were achieved, comparable with commercial transfection agents (13% of cells expressing green fluorescent protein) in similar assays (S Pennadam, MD Lavigne, C Alexander, DC Gorecki, unpublished observations).

Similar polymers have been prepared by the group of Piskin *et al.*, who found that high molecular weight linear PEI–graft–PNIPAm copolymers were the most effective in transfecting HeLa cells, and a branched PEI–graft–PNIPAm was effective at transport, but relatively poor in transfection [23-25]. Importantly, these studies demonstrated that incorporation of PNIPAm into the PEI-based polymers enhanced cell viability, compared with PEI alone, and in some cases this was without significant decreases in transfection efficiency.

The results from the different studies by the above groups all indicate a possible role of vector architecture and complex compaction/structure, which is affected by phase transition in the formation of complexes with nucleic acids, and also a significant difference in transfection efficiencies with different cell lines. Control of the polymer architecture through increased temperatures above phase transitions has thus been demonstrated to enhance polymer–DNA complex stability

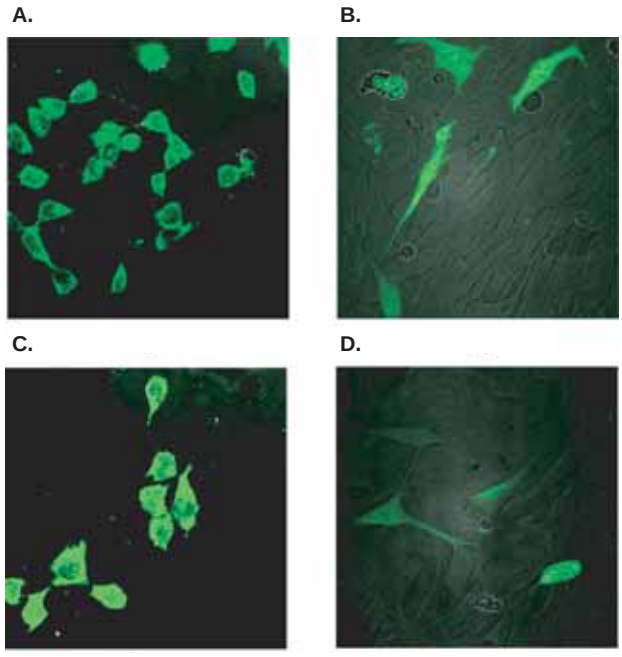

Figure 2. Confocal micrographs of dye-labelled DNA in mouse muscle C2C12 cells after 3 h (A) and 24 h (B) after incubation with PNDHA-1 at 37°C. C and **D.** C2C12 cells showing expression of GFP 24 h after incubation with PEI–PNIPAm-1 with GFP-encoding plasmid for 1 h at 25°C (below LCST), followed by 24 h at 37°C (above LCST). **C.** 1 h at 37°C followed by 24 h at 37°C (**D**).

GFP: Green fluorescent protein; LCST: Lower critical solution temperature; PEI: Polyethyleneimine; PNIPAm: Poly(*N*-isopropylacrylamide); PNDHA: PNIPAm-co-*N*,*N*-dimethylaminoethylmethacrylate polymer.

and intracellular trafficking of DNA *in vitro*. The reverse transition has been shown to promote DNA release in biophysical studies, and experiments have indicated a possibility of promoting DNA release during transfection experiments, although the detailed mechanisms remain elusive. However, it should be noted that the transfection efficiencies of the best responsive PNIPAm systems, when averaged across different cell lines, are still only about the same as commercially available transfection agents, indicating that improvements are still required for *in vivo* application.

3.3 Selected examples of pH-responsive polymers

A number of polymers developed for gene delivery have multiple ionisable functional groups that respond to pH changes. Indeed, many of the polyamine species that are used to form complexes with DNA display multiple pK_a values on account of their polyelectrolyte structure and close proximity of ionisable groups. PEI and PLL are the most widely used polymeric vectors that exhibit pH-responsive behaviour.

bPEI contains primary, secondary and tertiary amines, and the various pK_a values of the amines contribute not only to a strong buffering capacity but also to the ability of PEI to act intracellularly as a proton sponge [26]. Complexes containing bPEI are believed to become more extensively charged in late endosomes as the pH drops (typically reaching pH 5.5, compared with 7.4 in the cytosol) and less basic amine sites on the polymer are protonated. This, in turn, causes an influx of chloride ions, which results in bursting of the endosomal membrane due to osmotic pressure. However, this useful pH-response property may be a cause of the relatively high toxicity of bPEI, even though it is a very good transfection agent. In addition, bPEI is not biodegradable. On the other hand, unmodified PLL is biodegradable, but its transfection efficiency is several-fold lower than PEI; most likely to be due to the presence of only primary amine groups. These sidechain amine groups are fully protonated across the pK_a profile of cytoplasm to endosome (between 5 and 7), and, thus, there is no driving force set up in the endosome that brings in chloride ions to cause subsequent endosomolysis. Modified PLL and other variable pK_a polyamine derivatives are under investigation as better proton sponges to increase the transfection efficiency without increasing toxicity [27,28]. Synthetic polymers that are designed around this concept have included PLL-graft-polyhistidine. The PLL part remains positively charged at physiological pH, whereas the polyhistidine part (pK_a of polyhistidine ~ 6) protonates at endosomal pH, increasing the endosomolytic efficacy.

3.4 Membrane-disrupting pH-responsive polymers

Other pH-sensitive polymers have been developed to enable endosomal release of polymer–DNA complexes without using the proton-sponge effect. These have adopted the concept of viral fusogenic peptides, whereby a pH change causes a conformational change, which then exposes a hydrophobic domain and inserts into the endosomal membrane, forming pores [29-32]. In the case of viruses, pore formation in the membrane enables the viral nucleic acid to escape from the endosome before lysosomal fusion occurs. For synthetic polymers, the pH change must cause a conformation change in the polymer that disrupts the membrane, but this change must only occur when required (i.e., in the endosomes and not in the cytosol).

The pioneering groups of Hoffman *et al.* have investigated the use of poly(ethylacrylic acid), poly(propylacrylic acid) and poly(butylacrylic acid) as membrane-disruptive polymers [33,34]. These polymers are anionic; however, they can be carefully formulated with polycationic systems such that DNA condensation is not adversely affected, whilst retaining their endosomalytic/membrane fusion properties. Poly(ethylacrylic acid) and poly(propylacrylic acid) materials were shown to display a sudden conformational change at pH ~ 5 – 6, and were also able to lyse erythrocytes following a pH decrease from 6 to 5, confirming their membrane-fusion efficacy. Importantly, poly(ethylacrylic acid) was not found to be haemolytic at physiological pH, but became strongly active as the pH dropped. Increasing hydrophobicity of the alkylamide side chains, as with

poly(ethylacrylic acid) and poly(butylacrylic acid), enhanced haemolysis at higher pH and increased haemolytic efficiency at low pH, indicating a clear role for membrane disruption via lipid-like insertion. The same group showed that polymer composition could be varied to cause disruption of lipid membranes at specific pHs: while depending on the electron-withdrawing or -donating properties of the substituents, the pK_a of the polymer could be modulated so that conformational change occurred exactly at the endosomal pH. Other pH-sensitive polymer systems have been developed that contain acid-labile linkages. Polymer breakdown in the acidic environment of the endosome results in DNA release through loss of multiple charge–charge interactions. This is, in effect, a prodrug strategy and more properly falls in the category of triggered, rather than responsive, release. This is beyond the scope of this review. Nevertheless, some very elegant trigger systems have been developed, including acid-degradable moieties, such as acetals [35-37], cis-aconityls [38] and hydrazones [39,40], all of which have been shown to be stable at cytosolic pH but degradable at endosomal pH.

3.5 Encrypted polymers with acid-degradable side chains

A combination strategy has been developed by the groups of Hoffman and Stayton, which uses both triggered and responsive systems [41-43]. The concept involves masking a core cationic, membrane-disruptive polymer with PEG, in order to enhance solubility and provide stabilisation for the complexes in the presence of serum proteins. The PEG chains are conjugated onto the core polymer using acid-labile, acetal or reducible disulfide linkages. These bonds are degraded at the lowered pH of the endosome, unmasking the backbone, which disrupts the membrane (Figure 3).

These polymers were directed to hepatocytes via incorporation of lactose functionality and were found to be active in delivering oligonucleotides and a model macromolecular drug (PEG–fluorescein isothiocyanate) to the cytoplasm of the target cells. These highly sophisticated systems can, in limited ways, be considered as viral mimics as they contain targeting moieties and multiple cell penetration mechanisms. However, they offer considerable advantages over viruses in that their production may easily be scaled up and, of course, they are incapable of infection and replication.

4. Conclusions

Thermo- and pH-responsive polymers are now well established in gene delivery applications. Polymers of increasingly sophisticated chemistries and molecular architectures have been prepared that display responses over narrow temperature and pH ranges and are biologically relevant. Nearly all of the data has been obtained during proof-of-principle *in vitro* studies, but it is likely that *in vivo* studies will commence, once more detailed information concerning the exact mechanisms of the polymer response on DNA release and intracellular trafficking become established. There are still many unanswered questions concerning the escape of DNA from complexes and transport across the nuclear membrane. Other studies aimed at using delivered RNA to alter gene messages are yielding data on polymer–nucleic acid complex behaviour in the cytoplasm, but the added difficulties of DNA transport and expression do require more experimentation.

5. Expert opinion

The ideal gene delivery vector should be exclusively directed to the target tissue and then processed intracellularly. To achieve this, the polymer–DNA complexes must extravasate through the endothelium of the cell, and this is dependent on the size of the polyplexes and the permeability of the endothelia at the target sites. Most tissues have tight endothelia, thus larger complexes (> 150 – 200 nm) will only cross this barrier at organs (i.e., the liver, spleen, bone marrow) with larger endothelial spaces. In most healthy tissues, access to parenchymal cells is denied; however, the larger spaces in certain tumours are capable of being targeted by complexes with a diameter of > 200 nm. Thus, for non-tumour applications, thermoresponsive polymers that are able to reversibly compact DNA into complexes in the sub-200-nm range may be very advantageous in gene delivery. In addition, the enhanced hydrophobic binding above the LCST reduces the need for excess polycation in the complexes. High levels of positive charge at the surface of the complexes lead to non-specific interactions with cellular components, plasma proteins, complement factors, fibrinogen and red blood cells. The residence time of these positively charged complexes tend to be short, as interactions with plasma proteins lead to the formation of large polymer–protein aggregates, which are rapidly cleared from the bloodstream by phagocytic liver cells or removed via accumulation in fine capillary beds. Aggregate formation with blood components, especially erythrocytes, can cause obstruction of blood vessels and result in pulmonary embolism. Polycations can also activate the complement system.

The biodistribution of conventional polycation–DNA complexes is usually very high (80 – 90%) in the lungs immediately after injection. This is because aggregation with erythrocytes or plasma proteins results in trapping at pulmonary capillaries. Stability of these aggregates tends to be low, leading to the release of the polymer–DNA complexes into the circulation, after which they redistribute and show a high concentration in Kupffer cells of the liver. The endothelial tissue of other organs and tissues (e.g., the spleen, kidneys and, in particular, endothelia close to the site of injection) also accumulate significant levels of complexes. The site of gene expression is similar to the pattern of organ distribution. The highest level of gene expression occurs in the lungs, due to increased deposition, as well as more efficient gene expression in these organs. This is useful for the treatment of conditions such as cystic fibrosis and

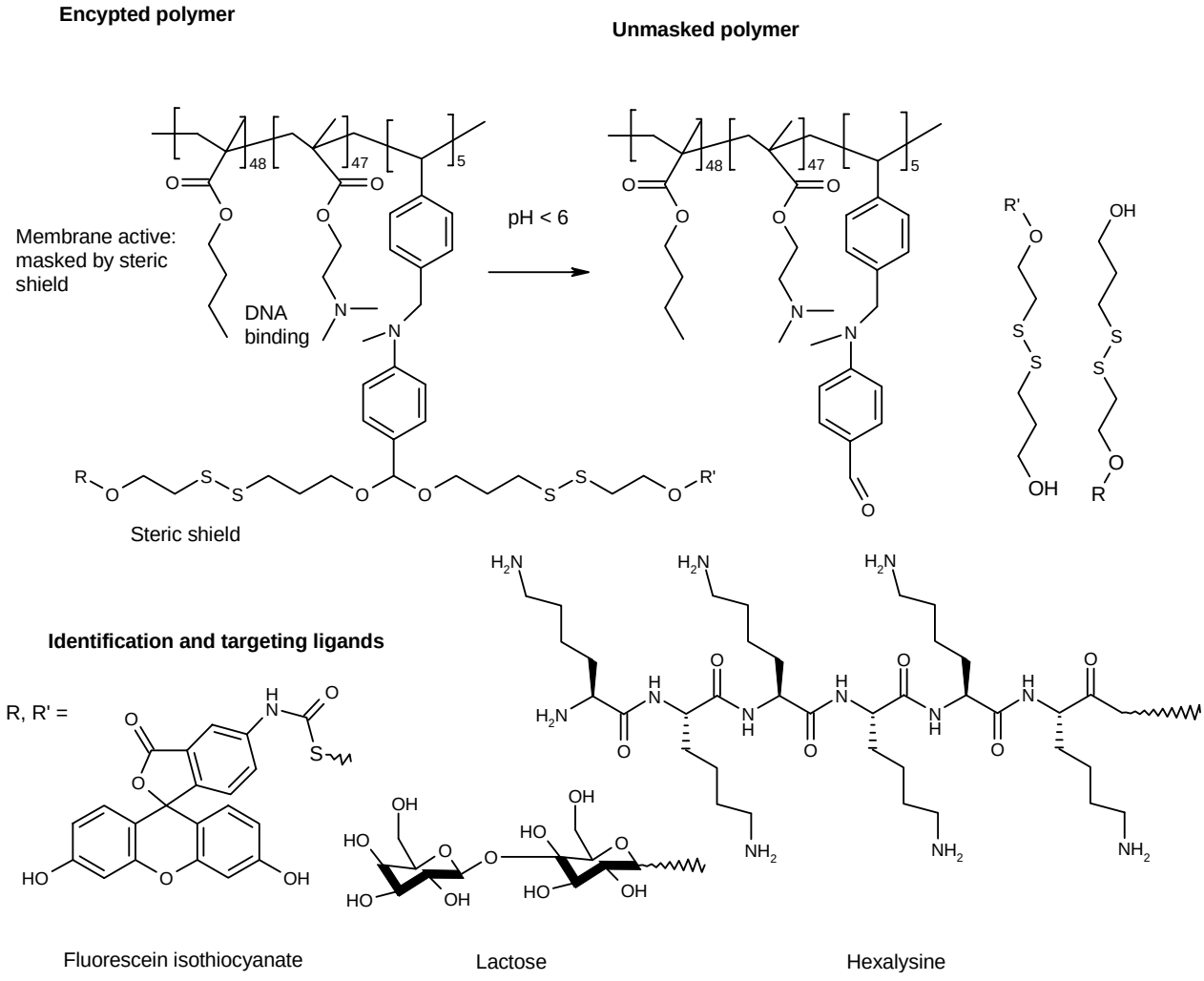

Figure 3. Chemistry and functionality of the pH-sensitive, membrane-disruptive polymeric gene delivery system, developed by Murthy *et al.* [42].

lung cancer, but, for other treatments, it is important that the polymer–DNA complexes can be prepared to be of suitable surface charge and size to be able to prevent aggregation, unwanted deposition at non-target sites and clearance.

For all of these reasons, any vector system that can strongly bind DNA without requiring a high level of polycation would be of significant benefit. The unique ability of a responsive polymer to enhance binding interactions through a mechanism such as increased hydrophobicity, and then to reduce this interaction through a stimulus and response, is perhaps the best way to overcome the above problems.

Responsive polymer strategies offer further possibilities to change the pattern of distribution, limit opsonisation and prolong circulation time of gene delivery vectors. Steric stabilisation via post-complex grafting of hydrophilic polymers to preformed complexes is now being widely adopted to prevent aggregation (e.g., blood components and complement factors). Macromolecules such as PEG, transferrin, and poly(*N*-[2-hydroxypropyl]methacrylamide) have been used for this stabilisation, but a responsive coating may be even more useful, as one can envisage switching the shield on or off, depending on whether the complex is near the cellular target or in transit. Targeting moieties could also be incorporated into the responsive vector, such that the homing signal is only activated close to the target region. The chemistries to do this are not trivial, but the recent demonstration of controlled display of antisense ligands to a polymer chain indicates that the approach is feasible. The ability to direct ultrasound for local hyperthermia, and the known local pH gradients in certain cell compartments and tumours, are also factors that are favouring the use of thermo- and pH-responsive polymers for highly specific therapies.

There is one further aspect to responsive polymers in gene medicine of particular note. The rapid development of

tissue engineering and regenerative medicine offers the prospect of wholly new modes of therapy. A significant practical factor for regenerative medicine will be the ability to deliver cells (the most efficient 'gene factories') to the required locations in the body. Responsive polymers are already being widely used as scaffolds for tissue engineering, and PNIPAm has again been the prototype material. More recent data has hinted that PNIAm may have a degree of biodegradability. For example, Stile *et al*. found that PNIPAm scaffolds for tissue formation showed clusters of cells after 90 days of culture, with large pores formed within the hydrogel [44,45]. This was attributed to aggregating cells deforming the matrix and/or slow hydrolysis of amide bonds within the PNIPAm gel. PNIPAm has traditionally been regarded as non-biodegradable, and, thus, if it were to be demonstrated that it is in fact biocompatible, the many proof-of-principle studies with this polymer may move towards preclinical studies. Of especial interest would be the use of this and related responsive polymers as cell delivery aids, either as cell-coating polymers or as responsive scaffolds. One could envisage a coating of sufficient bioadhesion to attach to a cell surface to provide protection, but able to respond to cell signals such that it either changes its bioadhesivity or simply degrades altogether. Similarly, scaffolds of defined but variable mechanical properties may be prepared that could release cellular cues (e.g., DNA, RNA and cytokines) in response to signals such that the matrix is either strengthened or weakened. These and many other fascinating applications are possible for the next generations of responsive polymers.

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