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Polymers and hydrogels for local nucleic acid delivery

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The potential of gene therapy for the treatment for chronic and life-threatening diseases has been seen for a long time, but widespread applications are still hampered by the difficulties to deliver the highly charged and large nucleic acid molecules to their intracellular targets. More recently, investigators have been aiming for local delivery of nucleic acids mostly by the use of hydrogels. In this way, *in vivo* efficacy can be enhanced by avoiding the target transport challenges and at the same time limit off-target effects. In these systems, nucleic acids are entrapped within hydrogels, either as conjugates or as polyplex particles, for local and controlled release. There are numerous design features in the selection of polymers, for both particle and hydrogel formation that should be considered to achieve efficient local nucleic acid delivery. Therefore, this review focusses on the rational design of polymeric and hydrogel materials for local gene therapy applications.

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1. Introduction

Nucleic acids encode and translate genetic information into proteins that in turn modulate and exert physical, chemical and biological functions. This offers virtually unlimited opportunities for therapeutic interventions. The cellular introduction of gene constructs encoding for therapeutically relevant proteins can in principle cure a number of monogenetic diseases. Moreover, with the discovery of the RNA interference (RNAi) gene silencing mechanism in 1998,[1] therapeutic interventions aimed at modulating and inhibiting pathological protein production gained a lot of interest, especially in the field of cancer treatment.[2–5] However, despite the huge potential, clinical translation of nucleic acid-based therapies has been limited.[6] One of the bottlenecks for successful medical development includes the delivery of the therapeutic nucleic acid molecules to their required cellular site.[7] In addition, achieving therapeutically active local tissue levels of the nucleic acids with acceptable off-target effects remains challenging. To facilitate the delivery and uptake by the target cells, complexation of nucleic acids with polymeric carriers into polyplexes is nowadays a recognized approach.[8–10] In recent years, hydrogels have been developed to act as local nucleic acid-releasing depots, facilitating sustained and efficient nucleic acid delivery.[11–13] Hydrogels are water-swollen cross-linked networks of hydrophilic polymers and have already been widely studied for biomedical and pharmaceutical applications.[14–18] This review will cover design features for the rational selection of polymers, for both polyplex and hydrogel formation to achieve successful local delivery of therapeutic nucleic acids.

1.1 Therapeutic nucleic acids

Therapeutic nucleic acids can act at different stages of the gene expression process, which is depicted in Fig. 1. The first concept of nucleic acids for therapeutic purposes was demonstrated by Zamecnik and Stephenson by the use of synthetic antisense oligonucleotides to inhibit virus growth in cell culture.[19,20] In addition, substitution of defect genes by functional genetic constructs, such as plasmid DNA (pDNA), has been extensively explored to treat genetic and other diseases. pDNAs are large molecules (generally more than 5000 base pairs) and can easily be designed to code for the protein of interest. To achieve translation into the therapeutic protein, pDNA must be delivered into the nucleus of the target cells, which is an extra barrier to overcome compared to other therapeutic nucleic acids which have their action in *e.g.* the cytosol.[21] In addition, the risk of inducing detrimental mutations in the host genome remains a biosafety issue for gene transfer systems that integrate into the genome.[22] Delivery of the product of DNA transcription, messenger RNA (mRNA), which does not require nuclear localization, can provide an interesting alternative strategy. However, mRNA is a single stranded molecule composed of ribonucleotides that is much more susceptible to enzymatic degradation than pDNA.[9] Another way to induce correct protein expression is by interfering with the natural mRNA biosynthesis and function. Mutations can result in mistakes during posttranscriptional

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Wim E. Hennink

Prof. Wim Hennink obtained his PhD degree in 1985 at the Twente University of Technology (The Netherlands) with a thesis on biomaterials research. From 1985 until 1992 he had different positions in the industry. In 1992, he was appointed as Professor at the Faculty of Pharmacy of the University of Utrecht. Currently, he is Head of the Department of Pharmaceutics, a position he took in 1996. From 1997 until 2004 he was European Editor of the Journal of Controlled Release. His main research interests are in the field of polymeric drug delivery systems. He has published ~550 papers and book chapters and holds 20 patents.

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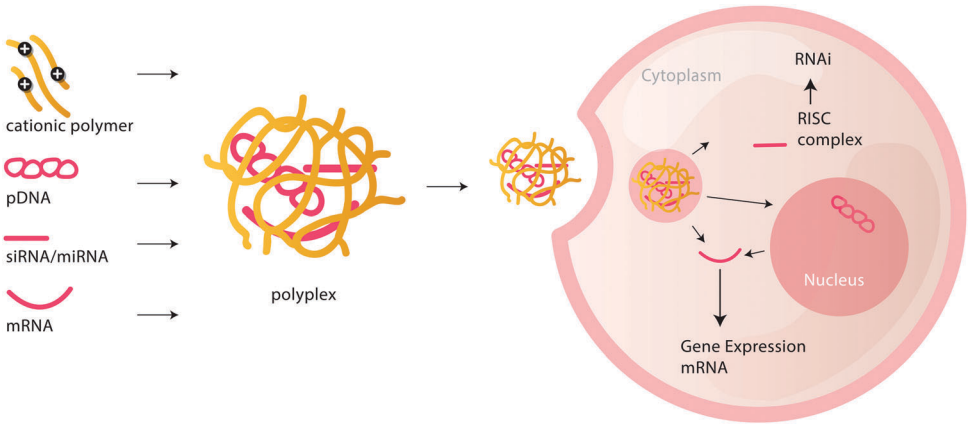

Fig. 1 Schematic representation of the different therapeutic nucleic acids and their intracellular site of action.

mRNA processing, like splicing, resulting in mRNA molecules that are translated into malfunctioning proteins. Exon skipping oligonucleotides or splice correcting oligonucleotides can interfere with this process and restore partly or completely mRNA processing. Such oligonucleotides, for example, have been evaluated for the treatment of Duchenne muscular dystrophy.[23] These strategies are aimed to make a therapeutic or corrected protein.

In contrast, knockdown of gene expression at the mRNA level can be achieved by single stranded antisense oligonucleotides. Antisense oligonucleotides are often DNA-based (because of reduced costs for synthesis and increased stability) and are designed to be complementary to a mRNA sequence of interest. Antisense oligonucleotides can sterically hinder the translation of mRNA into protein by forming a duplex with the mRNA strand.[24] In addition, antisense-mRNA duplexes can also recruit RNase that can cleave the matching mRNA. Finally, short double stranded RNA molecules can induce RNA interference on mRNAs to which they are complementary. They form another class of therapeutic nucleic acids aiming at knockdown of gene expression. This group includes micro RNA (miRNA), short interfering RNA (siRNA) and short hairpin RNA (shRNA), which are all around 18–23 base pairs in length.[25] Delivery in the cytosol of the target cells results in incorporation of one strand into the RNA-induced silencing complex (RISC), which suppresses translation of the target mRNA by mRNA degradation or disassembly of the translation machinery for the bound mRNA.[26,27]

2. Polymer source

2.1 Natural *versus* synthetic materials

Natural polymers are materials occurring in nature and can be extracted from natural sources. The advantage of using natural polymers for biomedical applications is related to their often inherent biodegradable properties and their ability to enhance biological interactions with host tissue.[28–30] The main classes of natural polymers studied for biomedical and pharmaceutical applications include polysaccharides and protein-based polymers. Polysaccharides, such as alginate, dextran and hyaluronic acid, are in general very hydrophilic polymers and therefore suitable for the design of hydrogels for biomedical applications. In addition, polysaccharides have a large number of reactive groups, such as hydroxyl, amine and carboxyl groups, which can be chemically modified to improve their functionality and to provide versatile properties.[31,32] Collagen, fibrin and gelatin are naturally occurring, enzymatically degradable proteins that have also been widely studied as depots for drug delivery purposes.[16,33] However, despite these attractive properties, also some concerns are associated with the use of naturally derived polymers for biomedical applications. The purification of these polymers can be complex, influencing also the batch-to-batch variation and they can potentially be immunogenic, depending on the source from which they are derived. Moreover, due to their natural origin, there is a risk of pathogen transmission.[30] These aspects should be considered when developing novel systems for drug delivery purposes.

The opposite approach is the use of synthetic polymers. Instead of being dependent on the polymer characteristics offered by natural sources from which the polymers need to be extracted, synthetic polymers can be produced with endless varieties under controlled circumstances. Therefore, the main advantage of this strategy is the possibility to control the polymer properties, such as chemical structure and composition, and the ability to tailor the property profiles for specific applications. Tuning of the properties of the polymer can be achieved by using random or block combinations of monomers with specific chemical functionalities in their main chain or in their side groups. Particularly, block copolymers with well-defined architecture are an interesting category in this respect, since various multiphase structures on a nano- and macroscale can be formed when two or more polymeric chains, having different physical properties, are covalently linked to each other. Examples of such block copolymers include the diblock AB type copolymers, which have been extensively reported in the literature.[34–36] In addition, multiblock copolymers, such as ABA and ABC triblock copolymers, have attracted much attention because of their unique structure with multiple (homo)polymer blocks.[37–39] The size and shape of the obtained structures can

be tuned by varying the properties of the blocks, including hydrophobicity, charge and chain length. Moreover, stimuli-responsive polymers can be generated, where the self-assembly behavior of polymers is influenced by an internal biological or external physical trigger.[40,41] Finally, synthetic materials can be designed in such a way that they exhibit good mechanical properties and thermal stability, which are lacking in some of the naturally occurring polymers. For example, high temperatures during processing could destroy the native structure of natural polymers.[28]

2.2 Polymerization techniques

Different polymerization techniques can be used for the synthesis of tailor-made macromolecules with varying structural designs. Commonly used polymer synthesis methods include polycondensation and ring-opening polymerization.[42] Polycondensation reactions involve polymerization of monomers in which small molecules, usually water, are eliminated. In general, low molecular weight polymers are obtained with this technique, due to the difficulty in removing the byproducts completely.[42,43] Polyesters and polyamides are examples of polymers that can be synthesized by polycondensation reactions. However, most polyesters are prepared by ring-opening polymerization (ROP). ROP is a polymerization reaction of cyclic monomers initiated by an active species, which can be of cationic, anionic or radical nature.[44,45] Some important ROP reactions include the synthesis of poly(ethylene glycol), poly(lactic-*co*-glycolic acid), polycaprolactone and poly(phosphazene). Other commonly used synthesis methods include radical polymerization, which is generally tolerant of water and many functional groups. Conventional free radical polymerization (FRP) is widely used to produce polymers with numerous different compositions. However, the architectural control in these polymers is limited, due to the very short lifetime of the growing chains.[46] Because of termination of the growing polymer chains, it is difficult to prepare block copolymers. Furthermore, little control over the molecular weight and relatively high polydispersity indices are usually associated with FRP. The development of controlled/living radical polymerization (CRP) methods has revolutionized the polymer chemistry field and opened the access to well-defined polymers with precisely controlled molecular architectures.[47–49] Reversible addition-fragmentation chain transfer (RAFT) polymerization, nitroxide-mediated free radical polymerization (NMP) and atom transfer radical polymerization (ATRP) are the three fundamental techniques in controlled radical polymerization. Their common feature to gain control over the polymerization reaction is the rapid formation of an equilibrium between a small fraction of active polymerizing chains and a majority of dormant species. Because of this, all chains will grow at the same time resulting in the molecular weight ideally being a linear function of the monomer conversion with a narrow distribution. Finally, the polymer chains are still dormant after polymerization meaning that a second block of a different monomer can be polymerized. This has led to an enormous interest in CRP techniques for the preparation of multiblock copolymers of AB, ABA or ABC type.[49–51]

Today, ATRP and RAFT are the most powerful methods for controlling radical polymerization, as evidenced by numerous patent applications and journal articles published annually. Although RAFT has been shown to be more robust and versatile, selection of the appropriate RAFT agent and reaction conditions is crucial for successful polymerization. Besides having control over the polymer architecture, another advantage of these CRP methods is the possibility to apply chain end-group modifications. Polymers prepared by ATRP contain two chain ends: the α-end, derived from the initiator, and the ω-end, which is typically a bromine or chlorine atom.[52] These halogen end-groups can participate in several nucleophilic substitution reactions, which increases the types of end-functional polymers accessible through ATRP. For example, a one-pot procedure was developed in which the bromine end-group was substituted with an azide functionality, directly after the polymerization reaction. Subsequently, a fluorescent label was covalently attached to the polymer chain *via* a Copper-catalyzed Azide–Alkyne Cycloaddition (CuAAC) 'click' chemistry reaction.[53] The same strategy could be used for example to obtain a cross-linked polymer network. As in the case of ATRP derived polymers, polymers synthesized *via* RAFT can undergo post-polymerization modification reactions to introduce functionality at the ω-end of the polymer chain.[54,55] The thiocarbonylthio group, present at the ω-end of the polymer, can be cleaved *via* a number of methods. Nucleophiles and ionic reducing agents, such as amines, hydroxides and borohydrides, transform the thiocarbonyl containing group into a free thiol, which can be exploited in further coupling reactions.[56]

3. Polymer design for polyplexes

The traditional approach in nucleic acid delivery is encapsulation of the nucleotide cargo in a carrier or complexation with cationic polymers to form a nanoparticle to protect the nucleotide from degradation and to promote cellular uptake (Fig. 2). Since the first application of polymers as non-viral gene delivery systems in 1965 by Vaheri and Pagano,[57] a huge number of different polymers have been developed and compared for application as nucleic acid carriers. The rationale for using these materials for gene delivery is that cationic polymers are able to efficiently condense the, often expensive, negatively charged nucleic acids

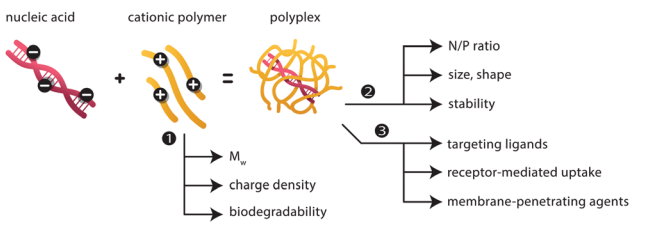

Fig. 2 Schematic representation of polyplex formation between negatively charged nucleic acids and cationic polymers. (1) Polymer characteristics that should be considered for polyplex design. (2) Critical physical parameters influencing polyplex properties. (3) Possible strategies to further optimize polyplex performance.

by electrostatic interactions into a polymer–nucleic acid complex (polyplex).[58] An important parameter in polyplex formation is the N/P ratio, which refers to the ratio of positively chargeable polymer amine (N) groups to negatively charged nucleic acid phosphate (P) groups in the aqueous mixture. Usually an excess of positive charges in the carrier is needed to form polyplexes which adequately encapsulate the nucleotide cargo, resulting in positively charged polyplexes. Besides effective encapsulation, further requirements for an ideal non-viral polymer carrier system include good stability in hydrophilic biological fluids, a low toxicity, a high transfection efficiency, and an appropriate degradation/clearance profile. However, it has proven difficult to develop such a polymer which fulfills all the desired tasks. In addition, some of the requirements, like high stability of the polyplex outside the cell to protect it from degradation while allowing efficient intracellular release of the cargo, appear to be qualities that are difficult to merge within one polymer.[9,21,59]

3.1 Synthetic cationic polymers

The cationic properties of the polymer are clearly of high importance as they form the basis for the interaction of nucleic acid with the polymer. However, balancing the charge density can be tricky due to the compromise between nucleic acid complexation, release as well as the level of cytotoxicity. In general, cationic polymers with high charge densities and high molecular weight are very effective in complexing nucleic acids and the resulting positively charged polyplexes are more readily taken up by cells *via* endocytosis due to interaction with the negatively charged phospholipids of the outer membrane of cells. At the same time, a polyplex with a large excess of positive charges can also cause disruption of the external and internal cellular membrane structures and ultimately cause cytotoxicity. High charge densities are also associated with blood compatibility issues such as platelet aggregation and complement activation.[60–62] Nonetheless, high cationic charge density polymers, including polyethylenimine (PEI), poly-L-lysine (PLL), polyamidoamine (PAMAM), and poly(2-dimethylaminoethyl methacrylate) (PDMAEMA), have all been extensively studied for delivery of nucleic acids with reasonable success (Fig. 3).

3.1.1 Polyethylenimine (PEI). Because of its chemical simplicity, high stability, and high transfection activity, PEI has been widely used as gene delivery carrier, either in branched (b-PEI) or in linear (l-PEI) form.[63] Several studies have attributed the transfection efficiency of PEI to the so-called proton sponge effect.[64,65] This hypothesis describes that after uptake of the polyplexes *via* endocytosis, the biological acidification of the endocytic vesicle (endosome) is counteracted by the fact that the protons that are pumped into the endosome become bound to free basic nitrogens of the PEI chains. The increasing positive charge of the PEI chains requires a concomitant influx of chloride counterions in order to keep electroneutrality, and the increasing osmotic pressure causes an influx of water into the endosome. Because of this osmotic swelling, the endosome can burst and release the entrapped polyplexes into the cytosol. In order to mediate this escape *via* the proton sponge effect, the cationic polymer needs to have a high buffering capacity in the endosomal pH range (from ~5 to 7.4).[66] Although significant evidence for the proton sponge effect was established by several studies,[65,66] the details of this endosomal escape mechanism are still under discussion and contradicted by others.[67,68] Moreover, other mechanisms such as membrane disruption caused by an interaction between the increasingly positively charged polycations and the endosomal membrane have also been proposed. In addition, other researchers claim that free PEI chains play a critical role in promoting gene transfection, depending on the chain length.[69] For PEI a clear relationship between molecular weight and toxicity has been established.[69,70] The primary amines present on PEI also allow for easy functionalization of the polymer with functional molecules, like fluorescent labels.[71–73]

3.1.2 Poly-L-lysine (PLL). PLL is a synthetic polymer obtained by polymerization of the *N*-carboxyanhydride of lysine, having protonable amine groups on the lysine side chain. Similar to PEI,

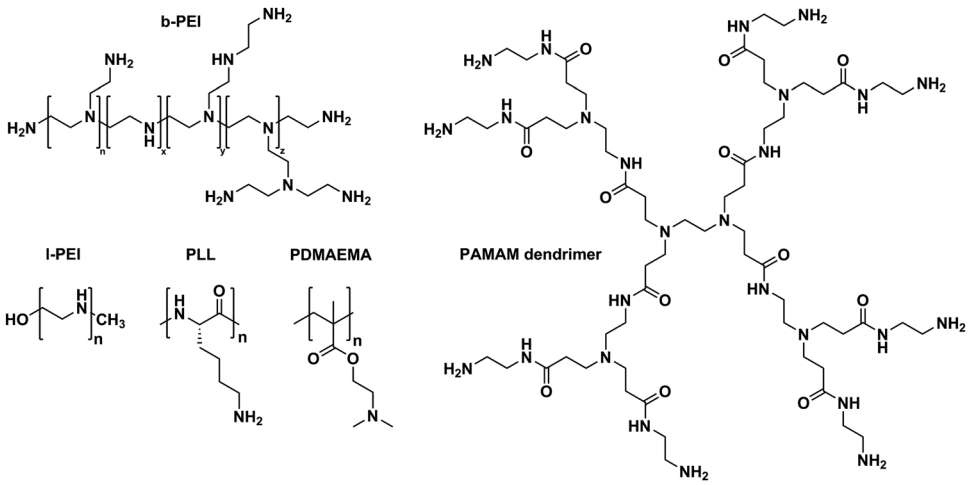

Fig. 3 Chemical structures of commonly used synthetic cationic polymers for nucleic acid delivery. b-PEI: branched polyethylenimine; l-PEI: linear polyethylenimine; PLL: poly-L-lysine; PDMAEMA: poly(2-dimethylaminoethyl methacrylate); PAMAM: polyamidoamine.

PLL is able to condense nucleic acids into nanosized polyplexes. Although PLL polyplexes show good cellular uptake, high transfection efficiencies are usually not reached when compared to PEI polyplexes.[74] At physiological pH, a large majority of the side chain primary amine groups are protonated ($pK_a \sim 10$),[75] resulting in a very low or absent buffering capacity because almost no free amino groups are available to absorb protons. The high permanent charge of PLL can lead to a tight binding between this polymer and nucleic acids and complexes which are not dynamic. Indeed, a study comparing the structural dynamics of PEI/pDNA and PLL/pDNA complexes showed that PEI in the polyplex shell can replace PEI chains in the polyplex core, while PLL cannot.[71] This more dynamic structure of the PEI polyplexes could also help explain the observed differences in transfection efficiency for these two systems. Moreover, since PLL has such a high permanent charge it is also very cytotoxic,[74,76] which has resulted in limited use of PLL as transfection agent.

3.1.3 Poly(2-dimethylaminoethyl methacrylate) (PDMAEMA). Another synthetic cationic polymer frequently used as a carrier and delivery system for nucleic acids is PDMAEMA. This polymer can be easily synthesized *via* radical polymerization of the corresponding vinyl monomer.[77] PDMAEMA contains only tertiary amines, which are approximately 50% protonated at physiological pH. Therefore, sufficient cationic charges are present to allow for effective nucleic acid complexation and high transfection efficiencies can be reached with PDMAEMA-based polyplexes.[77,78] As for all the cationic polymers, transfection efficiency and cytotoxicity are correlated to the molecular weight and for PDMAEMA the optimum was shown to be around 60 kDa.[35]

3.1.4 Polyamidoamine (PAMAM). Polycationic dendrimers, such as PAMAM dendrimers, are also known to be efficient nucleic acid delivery systems.[79,80] These PAMAM dendrimers have primary amine groups on the surface, participating in nucleic acid binding and condensation into small polyplexes. In addition, the tertiary amine groups inside the dendrimers are assumed to act as proton sponges in the endosome and thus promote the intracellular release of the nucleic acid molecules.[81,82] PAMAM dendrimers possess a high charge density, and therefore also show significant cytotoxicity. Linear polyamidoamines were found to have a more favorable cytotoxicity–transfection efficiency balance.[83]

3.2 Natural polymers and their derivatives

Chitosan is obtained by deacetylation of chitin, which is the structural element in the exoskeleton of arthropods, such as crustaceans (crabs, lobsters, shrimps, *etc.*), and cell walls of fungi. This biodegradable polysaccharide consists of D-glucosamine and *N*-acetyl-D-glucosamine units, linked *via* β(1–4) glycosidic bonds[84] (Fig. 4). Chitosan can be obtained in various molecular weights and degrees of deacetylation. Every deacetylated subunit of chitosan contains one primary amine group with a pK_a value of 6.5. Because chitosan can be easily degraded by lysozymes or chitinases in the physiological environment, it is an attractive polymer for use as a carrier for gene delivery. However, the transfection efficiency of chitosan was found to be insufficient,

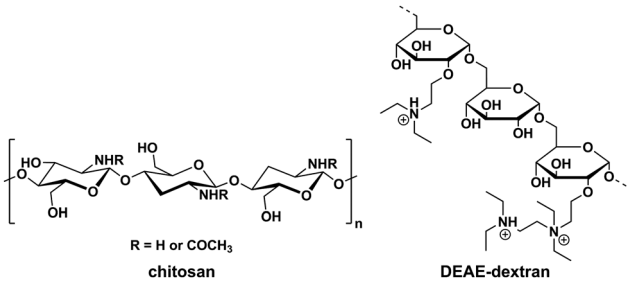

Fig. 4 Chemical structures of naturally derived cationic polymers used for nucleic acid delivery. DEAE: diethylaminoethyl.

which resulted in the development of various derivatives to overcome this issue. For example, galactosylated chitosan was used for enhanced uptake by liver cells[85] and histidine-modified chitosan showed improved endosomal escape.[86] Trimethyl chitosan, in which part of the amines are quarternized resulting in a permanent positive charge, is another chitosan derivative used for gene delivery.[87] Diethylaminoethyl (DEAE)-dextran was one of the first generation cationic polymers used for delivery of nucleic acids. It was reported that the transfection of poliovirus RNA was enhanced by DEAE-dextran, and the transfection of simian virus 40 DNA was even up to 100 000 times increased.[57,88] However, with the expanded knowledge of polymer-based transfection, the focus for polyplex improvement was more directed towards the use of synthetic polymers, such as PEI.

3.3 Optimizing polyplex properties

Introduction of additional functional domains into a cationic polymer can lead to improved properties of the polyplexes. For example, hydrophilic blocks or stimuli-responsive blocks with pH sensitive or thermosensitive properties can be added to the polymer structure. In addition, surface decoration of the polyplexes with targeting ligands can be used as a strategy to enhance cell specificity.

3.3.1 PEGylated polyplexes. One of the most used polymers to improve polyplex properties is poly(ethylene glycol) (PEG). PEG is an important component of various biomedical materials, because of its neutral and hydrophilic properties and generally recognized as safe (GRAS) status. Besides improving water solubility, shielding of the cationic charges of the polyplex with PEG chains is associated with reduced cytotoxicity. Upon complexation of PEGylated cationic polymers with nucleic acids, the PEG chains are exposed on the outside of the polyplex. In this way, the charges are shielded leading to a reduction in the zeta-potential of the polyplexes.[89] It was shown that PEGylation is also important for intracellular delivery of nucleic acids, since it can reduce the interaction of positively charged polyplexes with negatively charged extracellular matrix components.[90] PEGylation of polyplexes is also correlated with longer blood circulation times;[91–93] however, long circulation times are of less relevance for systems aimed for local delivery. As mentioned before, the cationic groups of the polyplexes can interact with the cell membrane facilitating cellular uptake. Complete shielding of the positive charges therefore may result in reduced cellular

uptake and insufficient release of the nucleic acids in the cytosol of the target cells. Besides having an effect on the net charge, PEG may also play a critical role in the formation of polyplexes and the resulting shape of the complex. Kataoka's group investigated the effect of PEG crowding on the packaging of plasmid DNA into polyplexes. PEG-*b*-PLL block copolymers with varying molecular weight of PEG and PLL were used and the results showed that rod-shaped polyplexes were preferentially formed when the PEG chains were dense enough to overlap one another.[94,95] On the other hand, condensed globular-shapes were formed when the PEG density was below the critical value. These results suggest that the steric repulsive effect of PEG influences the packaging of pDNA through folding into either rod- or globular-shaped polyplexes depending on whether the PEG chains overlap or not. This hypothesis was further supported by the preparation of polyplexes with a block copolymer having an acid-labile bond between PEG and PLL. Under acidic conditions, the PEG blocks could be removed from the polyplexes and this resulted in a shape change from rod-shaped particles to compact globular-shapes.[96] However, so far only gene expression was evaluated in a cell-free transcription/translation system, where rod-shaped complexes showed higher gene expression levels compared to globular structures. Additional studies, focusing on polyplex uptake and transfection efficiency, are required to further demonstrate the practical relevance of regulating the packaging of pDNA in these systems. Other researchers have similarly shown that for siRNA-loaded polyplexes the stability and size were clearly influenced by the length of the PEG chain and the PEG density on the cationic polyplex. The studies indicated that at a similar mass content of PEG, higher molecular weight PEG and less grafting density on chitosan favored the polyplex assembly with a smaller size and more condensed siRNA packaging.[92] Also, Mao *et al.* found in their systematic investigation of the effect of PEGylation on the properties of siRNA/PEI polyplexes that higher densities of shorter PEG grafting gave only relatively large complexes (300–400 nm) at a relatively high N/P ratio of 15.[97] PEGylation of cationic polyplexes has resulted in improved stabilization and reduced cytotoxicity of the polyplexes, but at the cost of lower polyplex uptake into cells. To overcome the so-called PEG dilemma, polyplexes with sheddable PEG chains have been investigated.[98] Wagner *et al.* prepared PEI polyplexes coated with the pH-sensitive PEG aldehyde-carboxypyridylhydrazone, *N*-hydroxysuccinimide esters (mPEG–HZN–NHS) or the corresponding stable (mPEG–NHS) reagent. It was shown that a 16-fold enhancement in gene expression with the reversibly shielded polyplexes as compared to stably shielded polyplexes was observed.[99]

3.3.2 PEG alternatives. Researchers have also been looking into alternatives for PEG, as illustrated by the synthesis of poly(hydroxyethyl methacrylate) (PHEMA)-based,[35,100] poly(2-oxazoline)s (POxs)-based,[101] and poly(vinyl pyrrolidone) (PVP)-based[102–104] cationic polymers. It was demonstrated for PDMAEMA that the use of high molecular weight polymers of 300 kDa resulted in high transfection efficiencies,[77] with the lower limit for efficient homopolymers being 43 kDa.[105] In order to improve the biocompatibility and reduce the cytotoxicity of these high molecular weight polymers, a PHEMA backbone was grafted with cleavable short PDMAEMA chains. Indeed, these brushed PHEMA–PDMAEMA graft copolymers showed lower toxicity as compared to the PDMAEMA homopolymer, while still able to transfect cells.[100] In another report, linear diblock copolymers of PHEMA–PDMAEMA were synthesized. It was shown that the addition of PHEMA blocks significantly increased the cellular interaction and transfection efficiency of low molecular weight PDMAEMA, without affecting the cytotoxicity.[35] Besides PHEMA, researchers have also used PVP as a hydrophilic block to reduce cytotoxicity and increase polyplex stability. Various PVP-based cationic polymers have been synthesized, including galactosylated-PEI-*graft*-PVP,[102] galactosylated chitosan (GC)-*graft*-PVP,[103] and PVP-*graft*-PDMAEMA.[104]

3.3.3 Surface decoration of polyplexes. Strategies for further improvement of the efficacy of nucleic acid delivery by polyplexes include the introduction of additional functional domains on the surface of the polyplexes, which is reviewed elsewhere.[9,106,107] To increase the interaction with the target cells and enhance cell specificity, polyplexes can be decorated on their shell with targeting ligands. Several cell-targeting ligands have been successfully incorporated into polyplexes, for example by conjugation of the ligand to the cationic polymer[108] or by post-functionalization of preformed polyplexes.[109] Other strategies are focusing on enhancing endosomal escape and nuclear localization, like the introduction of melittin, a membrane lytic peptide, into the polyplex.[110]

3.4 Type of cargo

Studies on the polyplex delivery of pDNA have resulted in the development of a variety of effective polymeric carrier systems and the knowledge obtained in this area can be beneficially used for the intracellular delivery of small silencing molecules, like siRNA or miRNA. However, the delivery of these smaller oligonucleotides also faces distinct challenges due to the differences in size and stability of the formed polyplexes, as well as the location and mechanism of action.[21] For a therapeutic effect, pDNA should be delivered in the nucleus of the cell, whereas RNA interference molecules exert their effect in the cytosol. One of the important parameters regarding *in vitro* and *in vivo* applications is the size of the polyplexes, as it can influence the mechanisms and specificity of uptake by cells. The polyplex size can be influenced by many factors as demonstrated by the numerous investigations of PEI-based complexes.[9,111–113] For example, it was found that the particle size of complexes of b-PEI and pDNA strongly depends on the charge ratio, where large particles of 1 μm were formed at N/P 2.5 and small polyplexes of 92 nm were formed at N/P 20.[112] The size of particles formed with l-PEI was less dependent on the used charge ratios. Similar trends were observed with siRNA formulations; however in general smaller polyplexes are formed with siRNA molecules compared to pDNA. Besides particle size, polyplex stability is crucial for their successful delivery, and this is one of the reasons for the different performance of a given polymeric carrier in pDNA or siRNA delivery. PEI is recognized as

an effective carrier and transfection agent for pDNA delivery, either in branched or in linear form.[113] Polyplexes of b-PEI and b-PEI with pDNA and siRNA were systematically compared in terms of size, charge, stability and transfection efficiency.[112] It was shown that complexes of b-PEI/pDNA were more stable than l-PEI/pDNA, since the latter ones could be completely dissociated with a 4-times lower amount of heparin. However, too stable polyplexes can result in lower transfection efficiencies, because of insufficient release of the nucleic acids from the complexes in the cytosol. The stability of b-PEI/siRNA complexes was similar to that of l-PEI/pDNA, whereas l-PEI/siRNA polyplexes were much more sensitive to heparin, a highly negatively charged polysaccharide.[111,112] This instability of l-PEI/siRNA complexes could also explain the lack of an efficient gene silencing effect. The less effective electrostatic interactions between the small oligonucleotides and cationic polymers are a common problem to achieve sufficient complex stability. In a recent study, it was suggested that the rigidity of double-stranded siRNA molecules (compared to single-stranded miRNA or mRNA compounds) is a critical parameter in polyplex formation.[114] The use of different cationic polymers for mRNA delivery has also been evaluated, especially since mRNA has been shown to be promising in the development of cancer vaccines.[115] Studies have demonstrated that in general PLL- and PEI-based polyplexes are too stable to release mRNA molecules to enable efficient translation.[116–118] Decreasing the electrostatic interaction by using low molecular weight PEI (2 kDa) resulted in the need to add chloroquine, an agent that enhances endosomal escape, to mediate mRNA expression. By conjugating the membrane-active peptide melittin to PEI, high levels of mRNA expression were demonstrated in the absence of chloroquine.[116] Another study investigated the use of histidine-rich reducible cationic polymers to increase mRNA transfection efficiency.[117] In conclusion, in order to select the appropriate polymer carrier for efficient delivery of nucleic acids, the type of therapeutic molecule used should also be considered.

3.4.1 Nucleic acid conjugates. As discussed above, delivery of nucleic acids into the target cells is promoted by using a polyplex carrier system. Alternatively, specially designed nucleic acid-conjugates can be used, which can be applied as non-complexed 'naked' molecules. These molecules are modified in such a way that transfection can take place in their naked form. A detailed overview of these chemical modification strategies can be found elsewhere.[119] One of the most commonly used modifications includes attachment with cholesterol. Several reports have shown that cholesterol modification of siRNA and miRNA can facilitate the uptake of these RNA interference molecules into cells resulting in effective gene silencing.[120–122] Other modifications include methylation or fluorination on the 2′ hydroxyl of the pentose ring and the introduction of phosphothiorate bonds between the nucleotides. Although these modifications can promote cellular uptake and improve stability, the presence of a carrier system can further enhance cellular entry and increase selectivity to the target cells.

4. Polymer design for hydrogels

Recently, hydrogels have been developed to facilitate local and sustained release of nucleic acids to reduce side effects and increase *in vivo* efficacy (Fig. 5). Various hydrogel characteristics, *e.g.* mechanical properties, degradation behavior, and release kinetics, are directly related to the choice of polymers that make up the network and the cross-link density, and therefore, selection of the appropriate building blocks is essential. Although some requirements are the same for all hydrogel delivery systems, such as low immunogenicity and low toxicity, others are more specific to the desired therapeutic application. Hydrogel administration can be performed either *via* surgical implantation or, preferably, *via* injectable formulations. In the latter case, the polymers should be designed in such a way that they can be injected and possess efficient *in situ* gelation behavior. For nucleic acid delivery, other features like compatibility of the hydrogel building blocks with the nucleic acids are important, and especially electrostatic properties play a significant role in this respect.

4.1 Charged hydrogels

Since nucleic acids have anionic charges and delivery approaches are based on complexes with cationic transfection agents, electrostatic properties of the hydrogel network are important to consider. For example, electrostatic interactions between polymers from the hydrogel network and the nucleic acids or polyplexes will have an effect on the release kinetics. Therefore, anionic hydrogels, such as alginate- or hyaluronic acid (HA)-based networks, are not the first materials of choice for nucleic acid delivery. A rapid release of the naked nucleic acids from alginate hydrogels[123,124] and HA-induced aggregation of the polyplexes resulting in reduced transfection[125] have been reported. On the other hand, hydrogels consisting of cationic polymers have the advantage that they can condense nucleic acids and promote transfection. For example, chitosan-based hydrogels have been studied for the local delivery of siRNA into tumor tissue.[126] Mice injected with siRNA-loaded chitosan hydrogels, targeting transglutaminase, showed a 48% reduction in tumor growth compared to control non-targeting siRNA/chitosan gels.[126] Hydrogels with a neutral charge are the most often studied systems since they do not directly have an electrostatic interaction with the polyplexes. In order to tune the release of the encapsulated polyplexes, the cross-link density of the hydrogel network as well as the degradation kinetics has to be optimized. It is assumed that the release of polyplexes from hydrogels is not governed by diffusion (they are simply too large) but rather by degradation of the matrix. Different cross-linking strategies are discussed in more detail in the next sections.

4.2 Cross-linking mechanisms of injectable, self-assembling hydrogels

The use of *in situ* forming hydrogels is often preferred over preformed hydrogels, because gelation can take place under physiological conditions upon injection without the need of an invasive surgical intervention. In addition, injectable hydrogels

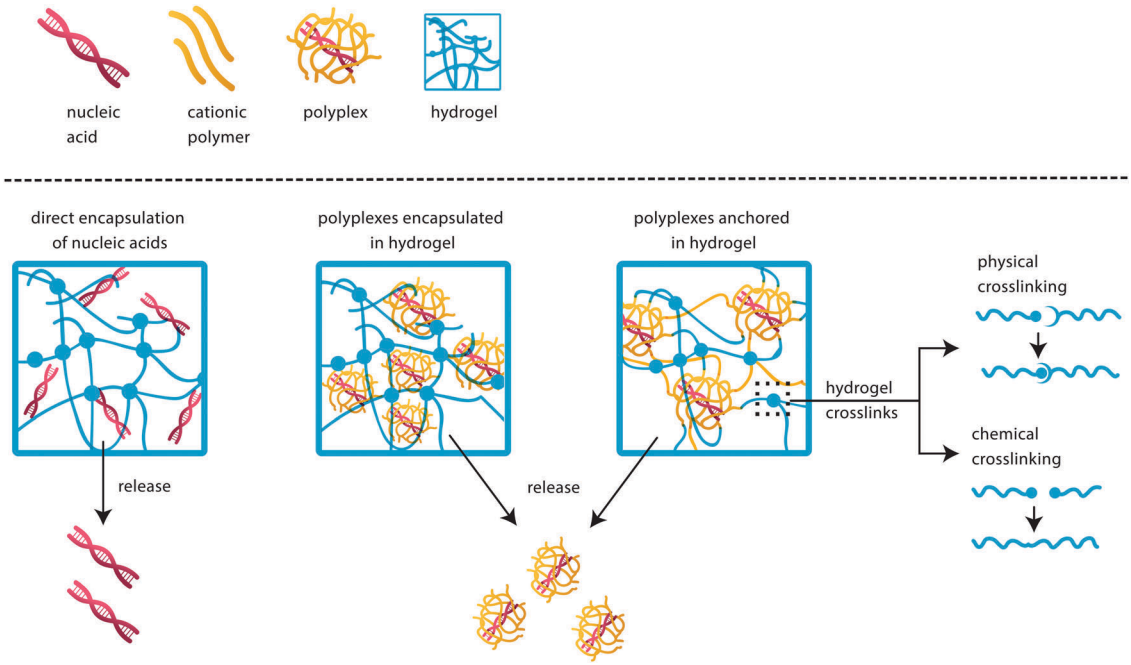

Fig. 5 Schematic representation of hydrogels releasing drug-loaded polyplexes for local delivery of nucleic acids.

may also access areas which are otherwise difficult to reach. Various strategies can be followed for the design of such self-assembling hydrogels, which rely on the selection of the type of cross-linking mechanism. Hydrogels can be physically cross-linked by noncovalent interactions or chemically cross-linked by covalent bond formation, or *via* a combination of both.[127] A special category of hydrogels is represented by the stimuli-responsive systems, or so called 'smart' hydrogels, which will be discussed in Section 4.4.

4.2.1 Physically cross-linked hydrogels. Hydrophobic interactions, ionic interactions and hydrogen bonding are the most commonly used interactions to form physical cross-links, which can be used under mild conditions, and are reviewed elsewhere.[15,16] Other interesting groups are represented by complexes formed by host–guest interactions (polymer inclusion complexes) and stereocomplexes.

4.2.1.1 Cross-linking by polymer inclusion complexation. Supramolecular hydrogels based on polypseudorotaxane formation between cyclodextrins (CDs) and polymers have attracted attention as injectable delivery systems. Cyclodextrins are cyclic oligosaccharides with an internal hydrophobic cavity, which can form a complex with linear polymer chains, such as PEG. Li *et al.* described such a cyclodextrin-based supramolecular hydrogel system for the controlled delivery of pDNA (Fig. 6). The triblock copolymer methoxy-poly(ethylene glycol)-*b*-poly(ε-caprolactone)-*b*-poly(2-dimethylaminoethyl methacrylate) (mPEG–PCL–PDMAEMA) was used to prepare pDNA polyplexes.[128] Upon addition of PEG (10 kDa) and α-CD, hydrogel assembly occurred, where the mPEG in the polyplex corona served as an anchoring segment for the polyplexes. Complete release of the complexed pDNA was observed after 6 days of incubation in PBS at 37 °C, compared to 100% release of free pDNA after 3 days. In a similar approach using mPEG–PCL–PEI as triblock copolymer, supramolecular hydrogels containing pDNA, encoding for the Nur77 protein to inhibit the overexpressed anti-apoptotic protein Bcl-2, were injected into tumor-bearing mice to reduce tumor growth.[129] After 7 days, the tumor volume was ~135 mm^3 for the nucleic acid-loaded hydrogel group, compared to ~687 mm^3 for the control saline group, indicating significant tumor tissue regression. The use of a supramolecular hydrogel to deliver MMP-9 shRNA plasmid to cancer cells was studied by Lin and coworkers. They synthesized a PEGylated arginine-functionalized poly-L-lysine dendron (mPEG–PLLD–Arg) to allow for shRNA condensation.[130] Through host–guest interactions with α-CDs, hydrogels were formed under mild conditions. The anti-tumor efficacy was evaluated in mice and the results showed that the one-time injection of the hydrogel system resulted in a comparative anti-tumor effect of a seven-time injection of PEI/MMP-9 plasmid polyplexes.[130] These results indicate that indeed such hydrogel systems have the potential to act as sustained release depots for gene delivery. Besides α-CD, β-CD has also been used to develop injectable, host–guest assembled hydrogels for local delivery of siRNA.[131] Branched PEI (25 kDa) was modified with β-cyclodextrin and this functionalized cationic polymer (CD-PEI) was used to allow for complexation with siRNA. As a complementary polymer, 8-arm PEG-maleimide (20 kDa) was functionalized with adamantane (AD-PEG), which is known to interact with β-CD through supramolecular chemistry to form an inclusion complex.[132] Hydrogels were formed upon mixing of CD-PEI with Ad-PEG, in the presence of siRNA to ensure encapsulation of the nucleic acid molecules (Fig. 7). When injected into rat myocardium, the localized hydrogels released polyplexes, as indicated by the uptake of Cy5.5-siRNA

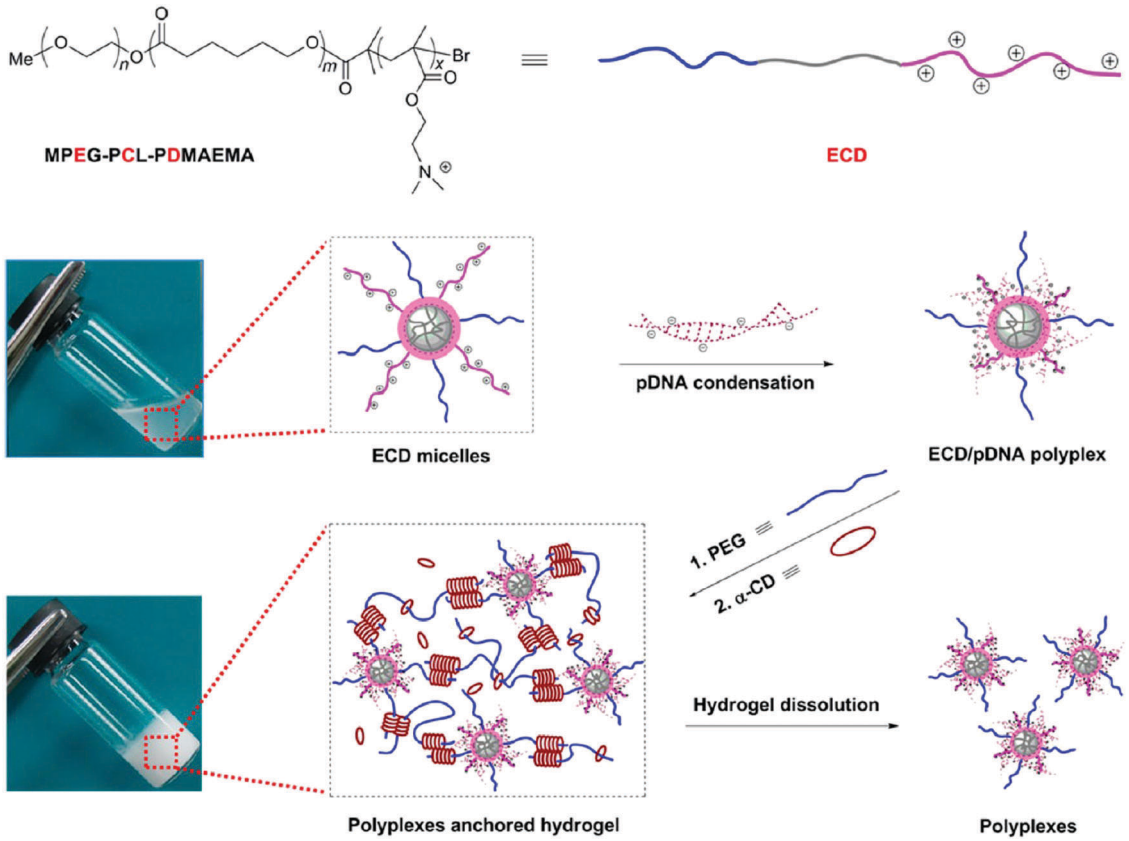

Fig. 6 Design consideration of polyplexes anchored in α-CD/PEG supramolecular hydrogels. Amphiphilic mPEG–PCL–PDMAEMA triblock copolymers (ECD) form micelles in an aqueous environment with a PCL core and mPEG/PDMAEMA corona. pDNA is complexed with PDMAEMA at the corona, while mPEG serves both as a stabilizing moiety for the ECD/pDNA polyplex and as a hydrogel anchoring segment. Addition of free PEG and α-CD resulted in hydrogel formation. Sustained release of pDNA polyplexes was achieved *via* hydrogel dissolution over time. Reprinted with permission from ref. 128. Copyright 2012 American Chemical Society.

and the silencing of GFP for 1 week in a GFP-expressing rat.[131] In a follow-up paper, a similar strategy was used for the localized delivery of miRNA molecules towards functional cardiac regeneration after an ischemic injury.[122] A mixture of CD- and AD-modified hyaluronic acids was injected together with cholesterol-modified miR-302 to allow for controlled release, since it is described that cholesterol can form inclusion complexes with cyclodextrins.[133] *In vivo* injection of miR-302-loaded hydrogels into a mouse model induced cardiomyocyte proliferation and resulted in recovery of the cardiac function.[122]

4.2.1.2 Cross-linking by stereocomplexation. Stereocomplexes refer to the formation of crystals upon mixing of two polymer enantiomers, which are mirror images of each other. This interaction has been exploited to create physically crosslinked hydrogels, and frequently used polymer enantiomers are poly(L-lactide) (PLLA) and poly(D-lactide) (PDLA). De Jong *et al.* first described this gelation mechanism for the development of protein loaded hydrogels,[134] where PLLA and PDLA were coupled to hydrophilic dextran polymers. In a more recent study, hydrogels were formed with a micelle mixture of two enantiomeric triblock copolymers, PLLA–PEG–PLLA and PDLA–PEG–PDLA, respectively.[135] It was shown that this stereomixture of micelles possesses a tunable sol-to-gel transition between 25 and 37 °C, depending on the length of the PEG mid block.[135,136] Taking it a step further, Mohammadi *et al.* described the formation of such a hydrogel system for the delivery of pDNA. In the first step, triblock copolymers PLLA–PEI–PLLA and PLLA–PEG–PLLA were used to form three-layered micelles (3LM) (Fig. 8). These 3LM were able to encapsulate pDNA in their core while they were shielded with PEG to increase the polyplex stability.[137] Next, complementary micelles consisting of PDLA–PEG–PDLA were prepared and mixed with the 3LM to form a hydrogel network at 37 °C.[135,137,138] It was shown that fluorescently labeled pDNA, released from hydrogels incubated in an acidic environment (pH 4.5), was taken up by macrophages *in vitro*. Nevertheless, convincing evidence showing that intact and bioactive polyplexes were released from the hydrogels has not been provided yet.

4.2.2 Chemically cross-linked hydrogels. Hydrogels based on only physical interactions are often easily degraded or rapidly dissolved, and this problem can be solved by the introduction of chemical cross-links. In addition, chemically cross-linked hydrogels usually have better mechanical properties, which may be relevant for certain applications. Various types of covalent cross-linking mechanisms have been used in the hydrogel field,

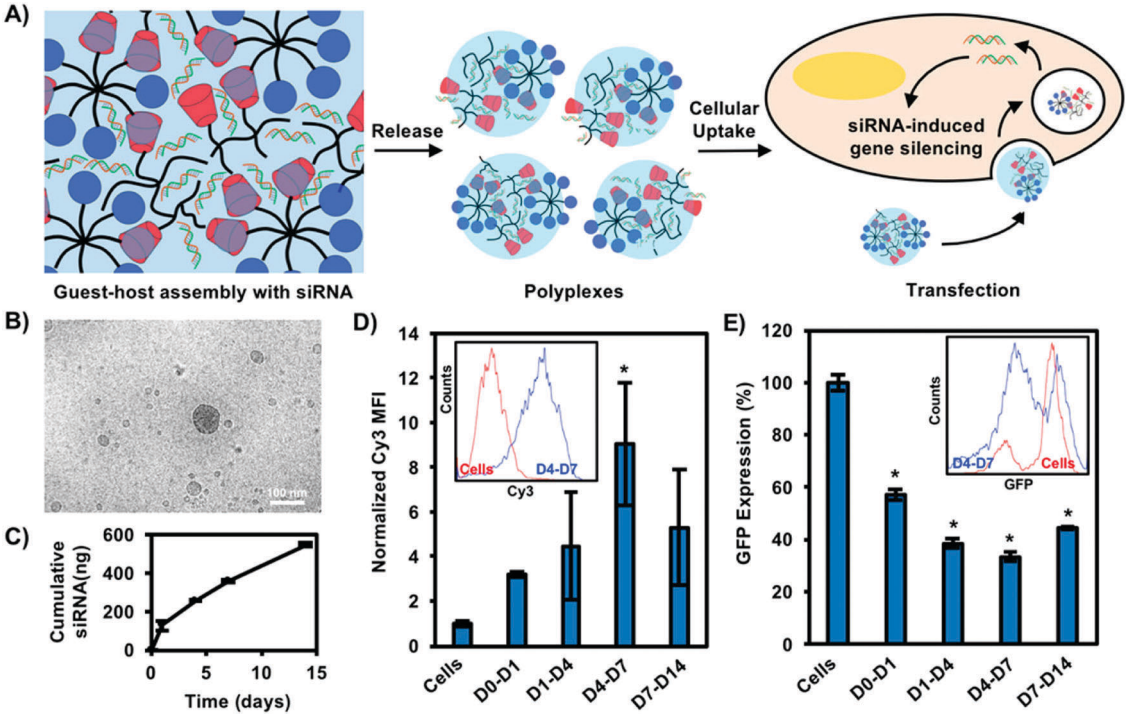

Fig. 7 Host–guest assembled hydrogels for local delivery of siRNA. (A) Schematic representation of hydrogel assembly with siRNA. Adamantine functionalized 8-arm PEG (blue) was mixed with β-cyclodextrin functionalized PEI (red) and siRNA, to form cationic gels in which siRNA is sustained through electrostatic interactions. Polyplexes released from the gel network are able to transfect the target cells. (B) Cryo-TEM images of siRNA polyplexes in release medium. Scale bar = 100 nm. (C) Cumulative siRNA release from the hydrogels over two weeks. (D) Cy3-siRNA transfection from release medium collected over two weeks in human fibrosarcoma cells quantified by flow cytometry. *$p < 0.05$ compared to untreated cells. (E) siGFP gene silencing from release medium collected over 2 weeks in GFP-expressing endothelial cells quantified by flow cytometry. *$p < 0.05$ compared to cells alone. Reprinted with permission from ref. 131. Copyright 2017 American Chemical Society.

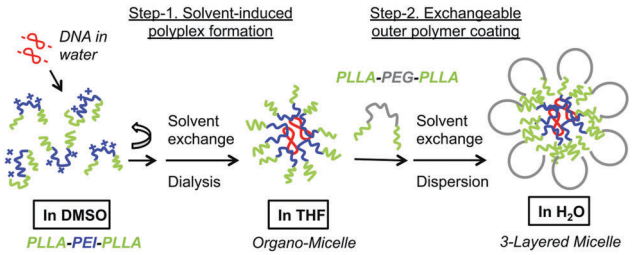

Fig. 8 Schematic preparation scheme of pDNA-loaded three-layered micelles (3LM). Step 1: polyplex formation by the solvent-induced process to yield the organomicelles in THF. Step 2: assembly of the PLLA–PEG–PLLA outer copolymer to form aqueous stable 3LM. Reprinted with permission from ref. 137. Copyright 2015 Wiley-VCH Verlag GmbH & Co. KGaA.

including click chemistry, photopolymerization and addition reactions, and have been reviewed elsewhere.[16,17,139,140] The ones relevant in nucleic acid delivery are described here.

4.2.2.1 Cross-linking by photopolymerization. Photopolymerization is a form of radical polymerization initiated by the decomposition of a photosensitive compound, called a photoinitiator, upon exposure to UV or visible light. This cross-linking reaction allows for hydrogel formation when hydrogel precursors have polymerizable groups, such as acrylate or methacrylate moieties. For example, siRNA delivery was studied using a photo-crosslinkable alginate hydrogel.[123] However, siRNA was rapidly released over one week from the hydrogel system, most likely due to the anionic properties of the alginate gel that accelerated the release. Important to consider for photo-crosslinkable systems is the limited penetration depth of UV radiation in living tissue and the potentially damaging effects of the generated radicals on the encapsulated nucleic acids. Therefore, *in situ* forming hydrogels utilizing covalent cross-linking between polymers with complementary functional groups have received more attention in the last few decades.

4.2.2.2 Cross-linking by Michael addition reactions. Michael addition reactions have become a very attractive strategy to introduce cross-links in hydrogel systems, because these chemical reactions can occur under mild conditions, namely in aqueous medium at room or body temperature, and at physiological pH. The reaction includes the addition of a nucleophile on an activated carbon–carbon double bond.[141,142] PEG-based hydrogels represent a good example of gel networks which can be formed *via* Michael type reactions by *e.g.* mixing two PEG derivatives with α,ω-functionalized thiol and acrylate groups, respectively. By introducing hydrolysable groups in the cross-links and changing the number of these functional groups, physical properties, like degradation behavior, could be tuned. In a publication of Nguyen *et al.*, degradation rates and thus the

release kinetics of encapsulated polyplexes were tailored by varying the density of the degradable ester linkages in the hydrogel network. Polyplexes consisting of branched PEI (25 kDa) and siRNA, targeting GFP and luciferase silencing, were prepared at N/P 10 and encapsulated in different PEG-based hydrogels.[143] For this, 8-arm PEG (10 kDa) was modified with either mono(2-acryloyloxyethyl) succinate (MAES) or acryloyl chloride (A) to obtain PEG-MAES and PEG-A, respectively. Hydrogel formation was achieved by mixing 8-arm PEG-SH (10 kDa) with the modified 8-arm PEG-MAES and/or PEG-A with a 1 : 1 stoichiometry ratio of acrylate and thiol groups to obtain a final concentration of 15% (w/v) (Fig. 9). The release rate of the polyplexes from the M gels (PEG-MAES + PEG-SH) was faster compared to that from the A and MA gels (PEG-A + PEG-SH and PEG-A + PEG-MAES + PEG-SH, respectively). After 19 days, more than 85% of the loaded siRNA was released from the M gel, while the A and MA gels released more than 90% siRNA over 42 and 35 days, respectively.[143] This was explained by differences in the concentration of degradable ester linkages in the macromolecular networks. On each arm of the 8-arm-PEG-M, three hydrolysable ester groups were present compared to one ester group on each arm of the 8-arm-PEG-A. Therefore, the faster degradation of the M gels was expected as a result of their higher ester density compared to the A and MA gels, which also resulted in a faster release of the polyplexes (Fig. 9b and d, respectively). The capability of the released siRNA/PEI complexes to silence GFP expression was assessed in HEK293 cells. Release samples from all hydrogel formulations

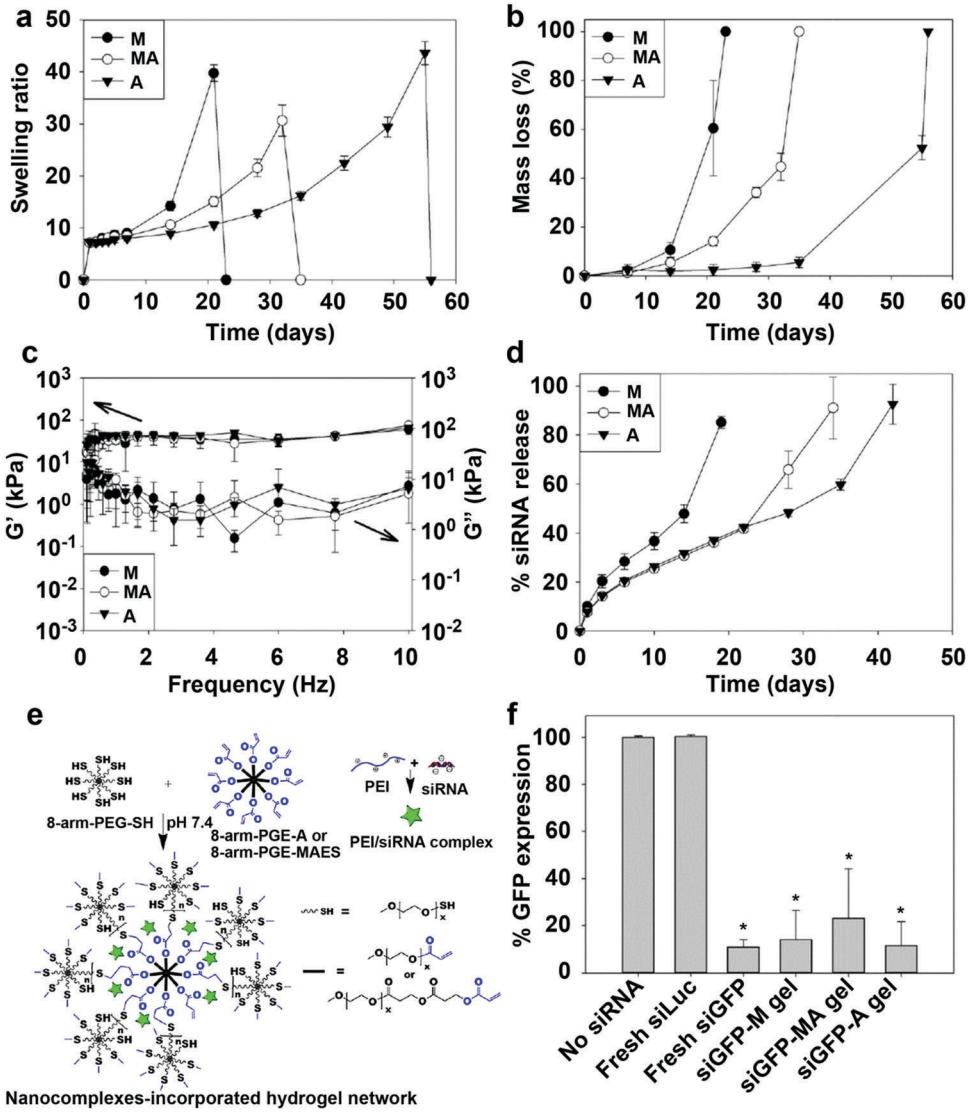

Fig. 9 The physical properties of the three degradable hydrogel systems were examined through measurement of their (a) swelling ratio, (b) degradation profiles and (c) rheological properties. (d) Release profiles of the siRNA/PEI polyplexes from the three different hydrogel systems. (e) Schematic representation of incorporation of siRNA/PEI polyplexes into the hydrogels. (f) Bioactivity of siRNA/PEI polyplexes released from the three hydrogels compared to freshly prepared polyplexes and non-targeting siRNA control (siLuc). *$p < 0.05$ compared with the no siRNA control group. Reprinted with permission from ref. 143. Copyright 2014 Elsevier Ltd.

showed ~80% GFP silencing, which was comparable to the silencing activity from freshly prepared polyplexes. These results indicate that indeed polyplexes were released from the hydrogels which still possess bioactivity.

4.2.2.3 Cross-linking by Schiff-base reactions. Schiff's reaction chemistry can be exploited to design *in situ* forming hydrogels in which amine and aldehyde groups are able to react and form an imine bond in aqueous solutions at physiological pH.[127,144] In addition, the imine bond is stable at physiological pH but labile at mildly acidic pH. In a publication by Segovia *et al.*, arginine modified-poly(β-aminoesters) (PBAEs) were used to form particles with nucleic acids (Fig. 10). To achieve sustained delivery of siRNA, the nanoparticles were embedded in hydrogels based on polyamidoamine (PAMAM) dendrimers cross-linked with dextran aldehyde. The Schiff-base reaction between aldehyde groups of oxidized dextran and terminal amines of PAMAM dendrimers resulted in a cross-linked network, which showed siRNA-loaded particle release for 12 days.[145] Interestingly, the polyplex release followed two phases, in which 30% of the encapsulated siRNA was released within 24 hours. Next, a more sustained release was observed reaching 100% release after 12 days.[145] Most likely, free non-encapsulated siRNA present was released first, since it is able to diffuse out of the hydrogel network, followed by release of the nanoparticles as the hydrogel degrades. Hydrogels loaded with anti-luciferase siRNA were implanted into a xenograft mouse model of human breast cancer. Tumor-associated luciferase expression was reduced by 70% at 6 days with siRNA-loaded hydrogels, whereas a single nanoparticle injection led to only 20% reduction in luciferase expression.[145] In follow-up reports, the same PAMAM-dextran aldehyde hydrogel was used for the co-delivery of two miRNAs, both having anti-cancer properties.[146,147] Hydrogels containing the two miRNAs were able to inhibit tumor progression, with almost 90% reduction in tumor size 13 days after hydrogel implantation.[146]

4.3 Protein-based hydrogels

Collagen and gelatin are biopolymers that have gained broad interest in the preparation of various biomaterials, because of their good cellular interactions and biodegradability.[31] Collagen is a fibrous protein, which can be found predominantly in connective tissues in the body, and gelatin is produced from collagen *via* partial hydrolysis. Gelatins are now also produced by biotechnological routes and have shown excellent opportunities for delivery of pharmaceutical proteins.[148] Fibrillar collagen hydrogels can be formed by entanglement of collagen fibers, but the thermal and mechanical stability of these protein-based hydrogels is relatively low.[33] Their performance can be improved by chemical modifications, and typically, glutaraldehyde or water-soluble carbodiimides are used to introduce covalent cross-links in these hydrogel systems.[149,150] Although collagen and gelatin hydrogels have been mainly exploited in tissue engineering, at present only a few studies exist in which such protein-based hydrogels are used for the local delivery of nucleic acids. Peng *et al.* encapsulated PEI/siRNA polyplexes in a type 1 collagen hydrogel for the treatment of gastric cancer. It was shown that polyplex-loaded hydrogels resulted in 60% siRNA-mediated

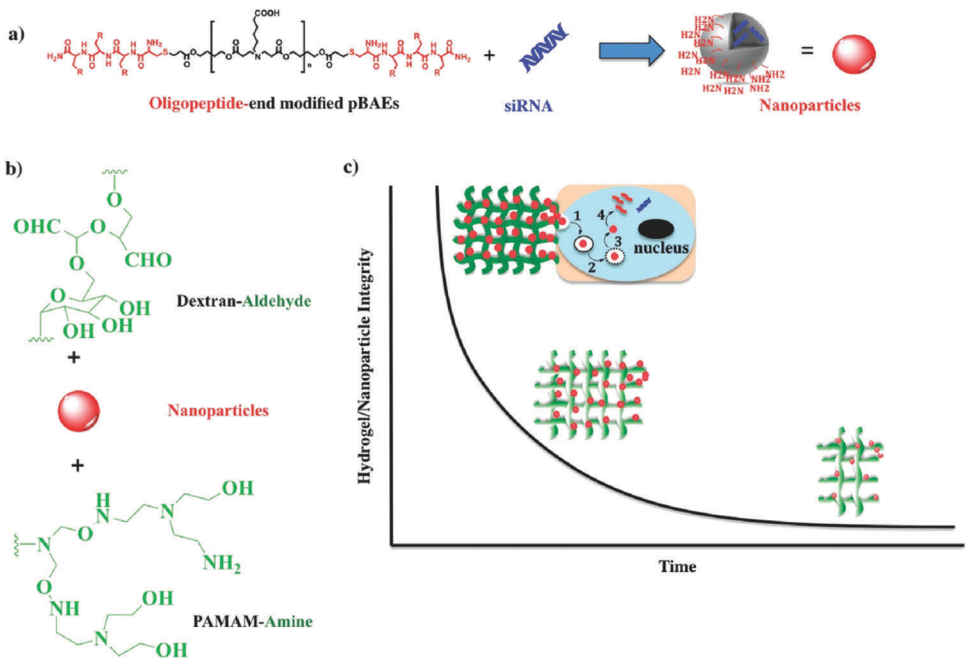

Fig. 10 Schematic representation of siRNA-loaded particles hydrogel system, cross-linked by Schiff-base reactions, for local and sustained release of siRNA. (a) Particle formation of arginine modified-poly(β-aminoesters) (PBAEs) and siRNA. (b) Hydrogel formation by Schiff-base reaction between aldehyde groups of oxidized dextran and terminal amines of PAMAM dendrimers, and encapsulation of siRNA-loaded nanoparticles. (c) Hydrogel disintegration with time, and subsequent siRNA-loaded particle release. Reprinted with permission from ref. 145. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA.

knockdown of Id1, which is involved in growth and migration of gastric cancer cells.[151] In a xenograft mouse model, gastric cancer cells were injected hypodermically together with the hydrogel formulations and after 4 weeks the tumors were examined. A four-fold decrease in tumor size was observed for siRNA polyplex-loaded collagen hydrogels compared to siRNA alone (Fig. 11). In addition, immunostaining of cellular targets of Id1 siRNA was in line with gene silencing activity. However, direct effects of the hydrogel formulation, like immune activation, or cytotoxic effects of cationic polymers themselves, could also play a role in the observed anti-tumor effect. This is not further discussed by the authors, and control formulations with only PEI/collagen could further support the conclusions. In another study, PAMAM dendrimers were used to form complexes with siRNA targeting Snail1, which is a transcription factor involved in tumor metastasis.[152,153] Subsequently, cross-linked collagen scaffolds were freeze-dried and reconstituted with the polyplex dispersion to obtain loaded scaffolds. The ability of a siRNA/PAMAM complex-loaded scaffold to control protein expression was evaluated by measuring Snail1 levels from NIH3T3 cells seeded on the scaffolds. A significant down-regulation of Snail1 gene expression was measured at both mRNA and protein expression levels.[153] Saito and coworkers used a gelatin-based hydrogel for the controlled release of siRNA. Here, gelatin was cationized by introducing ethylene diamine onto the carboxyl groups under different conditions to obtain varying cationization extents.[154] Polyplexes were formed with luciferase siRNA and cationized gelatin (CG) and mixed with non-cationized gelatin at a high concentration (20 wt%) to form the hydrogels, which were chemically cross-linked using glutaraldehyde. After 24 hours incubation at 37 °C in PBS, approximately 40% siRNA was released and a plateau was reached. After addition of collagenase, complete release of the loaded siRNA was measured within another 24 hours. Although gene silencing was observed when Colon26-Luc cells were incubated with the hydrogel supernatant, much higher transfection efficiencies were seen with freshly prepared polyplexes.[154] This might indicate that free siRNA was released from the hydrogels, instead of siRNA in a complex form. The collagenase added might not only result in hydrogel degradation, but also destabilize the siRNA/CG, which was not further discussed by the authors.

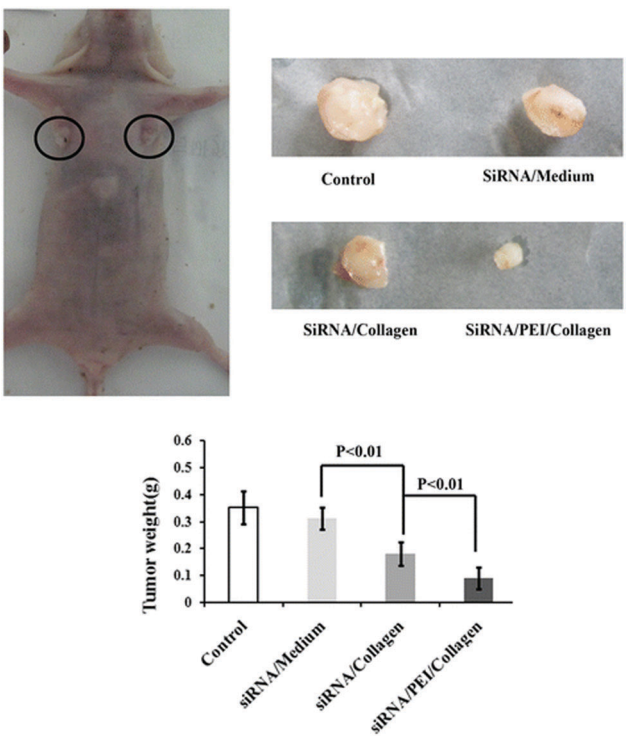

Fig. 11 Inhibitory effect of siRNA alone, siRNA encapsulated in collagen gels and siRNA-PEI polyplexes encapsulated in collagen gels on tumor growth in a xenograft mouse model. Top: Macroscopic images of tumors after 4 weeks. Bottom: Tumor weight after different treatments after 4 weeks. Reprinted with permission from ref. 151. Copyright 2016 Peng *et al.*

4.4 Stimuli-responsive 'smart' hydrogels

As mentioned above, polymers for hydrogels can be engineered in such a way that they can respond to external environmental triggers, like temperature, pH or light exposure. An applied stimulus can either trigger the assembly of the materials to form a hydrogel system, or, conversely, cause degradation of the network and subsequent release of the encapsulated polyplexes and nucleic acids (Fig. 12). The triggered nucleic acid release can provide an alternative mechanism for controlled delivery, allowing the release of desired doses at specific times. In this section, research on the role of these 'smart' hydrogel systems in nucleic acid delivery will be highlighted.

4.4.1 Temperature-sensitive hydrogels. Temperature-sensitive hydrogels are one of the most studied class of stimuli-responsive polymer systems. Self-assembly of this kind of polymers occurs at a critical temperature, the so-called cloud point (CP) in aqueous solution, due to hydrophobic interactions which results in phase separation. In general, thermosensitive behavior of these polymers is thought of as a balance between hydrophilic and hydrophobic moieties on the polymer chain.[155] When these thermosensitive polymers are combined with a permanently

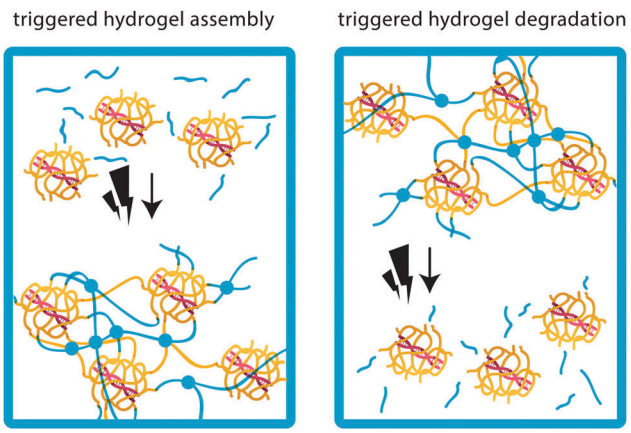

Fig. 12 Schematic representation of stimuli-responsive materials for triggered assembly into a hydrogel or triggered degradation resulting in polyplex release.

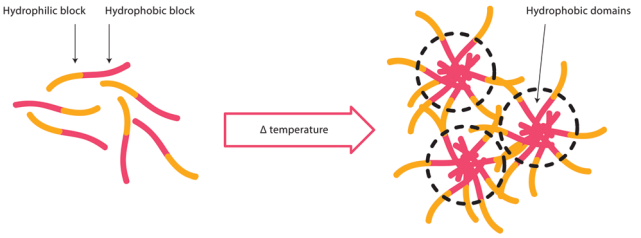

Fig. 13 Schematic representation of self-assembly of thermosensitive polymers into hydrogel networks.

hydrophilic block (*e.g.* PEG) in the form of an amphiphilic block copolymer, self-assembly into supramolecular structures, such as hydrogels, will occur spontaneously upon heating[156] (Fig. 13). Thermosensitive amphiphilic block copolymers having a lower critical solution temperature (LSCT) between room temperature and body temperature are very attractive for the preparation of materials for biomedical applications. At low temperatures these block copolymers are soluble, allowing for easy injection, and hydrogels are formed *in situ* upon increase to body temperature after administration. Among the family of these temperature responsive polymers, poly(*N*-isopropylacrylamide) (PNIPAM) is one of the most widely studied polymers. Its attractiveness is mainly due to the fact that PNIPAM has a LSCT in water of approximately 32 °C.[157] The gelation behavior of amphiphilic block copolymers with PNIPAM can be easily tuned by copolymerization with other monomers.[157] Many different block copolymers have been synthesized with PNIPAM for different purposes and having additional chemical cross-linking mechanisms.[158,159] Interestingly, so far only a few studies have reported on the use of such PNIPAM-based hydrogel systems for nucleic acid delivery[3,160,161]

Poly(D,L-lactic acid-*co*-glycolic acid)-*b*-poly(ethylene glycol)-*b*-poly(D,L-lactic acid-*co*-glycolic acid) (PLGA–PEG–PLGA) triblock copolymers are known to self-assemble into micelles with hydrophobic PLGA cores and hydrophilic PEG shells in aqueous solution at lower temperatures.[162] By increasing the temperature, a sol-to-gel phase transition occurs due to the increase in the hydrophobic interactions of PLGA blocks and partial dehydration of the PEG shell.[162,163] In a study by Ma *et al.*, such a thermosensitive PLGA-based hydrogel was investigated for the release of both shRNA/PEI polyplexes and doxorubicin (DOX) to treat osteosarcoma. PEI was used to form a complex with shRNA targeting PLK1, which is involved in tumor growth. Next, PLGA–PEG–PLGA polymers, DOX, and the polyplexes were mixed in aqueous solution and intratumorally injected in tumor-bearing mice to form drug-loaded hydrogels *in situ*. After 16 days, *ex vivo* analysis of the tumor mass showed around 35% silencing of PLK1 expression when treated with shRNA/PEI-lys hydrogels.[163] Tumor tissue analysis suggested that PLK1 mRNA levels were significantly lower in mice treated with hydrogels containing both doxorubicin and shRNA.

As an alternative to encapsulate polyplexes into hydrogels, the research group of Song proposed a system in which siRNA-loaded polyplexes directly assemble into hydrogels upon a temperature trigger. For this, l-PEI–poly(organophosphazene) conjugates were synthesized to enable hydrogel formation at body temperature.[12] Release data showed that Cy5.5-labeled siRNA was completely released from the gel in 28 days *in vitro.* In addition, siRNA targeting Cyclin B1, a regulator of the cell cycle, was encapsulated into hydrogels and upon intratumoral injection reduced tumor growth at 30 days was observed.[12] In follow-up papers, PEI was replaced with the cell penetrating peptide protamine to optimize the intracellular delivery of the polyplexes[164] or the PEI–poly(organophosphazene) polymers were modified with folate to specifically target the folate receptor (FR).[165] A selective uptake of folate-targeted polyplexes in the FR-over-expressing human breast cancer cell line compared to the FR-deficient human lung carcinoma cell line was observed.[165] It should be noted that the *in vivo* anti-tumor effect was not compared with the non-folate targeted polymers, which would be essential to demonstrate the beneficial effect of folate targeting. The most recent report evaluated the use of PEI–poly(organophosphazene)-based hydrogels for the dual delivery of siRNA and docetaxel (DTX).[166] Release of siRNA from the dual-loaded hydrogel was detected up to 40 days,[167] compared to 28 days in earlier reports of a similar hydrogel system without DTX.[12]

4.4.2 Light-sensitive hydrogels. Externally applied stimuli for triggering nucleic acid release can provide an alternative mechanism for controlled delivery, allowing the release of desired doses at specific times and sites. Light has been widely applied for control over the release of drugs as it can regulate the degradation of the polymer backbone of nanoparticles or linkages between polymers and bioactive agents.[168–170] Hunyh *et al.* engineered PEG-based hydrogels with photodegradable groups to allow for light-mediated degradation and subsequent release of siRNA complexes (Fig. 14). In this work, poly(ethylene glycol)–di(photolabile acrylate) (PEG–DPA) was synthesized containing two *ortho*-nitrobenzyl photolabile groups, which are known to be converted into acetal and acid groups upon UV light exposure.[11,171] Hydrogels were formed *via* free radical polymerization of PEG–DPA and the cationic molecule 2-amino ethyl methacrylate (AEMA) in the presence of ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) as redox initiator and catalyst, respectively. AEMA molecules were built in the hydrogel network to permit encapsulation of siRNA silencing GFP. Release of siRNA complexes could be tuned over two weeks by varying the exposure time and UV intensity.[11] Moreover, up to 80% GFP silencing was observed when HeLa cells were treated with released medium from siRNA-loaded hydrogels exposed to UV light. In another report, this approach was further developed for the co-delivery of siRNA and miRNA oligonucleotides.[171] Here, 8-arm PEG-thiol was reacted with PEG–DPA by Michael addition to yield networks in which siRNA/miRNA/PEI polyplexes were loaded by dispersing them in the solution of hydrogel precursors. It was shown that the released complexes of Noggin siRNA and miRNA-20a induced osteogenic differentiation of human mesenchymal stem cells to a similar extent as freshly prepared polyplexes.[171] This report demonstrated that such a photodegradable hydrogel system can provide 'on-demand' delivery of nucleic acid/PEI complexes, without affecting the bioactivity of the released RNA molecules.

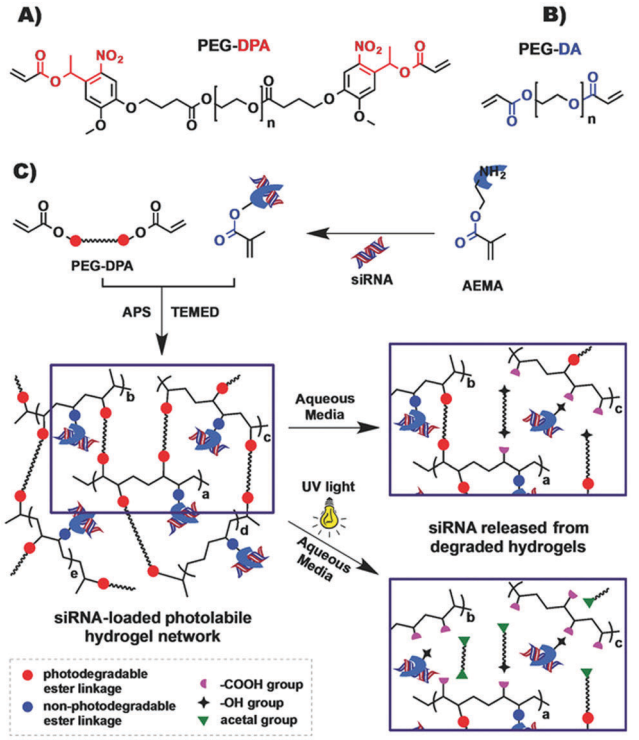

Fig. 14 UV light-triggered siRNA delivery through hydrogels formed from poly(ethylene glycol)–di(photolabile acrylate) (PEG–DPA) polymers. (A) Structure of photolabile PEG–DPA. (B) Structure of non-photolabile PEG-DA. (C) Hydrogels were formed *via* radical polymerization in the presence of 2-amino ethyl methacrylate (AEMA) to allow for siRNA complexation. Subsequently, siRNA was released upon degradation of the hydrogel network in aqueous media in the absence and presence of an external UV light source. Reprinted with permission from ref. 11. Copyright 2015 Wiley-VCH Verlag GmbH & Co. KGaA.

4.4.3 Enzyme-sensitive hydrogels. Another elegant example of recent development in smart hydrogels involves the design of peptide cross-linking resulting in enzymatically degradable networks. In this way, proteolytic enzymes secreted by surrounding cells determine the rate of degradation of the hydrogel and thus the release of nucleic acid-loaded polyplexes. In a recent report, polycationic 2-(diethylamino)ethyl methacrylate-*co*-*tert*-butyl methacrylate (DEAEMA-*co*-*t*BMA) nanogels (~100 nm diameter) were prepared and used to form complexes with siRNA targeting TNF-α.[172] Subsequently, the siRNA-loaded nanogels were encapsulated within a poly(methacrylic acid-*co*-*N*-vinyl-2-pyrrolidone) [P(MAA-*co*-NVP)] hydrogel cross-linked with a trypsin-degradable peptide linker. Upon addition of trypsin, the network degraded and the released particles were capable of inducing knockdown of TNF-α levels in murine macrophages, demonstrating the potential for such an enzyme-sensitive delivery approach.[172]

5. Discussion and outlook

As outlined by the presented examples in this review, the potential to achieve local and controlled delivery of nucleic acids by the use of hydrogels is evident. The applicability of this technology is promising for a broad field of gene delivery, as hydrogels can be tailored with various properties to address specific needs. This is demonstrated by the diversity of systems used to obtain *in vivo* anti-tumorigenic effects, improved cardiac function, and enhanced bone healing. Injectable hydrogels exhibit advantages towards clinical translation compared to implantable ones that require surgical interventions. Especially, the stimuli-responsive 'smart' hydrogel networks are expected to significantly influence the field of local nucleic acid delivery. Nevertheless, several limitations and challenges need to be overcome before clinical reality can be reached, which are not fully addressed in the literature.

Many systems that demonstrate *in vivo* release of polyplexes from hydrogel systems are proof of concept, leaving diverse fundamental and development questions still unanswered. First of all, an improved understanding of the release of polyplexes from hydrogels and subsequent uptake by target cells is needed. More specifically, understanding of how hydrogel bulk material properties affect the polyplex stability and release is of utmost importance. It has been reported in the literature that polyplexes can aggregate within hydrogels during hydrogel formation, resulting in deactivation of the carrier system.[173,174] Especially, when high concentrations of nucleic acids are loaded this phenomenon occurs,[175,176] which can limit clinical translation where sufficient amounts of nucleic acids are required for therapeutic efficacy. It highlights the importance of understanding the interactions that occur between polyplex and hydrogel for the design of such nucleic acid delivery systems. The group of Segura proposed a method called 'caged nanoparticle encapsulation' to overcome the aggregation problem.[175] For this, neutral saccharides (sucrose) and polysaccharides (agarose) were added before lyophilization to coat and stabilize the polyplexes. It was shown that coating of the polyplexes, only with both sucrose and agarose, prevented inactivation and aggregation during encapsulation within the hydrogel and up to 5 μg pDNA per μL hydrogel could be loaded.[175]

Furthermore, the structure and size of the released particles should be more carefully examined and compared with non-encapsulated ones. For example, many studies use DNA/RNA quantification methods to report *in vitro* release of nucleic acids from hydrogels. However, such results do not show the release of intact nanoparticles, which is required for intracellular nucleic acid delivery. In addition, the release of destabilized particles and thus the presence of free cationic polymers can influence the observed results, especially in studies investigating the potential of polyplex/hydrogel systems for anti-tumor treatments. It has been shown that cationic polymers have a pronounced anti-tumor effect themselves,[177] and not always is the control formulation cationic polymer/hydrogel included in these studies. Before encapsulation into hydrogels, polyplexes are typically characterized in terms of size and ζ-potential with analytical techniques like dynamic/electrophoretic light scattering (DLS/ELS) and nanoparticle tracking analysis (NTA). Analyzing release medium containing both released particles and hydrogel components is difficult with these techniques since they detect both polyplex and particulate degradation products of

the hydrogel matrix. Therefore, more effort should be made into developing methods to demonstrate particle release, instead of only nucleic acid quantification. For example, fluorescent labeling strategies could help in this respect.

Finally, local nucleic acid delivery *via* hydrogels raises certain questions related to issues such as biocompatibility, immunogenicity and medical safety. Inflammation and severe foreign body responses on the hydrogel systems themselves should be avoided. Interactions that take place at the tissue/material interface play a critical role in determining the level of biocompatibility. Different parameters like surface characteristics, chemical composition and degradation products of the hydrogels influence such interactions. In addition, degradation rate and clearance of the materials should also be included in further investigations, particularly in *in vivo* settings. Thus far, these considerations have received little attention in the research field, and should be addressed to move forward from proof-of-concept towards clinical implementation.

To summarize, with the need for advanced delivery systems for effective gene therapies and the continuous progress in the hydrogel technology field, it is likely that the role of hydrogels in nucleic acid delivery will expand and reach clinical applications.

Conflicts of interest

There are no conflicts to declare.

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