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Published in:
Journal of materials chemistry b

DOI:
[10.1039/c7tb02239e](https://doi.org/10.1039/c7tb02239e)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Ekkelenkamp, A. E., Elzes, M. R., Engbersen, J. F. J., & Paulusse, J. M. J. (2018). Responsive crosslinked polymer nanogels for imaging and therapeutics delivery. *Journal of materials chemistry b*, 6(2), 210-235. <https://doi.org/10.1039/c7tb02239e>

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Cite this: *J. Mater. Chem. B*, 2018, 6, 210

Received 21st August 2017,
Accepted 30th November 2017

DOI: 10.1039/c7tb02239e

rsc.li/materials-b

Responsive crosslinked polymer nanogels for imaging and therapeutics delivery

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Water-soluble, nano-sized crosslinked polymer networks, or nanogels, are delivery vehicles, which have highly interesting properties for therapeutic delivery and imaging. Nanogels may also possess responsive properties, depending on the employed polymers, allowing controlled release of therapeutics or image contrast generation upon exposure to physical or (bio)chemical cues. In this review, polymer nanogels are explored for application in imaging as well as for controlled drug and gene delivery. Moreover, nanogels are explored as responsive biomaterials and future applications are highlighted.

1 Nanomedicine

Technological advances in the past few decades have fueled a revolution in the world of medicine. New technologies, such as sophisticated biomaterials,¹ advanced biomedical imaging,² gene editing³ and targeted medicine⁴ enable detailed monitoring and controlling of physiological processes. Therefore, diagnostics and therapies are dealing increasingly with processes

occurring on a cellular level and consequently have also increased the need for diagnostic and therapeutic tools acting on the molecular level.^{5,6} Solutions to fulfill these needs are offered in the field of nanomedicine, where these problems are approached from a nanotechnology perspective.^{5–7} The application of macromolecules and polymers in nanomedicine is widespread, whether as a stabilizer for *e.g.* inorganic nanoparticles⁸ or as a nanocarrier.⁴ Due to their tremendous variability and versatility, macromolecular nanostructures play a pivotal role in the areas of controlled drug delivery and imaging.^{7,9} Nano-sized macromolecular architectures, such as polymer nanospheres,^{4,9} polymersomes,^{10,11} dendrimers^{12,13} and polymeric micelles,^{14,15} show great variety in structure, physical properties and ample opportunities for modular functionalization.¹⁶ Their variability in size (Fig. 1) and physiochemical properties^{17,18} makes these structures suitable for a wide range of biomedical applications.^{7,9}

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Fig. 1 Macromolecular nanostructures and their size-range.

However, combining biocompatibility and stability under physiological conditions in an efficient and stable macromolecular nanocarrier still remains far from trivial.¹⁹

Hydrogels have biocompatibility and stability on a large scale and similarly, hydrogel nanoparticles, or nanogels, may hold a similar potential.^{20–22} Nanogels are nano-sized aqueous cross-linked polymer networks and are, like hydrogels, highly biocompatible materials with high loading capacities for therapeutics, and can be synthesized through several strategies. Moreover, appropriately designed nanogels, with their unique physical properties and size, ranging from tens to hundreds of nanometers, are able to remain in the bloodstream for extended periods of time, to penetrate targeted tissues, visualize a diseased area, or release a therapeutic cargo in response to a local trigger. Stability is an important factor for *in vivo* applications, and can be provided by chemical crosslinks within the nanogel. Furthermore, the hydrophilic nature of nanogels makes the nanogel network accessible for (bio)-chemical stimuli, uniquely permitting the incorporation of reversible responsive properties into the polymer network. Therefore, crosslinked nanogels show tremendous potential in therapeutic and diagnostic applications.^{23,24}

2 Nanogels

The formation of large intramolecularly crosslinked macromolecules was recognized as early as the late 1940's in synthetic

rubber emulsions.²⁵ However, nano-sized hydrogels did not emerge until the mid-1980's,²⁶ and the potential for controlled release of therapeutics was indicated in the first decade of this century.²³ Similar to hydrogels,²⁷ nanogels can – depending on their chemical composition – respond to stimuli in a reversible or irreversible manner. The incorporation of degradable or responsive moieties endows the nanogel network with responsiveness. Moreover, in their aqueous state, nanogels may swell or shrink in response to reversible chemical processes (Fig. 2A), owing to repulsive and attractive forces within the nanogel network, respectively.²³ As a consequence, nanogels can be designed to be responsive to physical stimuli, such as pressure,²⁸ ionic strength,²⁹ temperature^{30–32} and pH.^{33–36}

Nanogels have tunable mechanical properties depending on their crosslinking density (Fig. 2B) and have been shown to markedly influence the cellular uptake and biodistribution properties of nanogels.^{23,37,39} *In vitro* studies with phagocytotic cell lines have shown that nanoparticles with a higher elastic modulus are taken up at a higher rate than their more rigid counterparts.⁴⁰ Whereas *in vitro* experiments on different cell types do not always show this trend,⁴¹ *in vivo* studies uniformly show that nanoparticles with increased elasticity are more slowly removed from the bloodstream as compared to more rigid nanoparticles.^{37,39,41,42} This is attributed to lower macrophage uptake and possible flow effects.⁴¹ Theoretical modeling of cellular uptake of nanoparticles has shown that the energy barrier to engulf elastic nanoparticles is higher,⁴³ while the same model also shows increased uptake of receptor-targeted elastic particles.⁴⁴ This has further been demonstrated through *in vivo* studies with poly(ethylene glycol) (PEG)³⁹ and zwitterionic poly(carboxybetaine) (pCB) nanogels,³⁷ which showed that nanogels with a lower crosslinking density were increasingly retained within the blood circulation, as depicted in Fig. 3. This suggested that the lower particle rigidity decreased the tendency towards particle uptake by immune cells and subsequent removal from the blood stream. It was recently shown by Haag and coworkers that nanogels are even able to penetrate the skin

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Jos M. J. Paulusse

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2 start-up companies and chairs the biennial European Symposium on Controlled Drug Delivery. His research interests include novel drug delivery systems and hybrid imaging modalities.

Fig. 2 Swelling of nanogels is influenced by the nanogels' composition, such as presence of weakly acidic and basic groups, the crosslinking density and incorporation of thermoresponsive moieties (A). The crosslinking density also influences the nanogel's swelling ability and particle rigidity (B). Jiang *et al.* illustrated this with 250 nm gels with high crosslinking density, which could not pass through a 0.22 μm syringe filter (shown on the left), whereas 250 nm gels with a low crosslinking density did successfully pass (shown on the right). Moreover, incorporation of thermoresponsive moieties in the nanogel matrix causes the nanogel to collapse upon heating above the volume-phase transition temperature (VPTT) (C). (A) Reproduced from ref. 23. (B) Reprinted (adapted) with permission from ref. 37. Copyright 2012 American Chemical Society. (C) Reprinted (adapted) with permission from ref. 38. Copyright 2012 American Chemical Society.

deep into the stratum corneum, which was attributed to the hydrophilic nature of the employed nanogels as well as their mechanical properties.^{45,46}

2.1 Nanogel formation

Nanogels can be obtained *via* various synthetic routes, ranging from emulsification to self-assembly. Several methods to obtain covalently crosslinked nanogels with different mechanical properties and responsiveness are discussed below. Physically

crosslinked nanogels can also be obtained,⁴⁷ but are outside the scope of this review.

2.1.1 Water-in-oil emulsions. A commonly applied technique for nanogel preparation is radical (co)polymerization in water-in-oil emulsions, which can be categorized into inverse (mini)emulsions and microemulsions.^{48,49} An aqueous solution of monomers and bifunctional crosslinkers is emulsified in a nonpolar solvent, resulting in a surfactant-stabilized emulsion of monomer-containing water droplets in an oil phase and subsequently the monomer-containing aqueous phase is polymerized.

Fig. 3 Depending on their crosslinking density, and consequently their rigidity, nanogels show differences in blood circulation times, as demonstrated with PEG (A) and pCB nanogels (B). PEG nanogels with a low crosslinking density (circles) were more slowly removed from the bloodstream, than their counterparts with higher crosslinking density (squares) (A). Similarly, zwitterionic pCB nanogels with decreasing mechanical stiffness: 1.35, 0.87, 0.58, 0.26 and 0.18 MPa, denoted by pCB 15%, pCB 10%, pCB 5%, pCB 2% and pCB 2%-, respectively (B) showed retention of all pCB nanogels for extended periods in the bloodstream, although lower crosslinking densities correlate with longer retention in the bloodstream. (A) Reprinted (adapted) with permission from ref. 39. Copyright 2015 American Chemical Society. (B) Reprinted (adapted) with permission from ref. 37. Copyright 2012 American Chemical Society.

After polymerization and subsequent removal of the oil phase surfactants and unreacted monomer, a crosslinked polymer network is obtained, which can be resuspended in water as a nanogel.^{50–53} Therefore, emulsion polymerization is compatible with a wide variety of polymerization techniques.^{48,54} Water-in-oil emulsions to obtain hydrophobic polymer nanoparticles generally result in hydrodynamic radii ranging between 50 and 300 nm.⁵⁴ However, due to swelling the hydrodynamic radius of nanogels will increase post-synthesis, where the extent of swelling is dependent on the crosslinking density.⁵⁵ Considering the ideal size for therapeutic and diagnostic applications, nanogels produced *via* this method typically have a hydrodynamic diameters of 50–150 nm.^{48,56–59} Polymerization in water-in-oil emulsions is a scalable process, allowing nanogel fabrication in large quantities. However, emulsions require the addition of surfactants, which may hamper the purification process. To obtain meticulous control over nanogel formation, Oh *et al.* used atom-transfer polymerization (ATRP) to prepare nanogels in a mini-emulsion, which resulted in nanogels with highly uniform molecular-weight distributions.⁶⁰ Moreover, ATRP allowed the formation of a uniform network, where degradation products maintained an $M_w/M_n < 1.5$, and precise control over end-group functionalization.

2.1.2 Precipitation and dispersion polymerization. Another technique involving two phases for nanoparticle preparation is precipitation polymerization.^{48,49} In precipitation polymerization and dispersion polymerization a solvent is selected which can dissolve both the monomer and initiator, but in which the formed polymer becomes insoluble above a critical degree of polymerization.⁶¹ Precipitation polymerization leads to complete precipitation of the growing polymer particles, whereas in dispersion polymerization a colloidal dispersion is formed upon polymerization. A stabilizer is added to achieve a stable colloidal dispersion.⁶² Opposed to water-in-oil emulsions, precipitation and dispersion polymerizations do not require an oil phase or a high concentration of surfactants, which greatly simplifies the polymerization and purification processes. However, precipitation and dispersion polymerization do require careful selection of specific monomers, which respectively precipitate or disperse upon polymerization.⁶¹

With classic dispersion polymerization, micrometer-sized particles can be readily prepared.^{48,61} This method has also been adapted in the synthesis of nanometer-sized structures, often by applying controlled radical polymerization techniques, such as reversible addition–fragmentation chain transfer (RAFT).^{63–66} Dispersion polymerization has been performed using a macromolecular RAFT agent that simultaneously acts as a chain transfer agent and a steric stabilizer for the formed polymer block.^{63–68} When a bifunctional monomer is added during the polymerization process, the growing fraction of the block-copolymers are crosslinked and core–shell nanogels are obtained.^{69–75} The formed nanogels generally have diameters of around 30–150 nm. Thermoresponsive monomers such as *N,N*-diethyl acrylamide (DEAAM), *N*-isopropyl acrylamide (NIPAM) and short oligo-ethylene glycol chains, as well as the non-responsive monomer *N*-(2 hydroxypropyl) methacrylamide, are

inherently miscible with water, while the corresponding polymers precipitate at higher temperatures owing to their lower critical solution temperature (LCST). These properties make aqueous dispersion polymerization particularly attractive for the preparation of thermosensitive nanogels.^{67,69,72,74}

2.1.3 Controlled polymerization with multi-functional monomers. Nanogels, and nanoparticles in general, are often prepared by interphase mixing with the addition of surfactants. These components are usually hard to remove and potentially complicate the purification process. Furthermore, the particle size is controlled by factors such as interphase mixing and micelle formation. Another interesting approach to prepare nanogels is by crosslinking monomers or polymers in a controlled manner, where the crosslinking process grants control over particle size.^{76–78} Since this process is not limited by the formation of emulsion droplets or polymer precipitation, it is particularly suitable for the preparation of small (sub-100 nm) nanostructures. For example, Harth and coworkers prepared several nanostructures from low molecular weight linear polyesters bearing alkene, alkyne or epoxide functionalities, whose crosslinking degree was controlled by the amount of added crosslinker.⁷⁸ Epoxide crosslinking using a diamine yielded particles with good control over size.

Controlled radical polymerization simultaneously allows polymer growth and branching/crosslinking, which leads to formation of polymer nanostructures and nanogels (Fig. 4) in a single step.⁷⁹ In this controlled radical crosslinking copolymerization, size and structure of the formed nanogels is governed by reaction conditions such as temperature, monomer concentration and reaction time. Often, this approach involves ATRP or reversible-addition fragmentation polymerization (RAFT) polymerization of a monovinyl monomer and a divinyl crosslinker.^{80–83} Other approaches include the use of inimers⁸⁴ or multifunctional initiators.⁸⁵ Since ATRP and RAFT both offer a large degree of freedom concerning monomer selection and control over polydispersity, these methods allow for the formation of uniformly crosslinked structures. In a recent review, the formation of intramolecularly crosslinked polymer particles has been described and it has been shown that a slow polymerization rate promotes the formation of intramolecularly crosslinked structures with well-defined and uniform structure.⁸⁶ This method however does require in-depth knowledge of and meticulous control over the polymerization process, as well as judicious selection of multifunctional monomers. In addition, these systems

Fig. 4 Examples of polymeric structures: star copolymers with crosslinked core (left), (hyper)branched polymers (middle) and crosslinked nano-networks (right).

remain rather sensitive to environmental factors and impurities, while the carbon-based polymer backbone renders these nanogels largely non-degradable.⁸⁶ Incorporation of degradable linkages through radical ring opening polymerization of cyclic vinyl monomers was shown to circumvent this latter issue.⁸⁷ The resulting nanogels contain unreacted double bonds, which may be used for functionalization. For example, Wang and coworkers investigated the degree of branching in nanogels based on a disulfide divinyl crosslinker and 2-(dimethylamino)ethyl methacrylate (DMAEMA) for gene delivery, where the unreacted vinyl groups were used for post-functionalization.⁸³

2.1.4 Inverse nanoprecipitation/solvent displacement. Polymer nanospheres are frequently prepared by nanoprecipitation of water-insoluble polymers: a polymer solution in an organic, water-miscible, solvent is added to a large volume of water.^{88,89} Under specific conditions the polymers will aggregate into clusters ranging from 100 to 1000 nm and surfactants are often used to stabilize the formed solution, as shown in Fig. 5.⁹⁰ After the organic solvent is removed by dialysis, ultrafiltration or similar techniques, a solution of solid polymer nanoparticles is obtained.^{91,92} Similarly, hydrophilic polymers can also precipitate into nano-sized clusters in an inversed system: the polymer is dissolved in an aqueous solution, which is added to a large volume of a water-miscible organic solvent.^{35,93,94} After chemical crosslinking and subsequent removal of unreacted polymer, crosslinker and used surfactants, a solution of nanogels is obtained. Nanoprecipitation has been demonstrated with natural polymers such as gelatin,⁹⁵ peptidomimetic polymers³⁶ and highly hydrophilic polymers, such as dendritic polyglycerol,^{93,96} polyvinylalcohol⁹⁷ and zwitterions.³⁵ Compared to inverse emulsions, nanoprecipitation does not require the use of high-shear mixing, which is known to damage proteins and DNA.⁹³ Surfactants are not required or only at low concentrations and nanoprecipitation can easily be performed on a laboratory scale. However, since the polymer concentration during nanoprecipitation requires large amounts of solvent, application on industrial scale becomes impractical. Furthermore, the process depends on solvent compatibility and concentration of the polymer, which has to be determined experimentally for every system separately.⁹⁰

2.1.5 Self-assembly. Fabrication of nanogels by self-assembly requires the polymeric components to spontaneously aggregate in an aqueous environment.²³ This can be achieved by incorporating functional groups into a polymer, which can form physical crosslinks with each other, with other polymer chains or with crosslinkers. Nanogels can also be stabilized by physical crosslinks that are assembled through hydrophobic interactions⁹⁸ or inclusion complex formation.^{99–101} In a similar fashion, covalently crosslinked nanogels can be obtained by aggregation and subsequent crosslinking, such as disulfide formation^{102–104} and chemical conjugation (Fig. 6).¹⁰⁵ In this manner, an amphiphilic micelle structure is converted to a hydrophilic nanogel matrix, which similar to polymeric micelles enables the formation of very small nanogels (10–100 nm).^{102,106,107} Another self-assembly process followed by photo-crosslinking was described by Wang and coworkers, who prepared acrylate functionalized triblock copolymer of enzymatically degradable polyethylene ester phosphate (PEEP) and PEG, p(PEEP-*b*-PEG-*b*-PEEP).¹⁰⁸ This triblock copolymer self-assembled into nanoaggregates upon the addition of salt, after which the aggregates were photo-crosslinked into nanogels between 70 and 550 nm in size for doxorubicin delivery.

The process of chemical crosslinking provides additional stabilization, as compared to physical crosslinking of nanogels.¹⁰⁷ The available amount of suitable monomers is however limited, since this process requires a block copolymer, which can form micelles under certain conditions, but must also be crosslinked to form a hydrophilic nanogel matrix.¹⁰⁷ The self-assembly process does not require organic solvents, surfactants or interphase mixing, which simplifies the purification process. However, the process is entirely dependent on the critical micelle concentration, which is influenced by the molecular weight and physical properties of the polymer.^{102,106} Other points of interest are the possible remainder of hydrophobic residues within the nanogel, which may alter properties such as responsiveness and drug release rate and the existence of non-incorporated polymers, which need to be removed.

2.1.6 Template-assisted nanogel formation. Etching of nanostructures on inorganic substrates, such as silicon and glass, has revolutionized the field of micro-electronics.¹⁰⁹ The templating of

Fig. 5 Nanogel preparation by inverse nanoprecipitation involves adding an aqueous polymer solution to a water-miscible non-solvent. Nano-sized polymer clusters form upon solvent and non-solvent mixing. Crosslinking of these polymer clusters and subsequent non-solvent removal results in an aqueous nanogel solution.

Fig. 6 Disulfide-crosslinked nanoparticles prepared by self-assembly of amphiphilic block-co-polymers containing hydrophobic cyclic carbonate (A) and pyridyl-disulfide (B) moieties. Hydrophobic self-assembled micelles were crosslinked by cystamine (A) and a catalytic amount of dithiothreitol (DTT) (B). Crosslinking of these micelles causes conversion of the hydrophobic micelle core into a hydrophilic nanogel matrix for protein (A) and drug delivery (B). (A) Reprinted (adapted) with permission from ref. 103. Copyright 2013 American Chemical Society. (B) Reprinted (adapted) with permission from ref. 102. Copyright 2010 American Chemical Society.

etched nano and microstructures allows the fabrication of structured biomaterials, which have been used for guiding cell attachment and promoting proliferation.¹¹⁰ DeSimone and coworkers have employed templating techniques (particle replication in non-wetting templates, PRINT) using etched nanostructures for the fabrication of nanogels from a wide variety of polymeric materials including PEG and poly(*D*-lactic acid).¹¹¹ A major advantage of PRINT over the aforementioned preparation techniques is that it grants unique control over particle size, dispersity and shape,¹¹² which also influences its interaction with biomolecules, proteins and cells.¹⁸ In addition, PRINT has been demonstrated to be a scalable process, through roll-to-roll production (shown in Fig. 7).¹¹³ However, the size and shape of these nanogels is determined by the applied etching methods to produce master wafers, which require advanced cleanroom facilities. Another template-assisted method was reported by Harth and coworkers, who used liposomes as a template to prepare polyglycidol

nanogels in a one-pot synthesis, resulting in drug carriers with improved release characteristics.¹¹⁴

2.2 Prospects on nanogel formation

A large variety of methods and modified methods exists to prepare nanogels, which present their own specific advantages and drawbacks. Water-in-oil emulsion-based polymerization offers a large degree of freedom in terms of monomer choice, as well as the incorporation of chemical functionality. Yet, the required high-shear mixing and posterior purification may be problematic when encapsulating sensitive compounds, such as proteins. In this respect, methods driven by physical aggregation, such as nanoprecipitation⁹³ and self-assembly¹⁰³ have been shown to be more effective. However, self-assembly as well as nanoprecipitation require dilute conditions, hampering large-scale application. Furthermore, PRINT provides interesting opportunities to prepare nanogels with excellent control over

Fig. 7 Illustration of PRINT nanogel production to roll-to-roll production. Clockwise, a perfluoropolyether mold is prepared via a nanostructured silicon wafer template, then polymer precursor added to the PRINT mold and the cavities are filled by rolling out the precursor material through roll-to-roll process. Nanogels are formed by photocuring and additional processes, and removed from the mold by a degradable adhesive layer, which is dissolved to obtain PRINT nanogels. Reprinted (adapted) with permission from ref. 113. Copyright 2013 by John Wiley Sons, Inc.

size down to approximately 50 nm and, importantly, unique control over nanogel shape, though high-precision laser-etching techniques are required to prepare the master silicon wafers.¹¹³ Current and future prospected biomedical applications of nanogels require robust and versatile synthetic methods, minimization of non-specific *in vivo* interactions and incorporation of responsive chemical moieties. Controlled crosslinking polymerization provides a generally applicable technique that allows facile formation of uniform nanogels with exquisite control over size, chemical structure and physical structure.⁸⁶ However, the technique is still in its infancy and requires intricate understanding of the polymerization and nanogel formation process, which still limits the applicability of this promising technique. During preparation of nanogels attention is predominantly drawn to physical properties, such as size, polydispersity and surface charge. However, more knowledge on the chemical composition of nanogels is required for their prospected applications. For example, the incorporation of targeting ligands at a desired density requires accurate control over the chemical composition of the nanostructure. The crosslinking of self-assembled polymeric structures and controlled radical polymerization methods

provide interesting advantages in terms of ease of incorporation and high modularity.^{60,108,115} Also the incorporation of drug molecules and the increased stability of the incorporated moieties remains an important point of interest.¹⁰² Other factors that are strongly dependent on the preparation method are the uniformity of crosslinking and the density of the nanogel network, as also observed for hydrogels.²⁰

3 Responsiveness

The field of controlled drug delivery has benefitted greatly from the application of responsive polymers, enabling spatiotemporal control over drug release.^{116–119} However, the application of responsive polymers in imaging/sensing has commenced only recently.^{24,118,120} In comparison to the number of responsive small molecular probes, the application of responsive polymers in molecular sensing is still in its infancy.^{118,120} The different modes of nanogel responsiveness, as summarized in Fig. 8, have been successfully employed both in controlled drug and gene delivery as well as biomedical imaging.^{121–123}

Fig. 8 Selected examples of responsive functional groups, which show responsiveness to temperature pH and molecules present in biological systems. The depicted reversible thermosensitive moieties undergo a conformational change upon heating above their LCST. Thermosensitive bonds formed by Diels–Alder additions have been applied in hydrogel chemistry, but not in nanogels.¹²⁴ The presented examples for reversible thermoresponsive polymers are (from left to right) poly(*N*-isopropylacrylamide), poly(vinylcaprolactam), poly(oligoethylene glycol acrylate) and poly(oligoethylene glycol methacrylate). The depicted examples for reversible pH-responsive polymers are (from left to right) poly(2-dimethylaminoethyl methacrylate), poly(2-diethylaminoethyl methacrylate), poly(2-diisopropylaminoethyl methacrylate) and poly(methacrylic acid). For more detailed information on responsive groups in nanogels ref. 72 and 138 are highly recommended.

3.1 Temperature responsiveness

Polymeric conformational changes in hydrogels result in volume phase transitions, effectively shrinking or expanding the hydrogel. Temperature-responsive polymers show lower (LCST) or upper critical solution temperature (UCST) behavior, becoming insoluble or soluble upon heating, respectively. The potential of poly(*N*-isopropylacrylamide) (pNIPAM) in biomedical applications already has long been recognized, because its LCST is close to body temperature and is independent of molecular weight.¹²⁵ Likewise, pNIPAM has been successfully incorporated in nanogels in multiple variations, often by copolymerization with other monomers to add functionality to the nanogels.¹¹⁹ Moreover, the volume-phase transition temperature (VPTT) of nanogels can be adjusted by copolymerization with other monomers, for example by acrylic acid to improve drug incorporation.^{32,34} Peng *et al.* studied the effect of the incorporation of PEG-methacrylate and methacrylic acid functional groups into a pNIPAM-based nanogel, which showed that acrylic acid lowers the VPTT of the nanogels, while PEG reduces the size and increases the solubility of the nanogels.³⁴ The tunability of the VPTT is important, since it optimizes drug encapsulation, since fully swollen nanogels easily release their drug content by diffusion,^{126–128} while fully collapsed nanogels can also expel their content.^{31,129,130}

Polymers based on oligo ethylene glycol acrylate (OEGA) exhibit lower immunogenicity than pNIPAM¹²⁷ and also display thermoresponsive behavior, but have an elevated LCST of 90 °C. However, the incorporation of functional monomers into the nanogel enables adjusting of the VPTT to the biologically more relevant temperature range of 25–35 °C.¹³¹ Poly(vinylcaprolactam) (pVCL) is a thermoresponsive polymer with an LCST in the temperature range 30–50 °C, depending on the pVCL concentration in solution and molecular weight.¹²⁵ Incorporation of other monomers, such as methacrylic acid¹²⁶ and hydroxypropyl-methacrylate¹²⁹ enabled altering the VPTT of pVCL nanogels, to physiologically relevant temperature ranges to efficiently encapsulate doxorubicin. Furthermore, pVCL nanogels, crosslinked with disulfide and ketal-containing crosslinkers, could efficiently release doxorubicin upon exposure to glutathione and pH levels below pH 7.4.^{126,129}

3.2 pH responsiveness

Throughout the body the local pH can vary considerably. Therefore, nanogels in biomedical applications have been designed to respond to these differences by swelling or degradation. Under normal conditions the physiological pH is maintained around 7.4 in almost all tissues,¹³² whereas the pH in the gastrointestinal tract, for example, can vary between 1 and 7.5.¹⁰⁷ Local pH differences are also observed on the cellular level, though to a much smaller extent. Whereas the extracellular environment is maintained around pH 7.4, the pH of endosomes can vary between pH 5–7.2.^{132,133} These variations in local pH can be used as triggers for release or response in therapeutic delivery or imaging, respectively. In many tumors, the pH of the tumor extracellular environment is acidified as compared to healthy

tissue, due to lactic acid production. This process is enhanced by poor tumor perfusion, which leads to lowering of the pH to 6.5–6.9 in the tumor microenvironment.¹³² Acidification to a pH between 6 and 7 is often also observed in inflamed and infected tissues.¹³⁴

3.2.1 Reversible swelling. As discussed earlier, swelling and shrinking are intrinsic properties of nanogels.²³ Incorporation of weakly acidic or basic groups grants pH-responsive swelling properties to the nanogel.¹³³ The generation of local charges within the network causes electrostatic repulsion within the polymer network, leading to swelling. This process renders the nanogel responsive to local pH changes, which is illustrated in Fig. 2A and 8.²³ The acidified microenvironment of tumors therefore offers possibilities for pH-responsive drug delivery and pH-responsive imaging.^{23,132} By choosing the pK_a of the polymer, the pH-responsivity of the nanogel can be tuned. An overview of pH-responsive monomers is shown in Table 1, which are applicable in different polymerization techniques, among which radical polymerization and *N*-carboxyanhydride (NCA) ring-opening polymerization.^{119,122,135} pH-Responsive nanogels prepared by radical polymerization are often prepared by incorporation of acrylic acid^{32,127,136} or methacrylic acid.¹³⁷ The pK_a of acrylic acid moieties lies around 7.3, which enables complexation of cationic drugs, such as doxorubicin, which is released upon protonation of the acrylic acid group below physiological pH.¹³⁶ 2-Diethylaminoethyl acrylate and 2-dimethylaminoethyl acrylate-based nanogels have been investigated by Oishi *et al.*^{138,139} and Wu *et al.*,¹⁴⁰ who demonstrated increased nanogel swelling in acidified media, enabling drug release and responsive imaging. However, incorporation of these cationic monomers also increases the toxicity of these nanogels.¹³⁹

3.2.2 Hydrolysis. Local pH differences within the body and cellular compartments can be utilized for degradation of specific chemical bonds. Since responses below physiological pH are interesting from a biomedical perspective,¹³² pH-sensitive hydrolysable bonds, such as ester, acetal and hydrazone bonds, are frequently incorporated into nanogels.¹²¹ pH-Mediated hydrolysis supports triggered release at target areas with a lower pH environment, such as endosomes, certain tumors or inflamed tissue. Hydrolysis however is a (slow) continuous process under physiological conditions, which is accelerated by acidification due to diseased tissue possibly causing premature release of encapsulated small molecules.^{129,151–153} Despite this drawback, hydrolysis remains an effective method to release drugs more selectively into an area of interest.¹²¹

pH-Triggered release of a nanogel's payload can be achieved by including bonds into the nanogel matrix which are hydrolyzed at a certain pH value, leading to swelling and ultimately degradation into macromolecular/polymeric fragments, depending on the design of the nanogel.^{129,151} These bonds are included into the nanogel matrix by incorporation in the polymer backbone, or by including pH-labile crosslinkers, as depicted in Table 1. The preparation of hydrolysis-sensitive nanogels can, for instance, be achieved *via* precipitation polymerization of NIPAM, OEGMA or VCL in the presence of hydrolysable crosslinkers, such as ethylene glycol dimethacrylate¹³⁹ and 2,2-dimethacroyloxy-1-ethoxypropane.¹²⁹

Table 1 pH-Responsive monomers and crosslinkers used for nanogel formation. Protecting groups of protected monomers are indicated in orange

Structure	Response	Ref.
<p>R = H or CH₃</p>	pH-Responsive	31, 32, 34, 126 and 141–145
<p>R = CH₃ or CH₂CH₃</p>	pH-Responsive, hydrophobic switch	140 and 146–149
pH-Responsive, protected NCA monomer	150
pH-Responsive, protected NCA monomer	150
pH-Degradable, switchable monomer	151
pH-Degradable crosslinker	56 and 147
pH-Degradable crosslinker	129

A brief overview of hydrolysable crosslinkers is given in Table 1. Another strategy is to combine functional groups within a polymer is shown by Lu *et al.*, who prepared zwitterionic poly(succinimide) polypeptide nanogels, crosslinked by pH-sensitive hydrazone bonds.¹⁵³ Qian *et al.* included hydrophobic monomers with hydrophobic *ortho*-ester bonds into nanogels, which hydrolyzed below physiological pH, rendering the nanogel hydrophilic, inducing swelling and facilitating drug release.¹⁵¹ Triggered release of drugs from a nanogel can also be achieved by utilizing a pro-drug strategy, where the drug is conjugated to the nanogel with a pH-labile bond, such as the conjugation of doxorubicin *via* a hydrazone linkage.^{154,155}

3.2.3 Charge conversion. Surface charge is an important parameter in directing cellular uptake of nanoparticles.^{156,157} Surface-charge switching has therefore received interest as a drug delivery mechanism.^{56,158–160} Charge conversion of anionic nanoparticles into cationic nanoparticles, greatly enhances their cellular uptake.¹⁵⁶ Du *et al.* reported a 2-aminoethyl methacrylate nanogel, of which the primary amine groups were functionalized with 2,3-dimethylmaleic anhydride, giving the nanogel an anionic surface charge at physiological pH.⁵⁶ However, below pH 6.8, which is typical for a tumor microenvironment, degradation of the amide bond releases dimethylmaleic anhydride and forms a protonatable primary amine on the surface of the nanogel.

The nanogels displayed a negative surface charge at physiological pH, but the charge changes to positive at pH 6.8, which

is typical for a tumor microenvironment. MDA-MB-435s (breast cancer) cells showed significantly more nanogel uptake at pH 6.8, compared to 7.4. Similarly, Chen *et al.* formed polyvinylalcohol nanogels by click chemistry, where unreacted azide groups were functionalized with dimethylmaleic propargylamide, to provide the charge-conversion properties to the nanogel.⁹⁷

3.3 Biomolecule sensitivity

3.3.1 Redox-responsiveness. In healthy tissue, the intracellular redox potential is regulated by an elevated concentration of glutathione (GSH) to regulate damaging reactive oxygen species, such as H₂O₂ and NO.¹³² Intracellular GSH concentrations are reported to vary between 2–10 mM,^{59,132} whereas the GSH concentration in the extracellular space is estimated between 2–20 μM.⁵⁹ Taking advantage of the elevated intracellular GSH concentrations, reduction-sensitive disulfide bonds are often incorporated in drug and gene delivery systems to achieve intracellular release.⁵⁹ A more recent development in this context is the stabilization of disulfide bonds *via* steric hindrance and charge repulsion, which enhances the stability of disulfide moieties under extracellular GSH concentrations.^{161,162} Drug and gene carriers which combine high buffering capacity with bioreducibility, have shown to be very effective due to the proton sponge effect.^{132,163} This effect involves protonation of the polymer in the endosome during endocytosis. The increased influx of protons, accompanied by chloride ions, eventually leads

to osmotic bursting of the endosome. Bioreducible nanogels can be prepared by including bioreducible, bifunctional, monomers (Table 2) into the reaction mixture during emulsion^{57,151,164} or precipitation polymerization.^{126,127,144} Nanogel crosslinking *via* NCA polymerization with bifunctional, bioreducible monomers, was also demonstrated by Chen and coworkers.¹⁶⁵ Another method to include disulfide bonds into the nanogel matrix is by crosslinking free thiols in a polymer backbone with each other,¹⁰³ for instance as demonstrated by Jayakumar and coworkers in chitin-hyaluronic acid nanogels.¹⁶⁶ Disulfide polymer crosslinking can also be achieved by polymerizing a pyridylsulfide monomer, which can then crosslink with itself *via* disulfide exchange.^{102,143,167}

3.3.2 Enzyme responsiveness. The incorporation of enzyme-cleavable sites into polymeric materials has been an interesting approach for the degradation of biomaterials.^{134,173,174} Enzyme-triggered release from polymer nanoparticles has the advantage that enzymatic cleavage is highly specific¹⁷³ and is strongly dependent on the type of cell and the state of the targeted tissue.^{134,173} Furthermore, upregulation of specific enzymes is commonly encountered in inflamed or cancerous tissues.^{173,174} However, enzymatic cleavage requires incorporation of specific peptide sequences into the nanogel matrix.¹⁷⁵ Thornton *et al.* reported a PEG-based nanogel for enzyme-triggered release of proteins, as shown in Fig. 9, where zwitterionic enzymatically cleavable peptides caused the nanogel to swell upon degradation by specific proteases.¹⁷⁵ The sites for enzymatic degradation were shown to be enzyme-specific, and subsequent protein-release was confirmed.

Polysaccharide materials provide an interesting alternative, since these materials are also subject to enzymatic degradation.¹⁷⁶ However, polysaccharides do require purification and modification for application in nanogel synthesis and limit functional

chemical design of nanomaterials.¹⁷⁷ Generally, nanomaterials are designed for hydrolase sensitivity, where proteases, lipases and glycosidases are currently the most relevant hydrolase targets in drug delivery.¹⁷³

Glycosidases are enzymes which degrade specific naturally occurring polysaccharides, making polysaccharide-based materials by definition enzymatically biodegradable.¹⁷³ One example is the hyaluronic acid degrading enzyme hyaluronidase, which activity is upregulated by several cancer types.¹⁷⁸ Hyaluronic acid has frequently been used to prepare nanogels, because of its biocompatibility.^{31,179,180} Moreover, the enzymatic degradation of hyaluronic acid is interesting for a variety of applications, including responsive imaging.¹⁸¹ Polyethylenimine (PEI)-pullan cationic nanogels loaded with paclitaxel were reported by Yim *et al.*, which were coated with hyaluronic acid to shield the positive surface charge in order to reduce non-specific uptake of the nanogel.¹⁸² Degradation of the hyaluronic acid coating resulted in charge conversion resulting in increased cellular uptake, as shown in Fig. 10. *In vivo* experiments showed dramatic reductions in tumor volume (Fig. 10B and C). Similarly, dextran has been investigated heavily as a material to prepare nanogels,^{53,183–186} which also contain other functional groups for nanogel degradation, such as disulfide bonds.^{53,184} Dextran degradation has also been investigated as a means of nanogel degradation.^{187,188}

Proteases can cleave specific peptide sequences. Matrix metalloproteases (MMPs) have been identified as target for controlled release, because of their upregulation in cancerous tissue.¹⁷³ Despite receiving considerable attention in the field of controlled drug delivery,^{173,189} MMP sensitive nanogels have only been reported sparsely, because of their activity towards specific peptide sequences.¹⁹⁰

Enzymatic activity of specific lipases has been identified as an interesting therapeutic target, since phospholipases are,

Table 2 Bioreducible monomers and crosslinkers used for nanogel formation. Protecting groups of protected monomers are indicated in orange

Structure	Responsiveness	Ref.
<p>R = H or CH₃</p>	Protected monomer for disulfide crosslinking	102, 143 and 167
<p>R = H or CH₃</p>	Bioreducible crosslinker	151 and 168
Bioreducible crosslinker	126, 127, 130, 144, 163 and 169–171
Bioreducible NCA crosslinker	165
Dual responsive crosslinker, bioreducible and lipase degradable	172

Fig. 9 PEG-based nanogels containing zwitterionic peptide moieties with a protease cleavage site between the anionic and cationic part of the moiety (A). Upon enzymatic degradation of the linker, the charge conversion within the gel leads to swelling and subsequent release of its protein payload (B). Reproduced from ref. 175 with permission from the Royal Society of Chemistry.

Fig. 10 Enzyme-responsive paclitaxel-loaded cationic nanogels, coated with hyaluronic acid to prevent non-specific cellular uptake: hyaluronidase, which is overexpressed by some tumors, degrading the hyaluronic acid layer and exposes the cationic nanogel, which is easily taken up by local cells (A). Paclitaxel delivery in a tumor-graft model led to a considerable reduction in tumor volume (B and C). Reprinted (adapted) with permission from ref. 182.

for instance, upregulated during infection and inflammation.¹⁷³ In this respect, polyphosphoesters are promising materials, since they are degraded by phospholipases, secreted by bacteria.^{191,192}

3.3.3 Glucose responsiveness. Monitoring glucose concentrations is critical in treating diabetes mellitus.¹⁹³ Responsiveness of a material towards glucose concentrations may allow on-demand release of insulin.¹⁷⁴ The design of glucose-responsive polymers is based on the incorporation of phenylboronic acid moieties in the polymer backbone.^{119,194,195} Boronic acids are hydrophobic in their neutral form, while the anionic

form is hydrophilic, which enables the formation of a boronate ester with 1,2-diols (as depicted in Fig. 8), as presented by glucose and polysaccharides. Phenylboronic acids are commonly used in biomedical applications, since its pK_a enables glucose-responsiveness at physiological pH.¹⁹⁴

4 Nanogels in imaging

Recent developments in contrast enhancement for medical imaging attributes an important role to nanoparticles,¹⁹⁶

among which polymeric nanoparticles.⁷ The versatile nature of polymer nanoparticles offers, for instance, control over their biodegradability and biodistribution properties.^{107,196} Furthermore, there are numerous strategies for conjugation of functional imaging or targeting groups to the nanoparticle^{9,197} and the combination of therapy and imaging, *i.e.* theranostics, focuses the attention towards multi-functional nanocarriers.^{7,196,198} Responsive polymeric materials are likewise gaining more attention,^{116,134,199} since stimuli-responsive nanoparticles can enable triggered release upon changes in local physiochemical parameters. Furthermore, stimuli-responsive imaging or sensing, can reveal information about the bio-environment of diseased areas in terms of pH, enzyme activity, and temperature, among others.^{7,24,118}

4.1 Magnetic resonance imaging

Magnetic resonance imaging (MRI) has developed to one of the most important imaging modalities since its introduction in the 1970's.²⁰⁰ Image contrast in MRI relies on the environment-dependent proton relaxation in magnetic gradients and is generated by influencing the spin-lattice (longitudinal, T_1) or spin-spin (transversal, T_2) relaxation times of water protons in the imaged tissue.^{201,202} Paramagnetic ions, such as gadolinium ions, shorten the T_1 relaxation time of water protons in its proximity and provide positive contrast, while superparamagnetic iron oxide nanoparticles (SPIONs) lengthen the T_2 relaxation time of water protons, and therefore give negative contrast. However, MRI contrast generation is also greatly influenced by many other factors, such as scan sequence.²⁰³

Due to their high water-content and the possibility to attach gadolinium chelates, nanogels are very suitable as carriers of MRI contrast agents. Li *et al.* synthesized co-polymers of oligo(ethylene glycol) methylether acrylate and pentafluorophenyl acrylate *via* RAFT polymerization to yield three different architectures (linear, hyperbranched and star-like nanogels), following functionalization with a chelator.²⁰⁴ Fig. 11 shows the relaxivity these architectures, where the hyperbranched

structure showed the largest relaxivity (Fig. 11B), despite the increased rotational diffusion time of the star-like polymers (794 ps *vs.* 651 ps for the hyperbranched structure). This was attributed to the enhanced exposure of the gadolinium complex to water, which was also shown by the same authors to be an important factor for increasing relaxivity.²⁰⁵ Lux *et al.* reported the formation of crosslinked poly(acrylamide) nanogels, where the crosslinker included the chelators gadolinium-loaded chelators.²⁰⁶ The resulting nanogels displayed a 3–4 times higher relaxivity than the separate crosslinkers. Furthermore, the chelator-based crosslinkers displayed a higher stability when they were incorporated into a nanogel. Similarly, well-defined single-chain polymer nanoparticles, crosslinked by chelator-containing crosslinkers, were reported by Perez-Baena *et al.*, combining fidelity of the nanostructure with ease of preparation.²⁰⁷

Zhang *et al.* showed the encapsulation of SPIONs in bio-reducible zwitterionic nanogels, which remained stable under physiological conditions and quickly released encapsulated SPIONs and drugs after exposure to dithiothreitol (DTT).⁵⁰

Gadolinium-based contrast agents as well as SPIONs present drawbacks, most notably associated nephrotoxicity for gadolinium compounds and the generation of negative contrast (signal removal). Therefore, other methods to generate MRI contrast, such as ^{19}F -MRI,¹⁴⁹ chemical exchange saturation transfer²⁰⁸ and hyperpolarization²⁰⁹ provide possible alternatives, which however require other MRI scanners than the current clinical standard.

4.1.1 Responsive MRI contrast agents. Nanogels are by nature responsive nanomaterials, which is advantageous in MRI, since MRI contrast is influenced by environmental factors. Nanogels offer the possibility of responsive MRI contrast by shrinking in response to their environment, while the resulting motion restriction of gadolinium chelates results in contrast enhancement.²¹⁰ This was shown in pH-responsive poly(acrylic acid) nanogels containing gadolinium chelates.²¹¹ An On/Off switchable nanogel-based MRI sensor has been reported by

Fig. 11 Investigated DOTA-functionalized polymer structures and the influence of the location of the gadolinium moieties (A), which revealed that hyperbranched structures offer the highest increase in relaxivity per gadolinium moiety (B). However, the star-shaped polymer allowed higher gadolinium loading. Reprinted (adapted) with permission from ref. 204. Copyright 2012 American Chemical Society.

Santra *et al.*²¹² (Fig. 12A). A poly(acrylic acid) nanogel with an iron oxide core was loaded with gadolinium chelates, of which the generated T_1 MRI contrast was quenched due to its proximity to the SPION core. Upon protonation of the nanogel matrix the gadolinium chelates were released and generate T_1 contrast. Similarly, Wang *et al.*²¹³ reported a glycol chitosan nanogel containing small SPION clusters and manganese ions (Fig. 12B). Upon acidification, the manganese ions were released and the MRI signal was switched on. These concepts show that triggered MRI contrast generation allows monitoring of local pH can also be incorporated into nanogels with other modes of responsiveness, for example as shown in Fig. 8. A pH-responsive ratiometric sensor based on a gadolinium chelate-coated SiO₂ core, surrounded by a poly(methacrylic acid) nanogel was reported by Okada *et al.*,¹⁴¹ as depicted in Fig. 12C. In its shrunken state, the nanogel restricts the mobility of bound water molecules, shortening the T_2 relaxation time of these protons. Since the T_1 relaxation time arising from the gadolinium chelates is not affected by the pH, the ratio between the T_2 and T_1 relaxation time can be used to determine the sensor's concentration. The use of nanogels with alternative contrast enhancement strategies, such as ^{19}F MRI¹⁴⁹ and magnetic nanocrystal structures,²¹⁴ has also been reported.

4.2 Nuclear imaging

Labeling of nanogels for nuclear imaging techniques offers the possibility to obtain highly sensitive imaging probes, since these techniques require much less imaging agent as compared to MRI. The use of colloidal gold nanoparticles is a promising new method to generate contrast in computed tomography (CT) images.^{215,216} Nanogels with embedded gold nanoparticles have been reported frequently, however, mostly for optical imaging techniques.³¹ Gold nanoparticle-loaded sucrosebutyrate acetate nanogels for image-guided radiation therapy have been reported by Jøllek *et al.*,²¹⁷ which can potentially enhance the effects of radiation therapy. Other nuclear imaging techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) require the labelling with radioactive tracers.²¹⁸ Majmudar *et al.* showed that 13 nm ^{89}Zr -labelled dextran nanogels can be used to target macrophages in arteriosclerotic plaque, which were imaged by PET/MRI to assess risks for plaque rupture.²¹⁹ Acrylamide nanogels with metal chelating crosslinkers were prepared by Lux *et al.* (as discussed earlier for MRI²⁰⁶) and loaded with ^{64}Cu for PET/CT imaging, revealing a higher accumulation of the nanoparticles in tumor tissue than the freely chelated ^{64}Cu tracer.²²⁰

Fig. 12 MRI-based nanogel pH sensors: pH-responsive nanogels, which contain SPIONs and gadolinium (A) or manganese (B), leaving the generated T_1 contrast by gadolinium and manganese ions in a quenched state due to the proximity of the SPIONs. However, release of the paramagnetic ions at a pH lower than physiological conditions, T_1 MRI contrast is restored. Ratiometric MRI pH sensor, consisting of a pH-responsive nanogels with a silicon core, with chelated gadolinium grafted to the surface. The relaxivity of the nanogel is influenced by swelling and shrinking of the pH responsive nanogel matrix. Measurement of T_1 -relaxivity, which remains constant, and T_2 relaxivity, allows for ratiometric measurement of the local pH via MRI. (A) Reprinted (adapted) with permission from ref. 212. Copyright 2012 American Chemical Society. (B) Reprinted (adapted) with permission from ref. 213. (C) Reprinted with permission (adapted) from ref. 141. Copyright 2014 by John Wiley Sons, Inc.

4.3 Fluorescence imaging

Integration of fluorescence probes into nanogels enables sensitive stimuli-responsive imaging.²²¹ The possibilities of fluorescent imaging are extended further by Förster resonance energy transfer (FRET) to visualize the proximity of two separate fluorophores.¹⁰² Since fluorescence imaging techniques are widely accessible, nanogels to measure temperature,³⁰ pH,²²² glucose concentration,¹⁴⁰ and enzyme activity²²³ *via* fluorescence imaging have been reported. Incorporation of fluorophores into a nanogel matrix can also provide an environment-sensitive sensor, which enables non-invasive monitoring of state of the local environment.

One strategy employed by Gota *et al.* was to prepare a nanogel based on a copolymer of NIPAM and a fluorophore-containing monomer.³⁰ Due to the thermosensitive chain collapse of pNIPAM, the water content of the nanogel was regulated by the environmental temperature. Another method is fluorescence quenching as demonstrated by Oishi *et al.*, who included gold nanoparticles into a pNIPAM nanogel, functionalized with FITC by a caspase-3 degradable linker.¹⁴⁷ Hyaluronic acid nanogels were functionalized with a fluorescent dye, of which the fluorescence intensity increased upon enzymatic degradation of the hyaluronic acid.¹⁸¹ Since hyaluronidase is overexpressed by a lot of cancers,¹⁷⁸ the nanogel can serve as an imaging tool for cancer detection. This mechanism was demonstrated in several cell lines, where signal intensity was increased

in cancer cell lines. Zhou and coworkers developed several core-shell nanogels with temperature,³¹ glucose¹⁴⁰ and pH-sensitive²²² optical imaging capabilities in combination with drug delivery. Metal nanoparticles^{31,140} and quantum dots²²² were included into the stimuli-responsive nanogels by dispersion polymerization. Responsiveness was included by copolymerization of acrylic acid, oligo(ethylene glycol)methyl ether methacrylate or 4-vinylphenylboronic acid, to achieve pH, temperature and glucose sensitivity, respectively. Fig. 13 shows the reversible sensitivity of quantum dot luminescence under influence of glucose concentration and temperature, where the sensitivity to glucose was shown to be only minimally influenced by other metabolites and ions. Similar nanogel systems, where quantum dots and metal nanoparticles embedded into a responsive nanogel matrix to achieve glucose sensitivity have also been reported.²²⁴

5 Nanogels in therapeutic delivery

Controlled delivery of molecules for therapeutic purposes is a central part of the nanomedicine field, which has originated bright perspectives for current and future clinical application.⁴ In this respect, nanogels promise to combine their advantageous properties, such as high water content, responsiveness and biocompatibility, for efficient therapeutic delivery.^{23,48}

Fig. 13 Photoluminescence response and reversible responsiveness of nanogels with a quantum dot core, sensitive to glucose (A and C, respectively) and temperature (B and D, respectively). The conformational changes of the nanogel matrix influences the photoluminescence of the quantum dot. Both nanogels proved responsive to glucose concentrations (A, decreasing photoluminescence upon exposure to glucose) and temperature (B, increasing photoluminescence upon increasing temperature), respectively. Moreover, both nanogels showed reversible responsiveness to glucose and temperature (C and D, respectively). (A/C) Reprinted (adapted) with permission from ref. 140. Copyright 2010 American Chemical Society. (B/D) Reprinted (adapted) with permission from ref. 31.

5.1 Drug delivery

Nano-sized drug delivery systems have been shown to be very effective, predominantly in delivering chemotherapeutics to tumors.¹⁰⁷ Some of these drug delivery vehicles have entered the clinical trial-stage of drug development.²²⁵ In this respect, application of nanogels for drug delivery is very interesting and depending on the used polymer, nanogels can release drugs upon exposure to specific stimuli.¹²¹ An important factor for (responsive) drug carriers is their drug retention until (triggered) release. Drug retention of disulfide-crosslinked p(OEGA-pyridyl disulfide methacrylate) nanogels was determined by Ryu *et al.*¹⁰² The importance of the crosslinking density of nanogels was further emphasized by examination of drug retention inside the nanogels in the presence of phospholipid membranes by FRET. Fig. 14 shows that encapsulation of a FRET pair in a densely crosslinked nanogel (37%) retains its FRET emission, whereas a lower crosslinking density (7%) results in the loss of FRET emission.

5.1.1 Chemotherapeutics. Targeted delivery of chemotherapeutics is of utmost importance in the treatment of cancer. The application of stimuli-responsive drug delivery vehicles, among which nanogels, has shown encouraging results.¹⁷⁴ For example, chitin nanogels containing acid-labile ester groups²²⁶ or redox-responsive disulfides¹⁶⁶ have been shown to effectively deliver doxorubicin to various cancer cell lines, exploiting the natural positive charge of chitin to facilitate cellular uptake. Furthermore, as described earlier, the VPTT behavior of thermo-responsive polymers is influenced by incorporation of hydrophilic monomers.^{34,102,126,130} Drug release is influenced by the VPTT,^{32,130} which is potentially useful under hyperthermia conditions.¹³⁰

Moreover, copolymerization with acrylic acid monomers facilitates binding of cationic molecules, such as doxorubicin^{34,136,144,145,227} and cisplatin³² at physiological pH, and triggering release at lower pH values. For instance, it has been demonstrated that p(NIPAM-co-acrylic acid) nanogels could be loaded efficiently with doxorubicin and cisplatin, respectively.^{32,144} Both systems showed an increased drug release rate at lower pH values and *in vivo* tumor graft models showed that both nanogels were able to achieve a considerable reduction in tumor volume, while having minimal influence on the body-weight of the used animals, demonstrating effective drug delivery without adverse health effects on the animals.^{32,144} Moreover, incorporating crosslinkers with disulfide bonds^{127,144,151,228} or crosslinking by disulfide formation,¹⁰² enables specific intracellular drug release, further increasing the therapeutic efficacy.¹⁴⁴

The biocompatibility and biodegradability of polypeptides makes these materials very interesting for application in nanomedicine and the variety of monomers and possibilities for post-modification allows the incorporation of stimuli-responsive functional groups.²²⁹ Di-block polypeptide-based polymers can be used to form polyionic complexes by counterion-induced self-aggregation, which has been researched extensively by Kataoka and coworkers.²³⁰ Nanogels can be obtained by crosslinking these polyionic complexes: Bronich and coworkers prepared PEG-polyglutamic acid nanogels, which were formed by initial self-aggregation by Ca^{2+} and subsequent crosslinking with cystamine.²³¹ The hydrophobicity of the nanogel was increased by partial phenylalanine modification of the polymer to increase drug loading efficacy. Combination therapy of doxorubicin and a chemotherapy-resistance inhibitor in a

Fig. 14 Disulfide-crosslinked nanogels containing a FRET pair (A) were incubated together with phospholipid vesicles. Nanogels with a low crosslinking density showed loss of FRET emission (B and D), while FRET emission remained stable in nanogels with a high crosslinking density (C and D). FRET emission was however lost after exposing the nanogels to a reducing agent (DTT). Reprinted (adapted) with permission from ref. 102. Copyright 2010 American Chemical Society.

nanogel formulation showed tumor size-reduction and a diminished loss of body weight in mice. Polypeptide nanogels have also been prepared by a single-step NCA polymerization of mono and bifunctional monomers and PEG-amine as initiator.^{232,233} The disulfide bond in the bifunctional crosslinker rendered the nanogels bioreducible and treatment of tumor-bearing mice with these nanogels resulted in a decreased tumor volume, while minimizing body-weight loss.¹⁶⁵ Furthermore, Chen and coworkers showed that incorporation of lysine and glutamic acid moieties gave the nanogels pH-responsive properties.¹⁵⁰ Similar to pNIPAM-acrylic acid nanogels,³⁴ lysine-containing nanogels encapsulated doxorubicin very effectively by ionic interaction and were able to deliver doxorubicin effectively into HeLa and HepG2 cells.¹⁵⁰ Doxorubicin release was shown to be facilitated by disulfide cleavage upon exposure to glutathione, as well as breaking the ionic interaction between doxorubicin and the nanogel below physiological pH. Doxorubicin-loaded zwitterionic polypeptide nanogels with pH-labile hydrazone crosslinks have also been reported.¹⁵³

Tightly crosslinked polypeptide nanogels were prepared by Lee *et al.*, who crosslinked a phenyl-alanine-co-histidine-*b*-PEG polypeptide by micelle formation and subsequent crosslinking of the outer PEG shell with bovine serum albumin (BSA).²³⁴ The histidine moieties caused swelling of the nanogel and concomitant doxorubicin release below pH 6.4. In a similar system, Ju *et al.* prepared a nanogel by crosslinking *N*-lysinal-*N'*-succinyl chitosan, *N,N'*-methylenebis(acrylamide) and NIPAM by radical polymerization. After purification, the nanogel was further crosslinked on the outer shell by BSA (Fig. 15A).³³ The resulting nanogel showed a reversible high degree of swelling and the nanogel was shown to be able to deliver doxorubicin sequentially to tumor cells. Furthermore, the research exemplified the possibilities of the reversible nature of the nanogels, which enabled sequential intercellular delivery of doxorubicin.

Fig. 15B shows that after co-incubation with previously infected cells, doxorubicin was continuously released in the freshly exposed cells.

Dendritic polyglycerol exhibits lower immunogenicity than PEG⁵² and hyperbranched dendritic polyglycerol-based nanogels have likewise been researched extensively, among others by Haag and coworkers and Frey and coworkers.^{58,235} Doxorubicin encapsulation was achieved by disulfide¹⁵⁴ or boronate ester⁹⁶ crosslinking. Furthermore, Sousa-Herves *et al.* used acrylate-functionalized hyperbranched polyglycerol and several acrylate-based monomers as well as bis(2-methacryloyl)oxyethyl disulfide to form a doxorubicin-loaded nanogel in a one-pot-synthesis, which is sensitive to bioreduction, as well as to pH changes.¹⁶⁸ Incorporation of therapeutics into nanogels can also be achieved by a pro-drug approach: dendritic polyglycerol-based nanogels have been used in the controlled release of doxorubicin.¹⁵⁴ Acid-labile hydrazine linkers were used to include doxorubicin on the nanogel exterior or inside the nanogel matrix. Furthermore, the nanogel was crosslinked by disulfide exchange, rendering the nanogel responsive to both pH and bioreduction.

Nanogels sensitive to bacterial-secreted lipases have been reported by Wang and coworkers, who developed a macrophage-targeted mannose-functionalized polyphosphoester-based nanogel, which proved sensitive to bacteria-produced phospholipase as well as to reducing environments to deliver antibiotics.¹⁹² To improve the targeting efficiency towards macrophages, the nanogels were functionalized with mannosamine (Fig. 16A). After loading with vancomycin, the nanogels were tested in a methicillin-resistant *Staphylococcus aureus* (MRSA)-infected RAW 264.7 culture and in an MRSA-infected zebrafish model; on both occasions the mannose-targeted nanogel performed significantly better. Fig. 16B shows that green fluorescent protein-expressing MRSA was completely quenched after 5 hours post injection. Furthermore, administration of low concentrations of

Fig. 15 Doxorubicin-loaded nanogels consisting of pNIPAM and *N*-lysinal-*N'*-succinyl chitosan and coated by bovine serum albumin, was shown to release doxorubicin below physiological pH (A). Moreover, cells, which were exposed to nanogels were sequentially introduced to new cell cultures (B, left-to-right). This revealed that doxorubicin was delivered intercellularly by the nanogels. Reprinted (adapted) with permission from ref. 33. Copyright 2014 by John Wiley Sons, Inc.

Fig. 16 Vancomycin-containing polyphosphoester nanogels, functionalized with mannose for targeting purposes (A) evaluated in a zebrafish methicillin-resistant *Staphylococcus aureus* (MRSA) infection model. Zebrafish infected with green fluorescent protein expressing MRSA and treated with PBS, empty mannose-functionalized nanogels (MNG), vancomycin (Van), vancomycin-loaded nanogels (NG-V) and vancomycin-loaded mannose-functionalized nanogels (MNG-V) (B). 9 hours post-infection MNG-V's treated zebrafish showed no fluorescence from MRSA infection. Survival-rate of zebrafish treated with 30, 15 and 3 ng vancomycin (C, D and E, respectively), showing significantly better survival rates for mannose-functionalized nanogels, at the lowest vancomycin concentration. Reprinted (adapted) with permission from ref. 192. Copyright 2012 by John Wiley Sons, Inc.

vancomycin encapsulated in the mannose-functionalized nanogels shows a substantially higher survival-rate when compared to free vancomycin and the non-targeted nanogel.

5.2 Gene delivery

Delivery of nucleic acids for therapeutic purposes is generally very inefficient and requires a suitable delivery vehicle to protect the nucleic acids against DNase and RNase mediated degradation.²³⁶ Furthermore, the therapeutic gene needs to be transported into the cells of interest. Despite their high efficiency, viral gene delivery vectors bring some critical issues, such as the required extensive purification, limited genetic payload, and safety issues. Hence, the use of polymeric gene delivery vectors as an alternative carrier is receiving ample interest.²³⁶ In this respect, nanogels offer superior properties

as gene delivery vehicles, since their crosslinked structure offers the required stability (opposed to non-crosslinked delivery vehicles) for encapsulation of small nucleic acid fragments – such as miRNA and siRNA.²³⁷ pNIPAM nanogels have therefore been applied in the delivery of siRNA.^{238–240} Although these nanogels lacked a specific release mechanism, nucleic acids were still readily released from the nanogel matrix. Incorporation of cationic moieties in these pNIPAM nanogels, enables stable and efficient condensation of nucleic acids and the crosslinked nanogel structure facilitates the condensation of small nucleic acid fragments very efficiently.^{139,237,241} Polysaccharide nanogels, such as dextran,⁵³ and hyaluronic acid nanogels,¹⁸⁰ have also been successfully applied in siRNA delivery for gene-silencing.²⁴²

pH-Responsive and degradable monomers have been included into the nanogel matrix to improve nucleic acid condensation and

triggered release.^{121,134,237} Tamura *et al.* prepared a 2-(*N,N*-diethylaminoethyl) methacrylate nanogel as a pH-responsive monomer with a pK_a around 7.0, which could facilitate endosomal escape as well as siRNA complexation. Ethylene glycol dimethacrylate was used as a pH labile crosslinker and PEG-methacrylate for incorporation of stealth properties.¹³⁹ These nanogels showed superior stability as compared to non-crosslinked polyplexes and highly efficient RNA interference at N/P ratios as low as 2.¹³⁹ The ability to condensate siRNA was further improved by methylation of the nanogels' tertiary amines.²⁴³ A quaternization degree of 10% proved to be very effective, exhibiting gene silencing properties comparable to b-PEI in HuH-7 cells.²⁴³ Raemdonck *et al.* used a similar approach, where quaternized 2-(*N,N*-dimethylaminoethyl) methacrylate was incorporated in pH-degradable dextran nanogels to facilitate siRNA-loading.⁵³ Averick *et al.* reported fully quaternized dimethylaminoethyl methacrylate bioreducible nanogels formed by ATRP in a miniemulsions (Fig. 17A).⁵⁷ These nanogels could be complexed with siRNA, as well as with pDNA, which resulted in a stable complex formation. Furthermore, no cell toxicity was observed and Fig. 17B and C show the transfection abilities of the nanogels for pDNA and siRNA.

Conjugation of siRNA to nanogels to further improve the complex stability has also been reported by Dunn *et al.* who initially prepared siRNA-loaded 2-aminoethyl methacrylate-based nanogels *via* a PRINT method.²⁴⁴ However, siRNA was readily released from the nanogel, limiting the RNA interference efficacy. Therefore, siRNA was conjugated to the nanogels by a disulfide linker, which dramatically improved RNA interference. A rather novel strategy is to use nucleic acids to crosslink delivery vehicles.²⁴⁵ Hong *et al.* crosslinked thiolated low molecular weight linear PEI with thiolated siRNA, which showed effective gene silencing.²⁴⁶ Moreover, the crosslinked

siRNA-PEI nanogel exhibited a condensed structure and caused minimal to non-observable cell toxicity.

Treatment of (chronic) inflammatory diseases with siRNA shows great potential, because of the ability to (temporarily) knock down pro-inflammatory cytokines.^{146,247} Using less invasive administration routes, such as oral delivery, expose the nanogel-siRNA complex to harsh conditions. Knipe *et al.* reported an oral formulation for inflammatory bowel disease treatment based on nanogels.¹⁴⁶ 2-(Diethylamino)ethyl methacrylate-*co-tert*-butyl methacrylate nanogels were prepared by ATRP in a water-in-oil emulsion. siRNA-nanogels were incorporated in trypsin-degradable peptide-crosslinked poly(vinylpyrrolidone)-poly(methacrylic acid) microgels to protect the nanogels during passage through the gastrointestinal tract. Fig. 18B shows that after microgel degradation, transfection in RAW 264.7 cells was comparable to non-encapsulated nanogels and Lipofectamine (a transfection agent for *in vitro* applications).

5.3 Protein delivery

Delivery of recombinant proteins can be a low-risk alternative to gene delivery, as long-term risks arising from genetic modification are avoided. However, direct administration often gives dissatisfying results due to protein instability, immunogenicity, short half-life and low cellular uptake.²⁴⁸ Encapsulation of therapeutic proteins into nanogel matrixes offers a hydrophilic and biocompatible environment, and is a very promising strategy to overcome these problems.^{51,93,248–250} Moreover, the responsive properties of nanogels offer possibilities for triggered release by incorporating enzymatically degradable,¹⁷⁵ bioreducible,^{103,251} and pH-labile bonds.^{93,160,248,252}

Enzyme encapsulation into an oligoethylene glycol matrix *via* mini-emulsion polymerization resulted in minimal enzymatic

Fig. 17 Bioreducible nanogels containing quaternized amines for controlled gene delivery (A). The nanogels were capable of delivering pDNA (B), as well as siRNA (C), with good transfection efficiencies. Reprinted (adapted) with permission from ref. 57. Copyright 2012 American Chemical Society.

Fig. 18 Microgel-encapsulated 2-(diethylamino)ethyl methacrylate-*co-tert*-butyl methacrylate nanogels for oral siRNA-delivery: after degradation of the microgel matrix, fluorescently labeled nanogels were added to murine macrophages, showing cellular uptake (A, cell nuclei are stained in blue, the cell wall in red and the nanogels in green). Delivery of TNF- α knockdown siRNA showed no significant difference between the nanogels and Lipofectamine (B). Moreover, nanogels released from degraded microgels, showed no significant change in TNF- α knockdown, compared to bare nanogels and Lipofectamine. Reprinted (adapted) with permission from ref. 146. Copyright 2016 American Chemical Society.

activity loss.^{51,252} After release from pH-degradable oligoethylene glycol nanogels, only 25% of the enzyme activity was lost.²⁵² Moreover, Steinhilber *et al.* showed the encapsulation of enzymes without loss of enzymatic activity into pH-degradable polyglycerol nanogels, using nanoprecipitation.⁹³ During nanoprecipitation, the nanogels were immediately crosslinked by click chemistry and after degradation of the benzacetal bonds enzyme activity was not noticeably reduced.

Delivery of apoptotic proteins into cancer cells can activate specific cell apoptosis pathways, such as apoptin. Zhao *et al.* prepared acrylamide-based nanogels where large anionic apoptin complexes served as a template material for cationic nanogels, as

shown in Fig. 19A.²⁵¹ Addition of *N*-(3-aminopropyl)-methacrylamide, acrylamide and *N,N'*-bis(acryloyl)cystamine and subsequent radical crosslinking resulted in 80 nm bio-reducible nanogels, which induced cell apoptosis after cellular uptake. Furthermore, *in vivo* studies in a mouse tumor graft model showed that intratumoral injections with the apoptin nanogels significantly reduced tumor volume (Fig. 19D). Encapsulation and release of cytochrome *C*, an apoptotic peptide, has been reported. Chen *et al.* prepared a block copolymer *via* RAFT polymerization consisting of a PEG block and a copolymer of 2-hydroxyethyl methacrylate and a cyclic carbonate monomer.¹⁰³ The cyclic carbonate was ring-opened with cystamine and

Fig. 19 Encapsulation of apoptin into a bio-reducible cationic nanogel (A). Nanogels effectively reduced cell viability in cancer cell lines by apoptosis delivery (B), but not on human fibroblasts (C). Moreover, the apoptin-containing bio-reducible nanogels also significantly reduced tumor volume in a mouse xenograft model (D). Reprinted (adapted) with permission from ref. 251.

immediately oxidized in the presence of cytochrome *C* to obtain disulfide crosslinked nanogels Boronic acid bearing polyglycerol nanogels, which were consecutively functionalized with primary amine and dimethylmaleic anhydride, giving them a zwitterionic character with charge conversion properties, have been reported for the delivery of cytochrome *C*.¹⁶⁰ Cytochrome *C* was encapsulated *via* nanoprecipitation and nanogel crosslinking was achieved *via* boronate ester formation with the polyglycerol backbone. Both studies revealed protein release as well as intracellular release of cytochrome *C* and the induction of apoptosis.^{103,160}

Enzymes may also be applied as a bioscavenger to remove toxins from the bloodstream. However, due to their short half-life and immunogenicity, application of recombinant enzymes as bioscavengers is not viable, not even after PEG-ylation.²⁵³ Zhang *et al.* crosslinked acrylate-modified butyrylcholinesterase into zwitterionic poly(carboxybetaine) nanogels for organophosphate removal.²⁵⁰ The incorporated enzyme showed superior blood circulation compared to the native enzyme due to the encapsulation into a zwitterionic matrix, which exhibited no observable immunogenicity.

5.3.1 Insulin delivery. On-demand delivery of insulin for treatment of diabetes mellitus may benefit from glucose-responsive delivery systems. Therefore, controlled release of insulin in the bloodstream has been investigated for phenylboronic acid-based glucose-sensitive nanogels.^{140,195} Poly-(4-vinylphenylboronic acid-*co*-2-(dimethylamino)ethyl acrylate) nanogel were templated on gold nanoparticles.¹⁴⁰ Insulin was loaded into the nanogel matrix, and released in response to increases in glucose concentration due to nanogel swelling.

Polypeptide nanogels for glucose-sensitive insulin release were prepared by crosslinking a glucose-functionalized PEG-polypeptide block copolymer with a bi-functional phenylboronic acid crosslinker in the presence of insulin.²⁵⁴ Insulin-containing nanogels were formed by crosslinking with a bifunctional, which showed insulin release upon exposure to glucose. Furthermore, cell viability in HELA cells was barely affected, while no significant nanogel-induced blood hemolysis was observed.

5.3.2 Nanogel-based vaccines. Delivery of proteins and peptides to antigen presenting cells – predominantly dendritic cells – plays a key role in the immune response modified by vaccines.²⁵⁵ Protection of these antigens and enhancements in specific uptake would promote the efficacy of vaccines, and provide important possibilities to trigger specific immune responses to cancer cells among others.^{98,256} Akiyoshi and coworkers have done extensive research on self-assembled cholesterylated pullan nanogels (CPNs).⁴⁷ For example, intranasal administration of antigen-containing CPNs in mice induced immunity against a *clostridium botulinum* type-A neurotoxin.⁹⁸ Covalently crosslinked nanogels have also been explored for vaccine delivery.^{184,257,258} Disulfide crosslinked PEI-alginate nanogels could effectively encapsulate ovalbumin, a model antigen.²⁵⁷ Ovalbumin-specific CD8⁺ T-cell counts were significantly increased after administration of disulfide crosslinked nanogels, compared to non-bioreducible nanogels or free ovalbumin. Li *et al.* incorporated thiol groups into dextran nanogels to covalently incorporate ovalbumin *via*

disulfide linkages.¹⁸⁴ Loading capacities above 10% were achieved and delivery of encapsulated ovalbumin into dendritic cells resulted in antigen presentation able to activate T-cells, which was not observed in dendritic cells exposed to ovalbumin. Synthetic poly(hydroxyethyl methacrylate-*co*-methacrylic acid) nanogels have been loaded with ovalbumin for oral delivery,²⁵⁸ where the ovalbumin was retained in the nanogel at low pH (gastric conditions) and released at physiological pH.

6 Conclusions

Polymeric nanostructures have opened up numerous avenues for the controlled delivery of therapeutics and targeted imaging. Nanogels are a relatively new delivery vehicle, which uniquely consist of a homogeneous hydrophilic matrix. Nanogels are characterized by their high water content, with potentially high drug-loading capacity and also offer interesting opportunities for the delivery of contrast agents for imaging. The preparation of nanogels is typically highly modular and the recently proposed controlled crosslinking with multifunctional monomers promises great versatility and control over particle size, chemical composition and crosslinking degree. However, it should be stressed that attention should also be directed towards biodegradability of the resulting nanogels. Currently, water-in-oil emulsions grant a large degree of freedom, with the major drawback of more extensive purification relative to other methods. Methods such as self-assembly and nanoprecipitation do not require high shear mixing and might therefore be more advantageous to encapsulate sensitive molecules, such as enzymes.

The use of highly hydrophilic materials to prepare nanogels, such as zwitterionic polymers, reduces non-specific interactions with cells and proteins and consequently improves their colloidal stability in biological environments. Manipulating the nanogel mechanical properties by controlling their crosslinking density, allows for prolonged blood circulation. However, low crosslinking densities, and thus high elasticity, make premature drug leakage from the nanogel more likely and elastic nanogels are likewise more interesting for imaging applications.

By careful monomer choice, responsive properties can be incorporated into nanogels. As a result, reversibly responsive nanogels have been successfully prepared for triggered payload release, as well as for responsive imaging. The cleavage of covalent bonds under specific conditions renders nanogels irreversibly responsive to pH, reducing environments and enzyme activity to trigger payload release, biodegradation and charge conversion. It has to be stressed that the responsive pH range should be in agreement with the targeted application and that bioreducible bonds (*e.g.* disulfides) may display cleavage already in the extracellular environment.

Besides triggered release, nanogels can also be used in responsive imaging to monitor, for instance, local pH, glucose concentration or temperature in biological systems. The application of nanogels with environmentally responsive properties for medical purposes still offers abundant opportunities, especially in the fields of MRI and optical imaging techniques.

Despite the promising results obtained in controlled drug delivery, nanogels require high crosslinking degrees for efficient encapsulation and the prevention of premature drug leakage. However, long-term stability of therapeutic nanogel formulations are yet to be evaluated before translation into clinical applications. Nevertheless, nanogels have shown great versatility in mechanisms for release and responsiveness, allowing specific design of nanogel structures for a wide range of applications. Prospected applications of nanogels in imaging include sensitive and location-specific measurement of pH, temperature, enzyme activity and metabolite concentrations, providing unprecedented levels of detail and localized information in a minimally invasive manner. Moreover, nanogels in therapeutics delivery promise efficient drug delivery and reversible responsiveness to physiological stimuli, allowing on/off switching of release to enable true on-demand delivery.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The funding of this work by the provinces of Overijssel and Gelderland, as well as the project consortium by the Center for Medical Imaging – North East Netherlands (CMI-NEN) is gratefully acknowledged.

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