Nanogels as Pharmaceutical Carriers: Finite Networks of Infinite Capabilities

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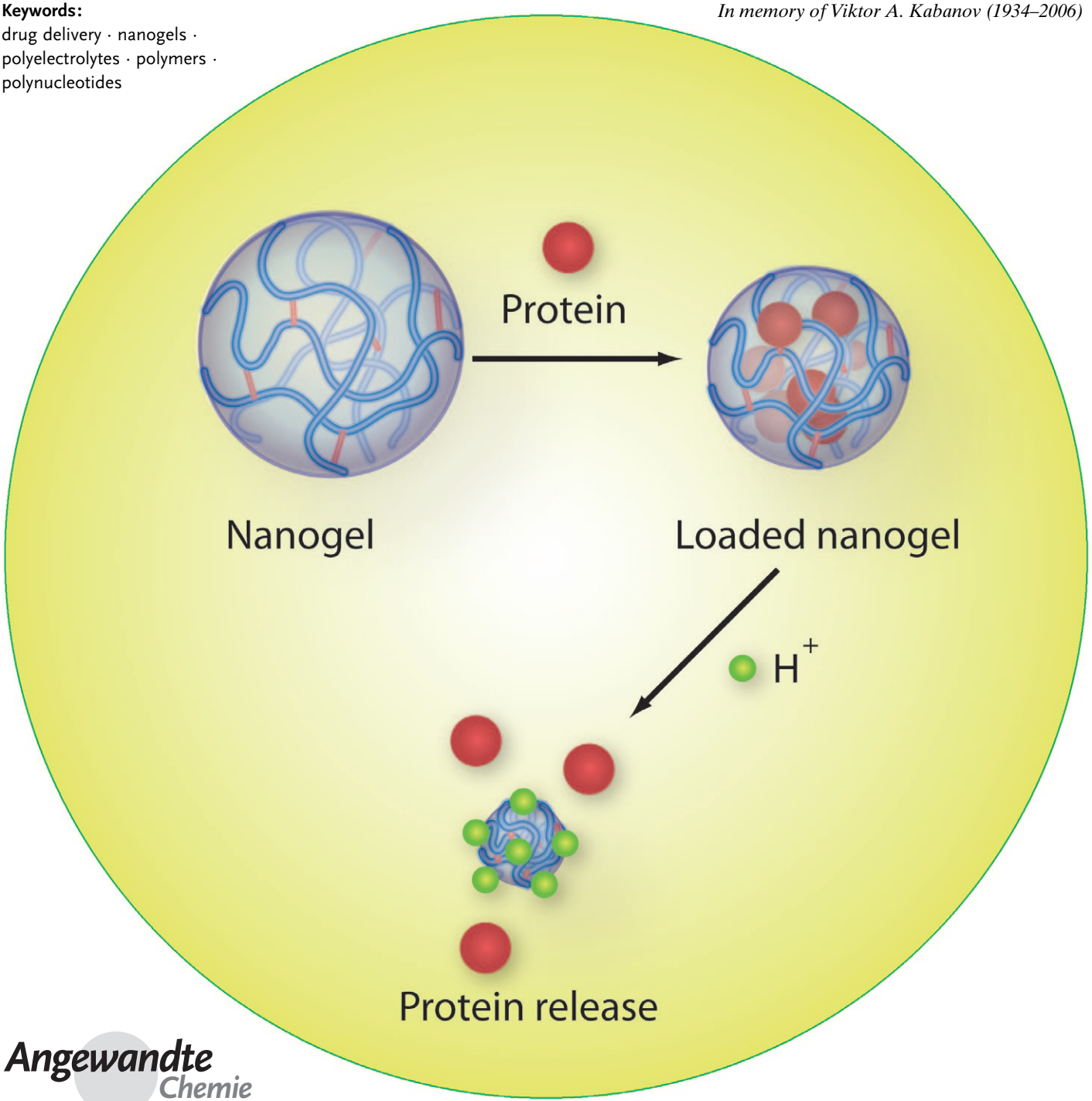
In memory of Viktor A. Kabanov (1934–2006)



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Nanogels are swollen nanosized networks composed of hydrophilic or amphiphilic polymer chains. They are developed as carriers for the transport of drugs, and can be designed to spontaneously incorporate biologically active molecules through formation of salt bonds, hydrogen bonds, or hydrophobic interactions. Polyelectrolyte nanogels can readily incorporate oppositely charged low-molecular-mass drugs and biomacromolecules such as oligo- and polynucleotides (siRNA, DNA) as well as proteins. The guest molecules interact electrostatically with the ionic polymer chains of the gel and become bound within the finite nanogel. Multiple chemical functionalities can be employed in the nanogels to introduce imaging labels and to allow targeted drug delivery. The latter can be achieved, for example, with degradable or cleavable cross-links. Recent studies suggest that nanogels have a very promising future in biomedical applications.

From the Contents

1. Introduction

The term "nanogels" usually defines aqueous dispersions of hydrogel particles formed by physically or chemically cross-linked polymer networks of nanoscale size. We introduced this term to define the swollen chemically cross-linked networks of cationic and neutral polymers such as the branched PEG-*cl*-PEI made from polyethylenimine (PEI) and poly(ethylene glycol) (PEG) which was initially designed for the delivery of antisense oligonucleotides.[1,2] However, prior to our work the research group of Sunamoto described the phenomenon of the physical cross-linking (self-assembly) of cholesterol-modified polysaccharides (for example, pullulan, mannan, amilopectin and dextran), which resulted in the formation of swollen hydrogels of nanoscale size;[3] a review on this type of systems was recently published.[4] Technically, these systems are also nanogels, and we will refer to them as nanogels here.

Nanogels are very promising as drug-delivery carriers because of their high loading capacity, high stability, and responsiveness to environmental factors, such as ionic strength, pH, and temperature that are unprecedented for common pharmaceutical nanocarriers. Since the first review on synthesis and application of nanogels published in 2002,[5] this novel family of nanoscale materials has attracted increasing attention for the delivery of drugs, biomacromolecules and imaging agents. The present paper provides an updated overview of pharmaceutical use and potential of nanogels. We also recommend recent reviews for additional reference on synthesis and applications of nanogels.[6,7]

Unloaded nanogels in a swollen state contain considerable amount of water. Loading of biological agent(s) is usually achieved spontaneously through electrostatic, van der Waals and/or hydrophobic interactions between the agent and the polymer matrix. As a result, the nanogels collapse forming stable nanoparticles, in which biological agent becomes entrapped. The aggregation of nanogels can be prevented by introducing dispersing hydrophilic polymers, such as poly(ethylnene glycol) (PEG) in their structure. During the collapse of the drug-nanogel complex such polymers become exposed at the surface and form a protective hydrophilic layer around the nanogel that prevents phase-separation. The functional groups at the nanogel surface can be additionally modified with various targeting moieties for site specific drug delivery in the body. Various nanogels have been shown to deliver their payload inside cells and across biological barriers. Such nanogels exhibit high stability and protect biological agents from degradation by cell's metabolic systems. Overall nanogels demonstrate excellent potential for systemic drug delivery and enhancing oral and brain bioavailability of low-molecular-weight drugs and biomacromolecules.

2. Preparation of Nanogel Networks

Current approaches used for the preparation of nanogels can be divided into 1) physical self-assembly of interactive polymers; 2) polymerization of monomers in a homogeneous phase or in a micro- or nanoscale heterogeneous environ-

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ment; 3) cross-linking of preformed polymers; and 4) template-assisted nanofabrication of nanogel particles. These methods are illustrated in Figures 1–4 and described below.

The physical self-assembly of polymers was used by several research groups to produce various nanogels. This method usually involves controlled aggregation of hydrophilic polymers capable of hydrophobic or electrostatic interactions and/or hydrogen bonding with each other. The preparation of nanogels is conducted in mild conditions and in aqueous media. Self-associating hydrophilic polymers allow encapsulation of biomacromolecules, and are useful for the preparation of protein-loaded nanogels. For example, Akiyoshi et al. prepared hydrogels by the hydrophobic association of cholesterol-modified pullulan in the presence of insulin (Figure 1 a).[8] The nanogels formed in a narrow range of cholesterol/sugar units ratio (1:40–1:100) with a diameter of 20–30 nm and contained up to five insulin molecules per particle. The sizes of the self-assembled nanogels are controlled by appropriate selection of the concentration of the polymers and environmental parameters, such as the pH value, ionic strength, and temperature. For example, Yu et al. prepared protein nanogels by temperature-induced gelation of oppositely charged proteins, such as ovalbumin and lysozyme or ovotransferrin.[9] Similarly, nanogels were obtained by pH- and temperature-induced gelation of chitosan and ovalbumin.[10] A study by Gref and co-workers described the self-assembly of nanogels of various sizes by association of a lauryl-modified dextran and a β-cyclodextrin polymer in aqueous media (Figure 1 b).[11] Gels of about 120–150 nm were obtained by variation of the two polymers over a wide concentration range. The resulting nanogels were stable and withstood freeze-drying, which was found to be a convenient method for their long-time storage.

Chemical synthesis in heterogeneous colloidal environments can generally provide opportunities to vary the structure and properties of the nanogel. Few studies used inverse water/oil (w/o) microemulsions as a medium for the polymerization of monomers, with bifunctional monomers added as cross-linkers to ensure formation of stable nanoscale networks (Figure 2 a). Speiser et al. were the first to carry out the copolymerization of monomers in reverse micelles.[12] Their approach was extended by Levashov and co-workers to covalently immobilize enzymes in polymeric nanogranules of acrylamide and *N*,*N*-methylenebisacrylamide copoly-

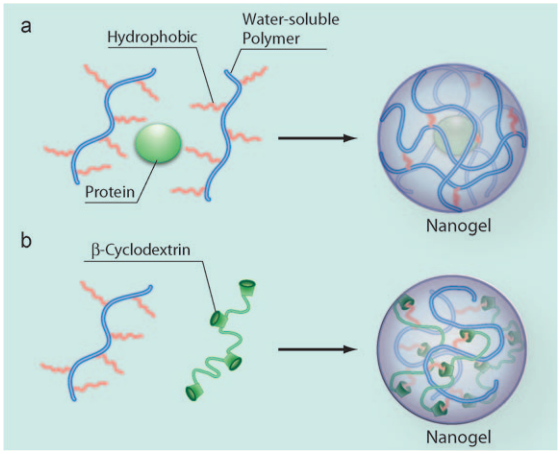

Figure 1. Physical self-assembly of nanogels in aqueous media. a) Aggregation of the hydrophobically modified polymer cholesterol-pullulan in the presence of insulin molecules results in nanogels containing entrapped protein. b) Mixing of lauryl-modified dextran and a β-cyclodextrin polymer results in the formation of nanogels stabilized through the host–guest binding of the β-cyclodextrin and lauryl moieties.

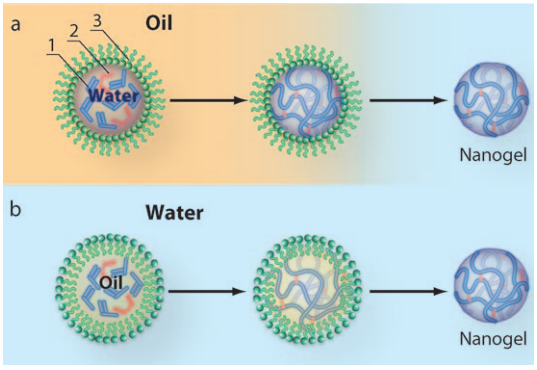

Figure 2. Chemical synthesis of nanogels by copolymerization in colloidal environments. a) Copolymerization of monomers (1) and bifunctional cross-linkers (2) in w/o microemulsions stabilized by surfactants (3) produces nanogels that can then be transferred into aqueous media after removal of the surfactants and organic solvent. b) Copolymerization reactions can also be carried out in o/w emulsions that can be additionally stabilized by surfactants.

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mers.[13] Subsequently, DeSimone and co-workers prepared cationic PAETMAC nanogels by inverse microemulsion polymerization of 2-acryloxyethyltrimethylammonium chloride and 2-hydroxyethylacrylate in heptane with PEG-bis-acrylate used as a bifunctional cross-linker.[14,15] Anionic nanogels with particle hydrodynamic diameters as small as 50 nm were also prepared by copolymerization of poly(dimethylacrylamide-*co*-2-acrylamido-2-methyl-1-propanesulfonic acid) and the *N*,*N*-methylenebisacrylamide cross-linker in an inverse microemulsion.[16]

Labile bonds are frequently introduced into nanogels during polymerization to make them degradable and facilitate drug release. For example, Fréchet and co-workers reported a free-radical polymerization in an inverse emulsion that led to the preparation of degradable acrylamide-based nanogels containing acid-labile acetal cross-linkers for protein, antigen, and DNA delivery.[17] The acetal group is stable at pH 7 ($t_{1/2} = 24$ h), but rapidly hydrolyzes at acidic endosomal pH values ($t_{1/2} = 5$ min), which results in degradation of the nanogel and facilitates the release of the payload.[18,19] The Matyjaszewski research group used atom-transfer radical polymerization (ATRP) in an inverse microemulsion for the synthesis of stable cross-linked nanogels of water-soluble polymers.[20] They used a disulfide-functionalized cross-linker to synthesize biodegradable nanogels. Disulfide groups are stable in extracellular media but cleave inside cells because of the presence of glutathione. This may also facilitate the release of the nanogel payload inside the cells. Matyjaszewski, Kataoka, and co-workers further extended this approach to the synthesis of biodegradable, cross-linked poly-[oligo(ethylene oxide)-methyl methacrylate] nanogels.[21]

Polymerization reactions that result in the formation of nanogels can also be carried out in o/w emulsions or aqueous suspensions (Figure 2b). Furthermore, the polymerization can be started in a homogeneous aqueous solution of water-soluble monomers, which results in the formation of a colloidal suspension of the growing polymer. For example, Peppas and co-workers synthesized a suspension of nanospheres composed of PEG-grafted poly(methacrylic acid) (PMA) in water by using a UV-initiated solution/precipitation polymerization method.[22]

In addition to these polymerization methods, covalent cross-linking of preformed polymer chains provide excellent opportunities for producing nanogels with large pore sizes.[23] The cross-linking method was widely used for the synthesis of a variety of functional nanogels for drug delivery. In particular, it was used to synthesize the first cross-linked cationic nanogel for polynucleotide delivery.[1] In this case, a doubly activated PEG was conjugated to a branched PEI in an o/w emulsion (dichloromethane in water), followed by evaporation of the solvent in vacuo and maturation of the nanogel in an aqueous solution (Figure 3a). Cationic PEI-containing nanogels of 80–200 nm diameter were also obtained by the photo-Fenton reaction in aqueous media.[24,25] Small (40–45 nm) nontoxic cross-linked pullulan nanogels were prepared in the reverse micellar system (aerosol OT/hexane).[26,27] Similar to the case of polymerization reactions, the cross-links that connect the polymeric chains in such nanogels can be made degradable. For example,

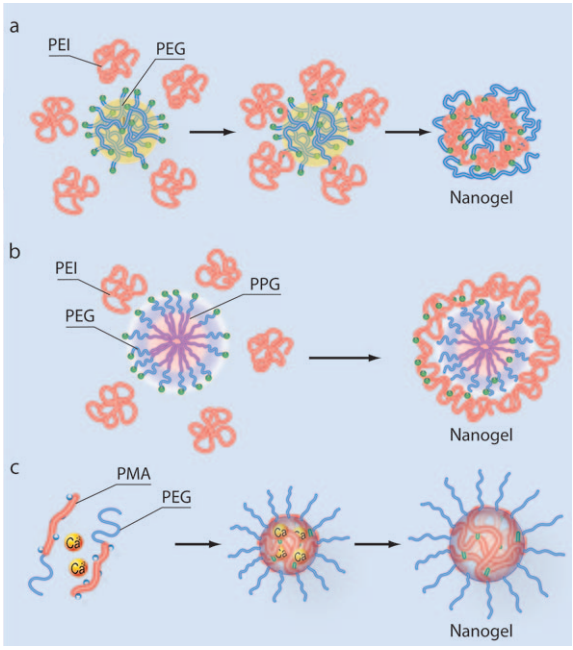

Figure 3. Synthesis of nanogels by cross-linking of the preformed polymer chains or self-assembled polymeric aggregates. a) Cross-linking of PEG and PEI chains with two activated termini in an o/w emulsion followed by evaporation of the organic solvent. b) Conjugation of PEI to an activated pluronic block copolymer (PEG-PPG-PEG) with two activated termini which self-assembles into polymeric micelles in aqueous solution results in nanogels containing hydrophobic PPG domains and a cross-linked PEI-PEG network. c) The diblock copolymer PEG-*b*-PMA contracts in the presence of divalent metal cations in an aqueous solution into a micelle with a polyion-metal core and a PEG corona. This is followed by cross-linking of the micelle core and removal of the metal cations, which results in nanogels with a cross-linked polyanion (PMA) core and a PEG corona.

a biodegradable segmented PEI connected by disulfide linkers was used to prepare cationic nanogels for polynucleotide delivery with reduced toxicity.[28,29] In another study hyaluronic acid (HA) nanogels containing biodegradable disulfide linkages were prepared by the inverse w/o emulsion method.[30] An interesting type of cross-linked nanogels containing DNA was obtained by mixing thiol-functionalized six-arm-branched PEG and DNA in dimethyl sulfoxide, which produced particles of 100 nm diameter. These nanoparticles were then cross-linked by oxidation to obtain DNA-loaded biodegradable nanogels.[31]

Unprecedented opportunities for the control of the spatial distribution of polymeric chains at the nanoscale are provided by combining the controlled self-assembly of polymeric micelles and cross-linking techniques. For example, Wooley and co-workers have chemically linked the shell layers of polymeric micelles to obtain various shell-cross-linked nanoparticles.[32–37] By adjusting factors that affect the morphology, such as solvents and organic counterions, they produced an array of different cross-linked nanostructures including spheres, rods, and even toroids.

In another study, PEI was cross-linked in aqueous solution to the micelles of doubly activated pluronic triblock copolymer (PEG-*b*-PPG-*b*-PEG; Figure 3b; PPG = poly(propy-

lene glycol)).[38] This produced nanogels with a hydrophobic PPO core surrounded by a swollen cross-linked PEI and a PEO shell (PEG-*cl*-PEI). Lee et al. photo-cross-linked the polymeric micelles of poly(D,L-lactic acid)-*b*-PEG-*b*-poly(D,L-lactic acid) with acrylate end groups.[39] As a result, nanogels were formed which contained self-assembled hydrophobic domains of micelles with insoluble poly(D,L-lactic acid) cores, which could be loaded with a hydrophobic anticancer drug.

A unique control of spatial distribution of polymer chains in a nanogel was achieved by Bronich et al.[40] They developed a procedure in which polyelectrolyte micelles were initially prepared by the self-assembly of ionic blocks of double hydrophilic block copolymers with an oppositely charged condensing agent. This was followed by chemical cross-linking of ionic blocks in the core and removal of the condensing agent (Figure 3c). The resulting nanogels made from PEG-*b*-poly(methacrylic acid) (PEG-*b*-PMA) diblock copolymers contained a hydrophilic PEG shell and a cross-linked hydrophilic PMA ionic core which swell in water and can incorporate hydrophilic drugs.[41] A similar technique was also used to prepare core-shell nanogels by the condensation and cross-linking of PEG-grafted poly(acrylic acid) (PEG-*g*-PAA).[40]

Finally, DeSimone and co-workers developed a novel method for the fabrication of polymeric particles with sizes on the order of tens of nanometers to several micrometers, which can be used for the synthesis of nanogels.[42] This imprint photolithographic technique (particle replication in Nonwetting templates PRINT) uses nonwetting elastomeric molds of a low surface energy perfluoropolyether network prepared on patterned silicon templates by photochemical cross-linking of dimethacrylate-functionalized perfluoropolyether oligomers. The nonwetting molds eliminate the formation of a residual interconnecting film between the molded objects, thus allowing the production of monodisperse, shape-specific nanoparticles from an extensive array of organic precursors (Figure 4). This method enables strict control over the particle size, shape, composition, and surface functionality, and permits the loading of delicate cargos, including pharmaceutical drugs and biomacromolecules. For example, monodisperse 200 nm PEG-based swellable particles were fabricated with the PRINT method by UV-induced copolymerization of several vinyl monomers such as PEG triacrylate, PEG monomethyl ether monomethacrylate, and *p*-hydroxystyrene.[43]

Many studies have used one of the basic procedures described above to prepare increasingly sophisticated types of nanogels. For example, a nanostructured thermosensitive hydrogel based on an interpenetrating network of poly-(*N*-isopropylacrylamide) (PNIPAAm) was immobilized on porous silica gel and hydroxyapatite.[44,45] Richtering and co-workers used the layer-by-layer deposition of polyelectrolyte multilayers at the surface of an anionic PNIPAAM-*co*-PMA nanogel to prepare thermosensitive core-shell materials.[46,47] Layer-by-layer deposition can be used to introduce various materials, such as magnetic nanoparticles, at the surface of the nanogels.[48]

Another interesting type of hybrid nanogels covered by a lipid bilayer was recently introduced by the Levon and Hennink research groups.[49,50] They prepared liposomes loaded with a water-soluble monomer, which was then polymerized by photoinitiation to form a network of polyacrylamide (PAA) within the inner cavity of the liposome. Yan et al. produced catalytic nanogels encapsulating horseradish peroxidase or bovine carboxyanhydrase by polymerization of PAA and*N*,*N*-methylenebisacrylamide on the surface of a single enzyme molecule.[51,52] The enzymes maintained their catalytic activity and exhibited high thermal stability as a result of immobilization within the nanogel particles. In other studies PEGylated nanogels embedded with metal nanoparticles were synthesized by Oishi et al.[53,54] Such hybrid nanogels containing Pt nanoparticles (<2 nm) have shown catalytic activity which is dependent on the pH value, and can be used as scavengers of reactive oxygen species in biological and medicinal applications. The nanogels containing Au nanoparticles (ca. 6 nm) exhibited a shift of the surface plasmon resonance (SPR) band in response to a change in the pH value. Such nanogels may be adopted as SPR probes in various types of sensors. Finally, the photochemical emulsion-free polymerization on the surface of superparamagnetic ferrous oxide nanoparticles in aqueous solution was used for the preparation of composite superparamagnetic nanogels,[55,56] which may be used for microwave ablation therapy.[57]

Future developments in the preparation of chemically or physically cross-linked nanogels may include the use of micropflidic techniques, which have shown promise in the preparation of micrometer-sized hydrogels and nanomaterials of different sizes, shapes, and morphologies.[58,59]

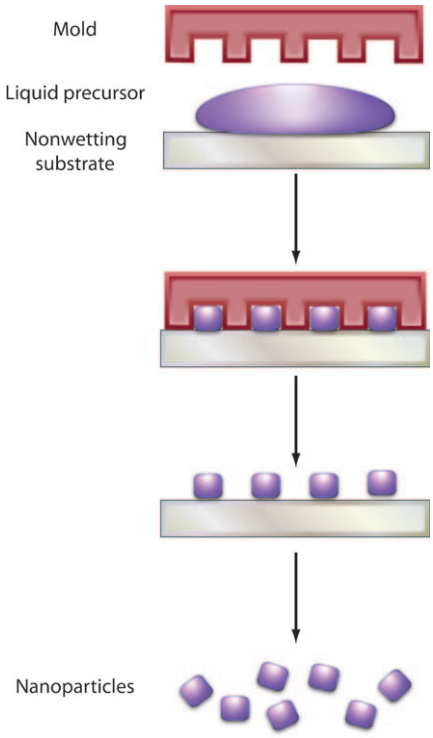

Figure 4. Nonwetting elastomeric perfluoropolyether molds are used in the photolithographic technique PRINT to produce monodisperse, shape-specific nanoparticles from various organic precursors.

3. Chemical Modification of Nanogels for Site-Specific Drug Delivery

Nanocarriers can be delivered to disease-affected sites after injection in body fluids. Major impediments to this delivery strategy include: 1) the interaction of nanocarriers with serum proteins, thereby resulting in opsonisation or agglutination; 2) the clearance of the nanocarriers by the reticuloendothelial system (RES) or through kidney glomerules, and 3) nonspecific accumulation in organs. To reduce the interaction with serum proteins and extend circulation time, the nanocarrier surface is often modified with inert hydrophilic polymers, such as PEG.[60] For example, drug-loaded PEG-*cl*-PEI nanogels have a core-shell architecture in which the core is surrounded by PEG chains.[61] Similarly, PEG chains can be tethered to polymethacrylate nanogels during the emulsion polymerization procedure.[62] Nanogels with cross-linked cores and PEG corona can be produced by the self-assembly of polyelectrolyte micelles followed by cross-linking of the cores.[40]

A recent study characterized the in vivo pharmacokinetics and biodistribution of PEG-based cross-linked nanogel cylinders (ca. 200 nm) obtained with the PRINT technique.[43] The particles were cleared relatively rapidly from the blood ($t_{1/2} = 17$ min) and accumulated in the liver. This result was not surprising since the nanogels contained relatively short PEG chains (ca. 1 kDa), while the optimal coating for long-circulating nanoparticles would have PEG between 2 and 5 kDa.[63]

The nanogel "surface" can also be decorated with biospecific targeting groups, which can enhance the site-specific delivery of the nanogels in the body. For example, we described biotinylated PEG-*cl*-PEI nanogels that were vectorized with (strepta)avidin by biotinylated ligands (transferrin or insulin).[64] Biotin groups were also attached to OH-functionalized poly[oligo(ethylene oxide)-methyl methacrylate] nanogels.[21] However, biotin-(strepta)avidin conjugation is not practical because of the biological activity of biotin. Hence, it is preferable to directly conjugate the targeting groups to the nanogels. For example, after carbodiimide activation, 1–5 % of the primary amino groups of a PEG-*cl*-PEI nanogel were modified with folic acid.[61] These folate-modified nanogels exhibited a noticeable increase in the transport across a gastrointestinal model barrier (Caco-2 cell monolayers) in vitro. In another study, polymethacrylate microgels modified with folate demonstrated increased and selective cellular uptake in cancer cells overexpressing folate receptors (FR).[65] To reduce problems associated with the accessibility of folate to its cellular receptors, several research groups recommended insertion of a polymer linker (for example, PEG) between the folate moiety and drug carrier.[66] For example, the terminal amino groups of PEG in a poly(aminoPEG-cyanoacrylate-*co*-hexadecyl cyanoacrylate) nanogel were modified with folic acid.[67] Such folate-decorated nanogels also demonstrated enhanced accumulation in FR-overexpressing cancer cells.

Nanogels were also conjugated with human transferrin (hTf), a tumor-targeting protein.[68] In this method, amino groups in hTf were first treated with the heterofunctional reagent sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate in an aqueous medium to obtain a maleimide derivative of hTf. In a second step, thiol groups were then introduced into the PEG-*cl*-PEI nanogels by reaction with 2-iminothiolane (Trout's reagent). Finally, the reaction between maleimide-hTf and the thiol groups of the nanogel led to the formation of hTf-decorated nanogels with a controlled number of hTf molecules (from 4 to 12) per particle. The large size of the conjugated hTf resulted in it being exposed at the nanogel surface, which facilitated its interaction with cellular transferrin receptors.

Additionally, peptide ligands can be attached to nanogels through a bifunctional PEG linker.[28] For example, a peptide with terminal cysteine groups was conjugated to a maleimide-PEG-*N*-hydroxysuccinimide linker. The product was then reacted with the amino groups of PEI to obtain a decorated nanogel with the required peptide density. Finally, a mono-*N*-acetylcystamine-PEG linker was introduced into nanogels by 1,1′-carbonyldiimidazole activation; the thiol groups on the nanogels were unmasked with dithiothreitol and treated with the thiol-specific (for example, maleimide) derivatives to yield the protein/peptide-modified nanogels.[69]

In summary, nanogels provide numerous possibilities for their surfaces to be decorated with various targeting groups. Initial evidence suggests that these approaches can be used to transport nanogels to selected cellular receptors.

4. Swelling: The Most Important Property of Nanogels

Nanogels are soft nanomaterials. The swelling of nanogels in an aqueous environment is controlled by both 1) the nanogel structure (chemical structure of the polymer, degree of cross-linking, charge density in the polyelectrolyte gels), and 2) environmental parameters, such as pH value, ionic strength, and chemical nature of low-molecular-mass ions for polyelectrolyte gels as well as temperature for thermoresponsive gels (Figure 5). It is well known that a balance between the osmotic pressure and the polymer elasticity determines the physical dimensions of a hydrogel particle.[70] The osmotic pressure of polyelectrolyte hydrogels results from the net difference in the concentration of mobile ions between the interior of the gel particle and the exterior solution. The ionized groups attract hydrated counterions. This favors the swelling of the gel, while the entropy elasticity of the polymer chains opposes the expansion. The ionization of weak polyelectrolyte gels depends on the pH value. A reduction in the total charge and number of counterions as the pH changes results in compression of the gel (because of decreased osmotic pressure) until the excluded volume of the polymer chains limits further compression. For example, cross-linked PEG-*b*-PMA nanogels compressed as the pH value decreased from 9 to 5 as a result of protonation of the carboxylate groups of PMA.[41] Likewise, the PEG-*cl*-PEI nanogel compressed as the pH value increased from 8.5 to 10 through deprotonation of the PEI amino groups.[71]

The swelling of polyelectrolyte hydrogels also depends on the ionic strength. For example, at high ionic strength, the

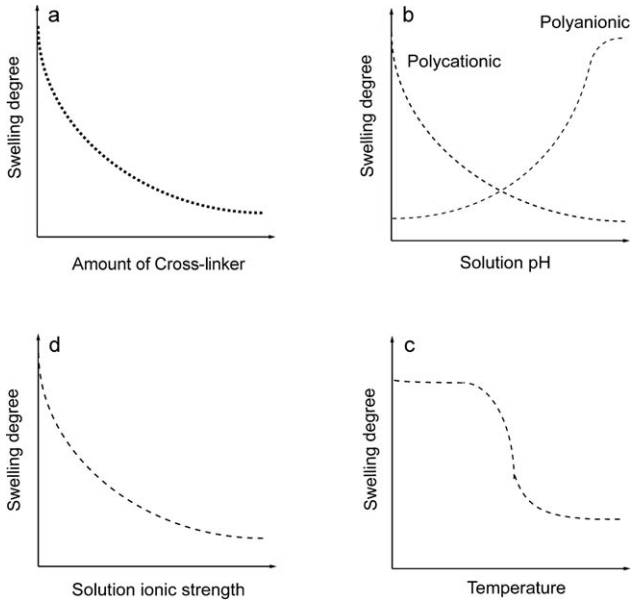

Figure 5. Factors affecting nanogel swelling. a) An increase in the amount of cross-linking decreases the swelling of the nanogels comprised of a hydrophilic polymer. b) An increase in the pH value results in the collapse of a nanogel comprised of weak polybase chains, and the swelling of nanogels comprised of a weak polyacid. c) An increase in the ionic strength decreases the swelling of polyelectrolyte nanogels. d) Nanogels comprised of polymers exhibiting LCST behavior collapse as the temperature increases above the LCST.

swelling of cationic PAETMAC nanogels is governed by the concentration of the cross-linker, while at low ionic strength the swelling is influenced by both the cross-linker and the concentration of the charge.[14] As a general rule, the swelling ratio of cross-linked hydrogels decreases as the number of cross-links increases.[14,41] This was shown by using cross-linked PEG-*b*-PMA nanogels as an example.[32]

In selected cases, the interactions between the solvent and the polymer chains of the nanogels are temperature dependent, and can lead to swelling or collapse of the gels. For example, since PPG chains in pluronic polymers exhibit a lower critical solution temperature (LCST), the pluronic-based nanogels are temperature-responsive. For example, small 120 nm nanogel particles were shown to swell drastically to over 400 nm upon a rapid decrease in the temperature below the LCST.[72] A temperature dependence of the swelling was also observed for nanogels of *N*-isopropylacrylamide (NIPAAm) copolymers.[45,73] This property can be used to engineer stimuli-responsive drug carriers. One recent example is glucose-sensitive phenylboronic acid conjugated NIPAAm nanogels which have a swelling behavior that depends on the glucose concentration.[74] One of the advantages of highly dispersed hydrogels is that they usually respond very rapidly to changing environmental conditions,[75] which facilitates the incorporation and release of biological agents in pharmaceutical applications. In contrast, the swelling equilibrium for bulky hydrogels requires periods on the order of days. In selected cases, the loading of drugs in the hydrogel can additionally reduce the volume. One reason for this is that the drug interacts with the hydrogel chains through electrostatic binding, hydrophobic interactions, and/or hydrogen-bond formation, which decreases the "solubility" of the hydrogel chains and results in contraction and collapse of the gel. The swelling and collapse of nanogels are unique properties for optimizing drug loading and release (see Section 5).

5. Loading Nanogels with Drugs and Their Release

Biological agents can be incorporated in nanogels by 1) physical entrapment, 2) covalent conjugation, or 3) controlled self-assembly. Physical entrapment was employed for the incorporation of insulin in cholesterol-modified pullulan nanogels[8] and siRNA in thiol-conjugated hyaluronic acid nanogels.[30] The physical entrapment of drugs can also be achieved by the complexation of dextrans with poly-L- and poly-D-lactide side chains into monodisperse biodegradable nanogel particles with an average diameter of 70 nm.[76] Such nanogels contain hydrophilic dextran chains joined by partially crystallized hydrophobic regions of noncovalently bound chains of polylactide stereisomers.

In addition, hydrophobic molecules can be solubilized in the hydrophobic domains present in some nanogels. For example, prostaglandin E_2 was solubilized in nanogels of cholesterol-modified pullulan.[77] Doxorubicin was also loaded in amphiphilic cross-linked nanogels based on pluronic F127[78] or poly[oligo(ethylene oxide)-methyl methacrylate].[21] Notably, in most cases loading arising from hydrophobic interactions alone results in relatively low loading capacities.

An example of the covalent attachment of a biological agent to a nanogel is the loading of cisplatin in PEG-*b*-PMA.[79,80] Such nanogels have a cross-linked polyanionic (PMA) core and a neutral polymer (PEG) corona. In aqueous solution cisplatin reacts with the carboxylic groups in the core of the gels, which leads to collapse of the drug-loaded core.

Modification of enzyme molecules with *N*-hydroxysuccinimidoacrylate followed by polymerization of the acrylamide in dilute aqueous solutions was used to obtain protein-encapsulating polyacrylamide nanogels.[51,52] Alternatively, polyacrylamide nanogels incorporating modified α-chymotrypsin were prepared by copolymerization in an inverse microemulsion.[13] Such nanogels which contain covalently bound proteins can increase the thermostability and shelf life of the protein.[56]

A different approach based on the controlled self-assembly of polyelectrolyte-based nanogels with oppositely charged solutes can produce nanomaterials with a high loading of biological agents. Various polyelectrolyte-based nanogels were shown to interact with oppositely charged surfactants, synthetic polyelectrolytes, polynucleotides, and proteins in aqueous solutions.[1,69,71,81,82]

The self-assembly of such materials is usually characterized by high binding cooperativity and efficiency. The binding of an anionic surfactant, such as sodium tetradecyl sulfate, with cationic PEG-*cl*-PEI nanogels has an onset at a "critical association concentration" (cac) which is two orders of magnitude lower than the critical micelle concentration

(cmc) of this surfactant alone.[71] Such a drastic difference in these values is explained by cooperative stabilization of surfactant aggregates as a result of electrostatic interactions between the surfactant head groups and the PEI chains of the nanogel. Charged and amphiphilic biological active molecules such as sodium oleate, indomethacin, and retinoic acid were also incorporated into PEG-*cl*-PEI nanogels.[71] For example, nanogels loaded with retinoic acid formed nanosized dispersions that were stable at physiological pH and ionic strength, and which could be lyophilized, stored, and then redispersed. A similar formulation of the antiepileptic drug valproic acid in PEG-*cl*-PEI nanogels was also obtained and investigated in vitro.[69] Furthermore, hydrophobic regions present in PEG-*cl*-PEI/surfactant complexes can serve as non-aqueous reservoirs for solubilizing water-insoluble molecules. Since a combination of polyionic and hydrophobic interactions stabilizes drug-nanogel formulations, anionic compounds bearing only a few charges, for example, 5′-triphosphates of nucleoside analogues, such as fludarabine, zidovudine, cytarabine, and floxuridine, can also be efficiently loaded into cationic nanogels.[29,38,61]

Recently, the properties and prospective applications of this type of nanoformulations were reviewed.[83] In summary, polyelectrolyte nanogels can be a versatile platform for the incorporation of various small drug molecules through the combination of electrostatic and hydrophobic interactions as well as hydrogen-bond formation.

One of the most important features of weakly cross-linked polyelectrolyte nanogels is their ability to incorporate biomacromolecules of the opposite charge. The accommodation of biomacromolecules in hydrogels is usually hindered by the excluded volume and cross-linking density. However, if the biomacromolecules and polymer network have opposite charges, they react effectively with each other to form a polyelectrolyte complex. In the cases when the polyelectrolyte chains penetrate the nanogels, the process develops as a frontal reaction between oppositely charged polyions and spreads from the exterior of the gel to its core.[84] As a result, efficient loading of biomacromolecules can be achieved even with bulk polyelectrolyte networks (Figure 6).[85]

This principle has been exploited to immobilize polynucleotides in cationic nanogels. Both PEG-*cl*-PEI and PAETMAC have been used to incorporate antisense oligonucleotides.[1,14] The addition of oligonucleotides to a PEG-*cl*-PEI nanogel dispersion at physiological pH resulted in the rapid formation of polyelectrolyte complexes between the oligonucleotide and the PEI. This process was accompanied by an approximately 10-fold reduction in the nanogel volume ("collapse") by neutralization of the charges in the network. Notably, the binding of the oligonucleotide with the nanogel was almost complete, and the loading capacity of the nanogel was 15 to 30 wt%. Interestingly, the oligonucleotide-loaded nanogels remained stable in the presence of negatively charged serum proteins, which can be explained by a higher binding cooperativity between the oligonucleotide chains and the polycation compared to the protein–oligonucleotide binding.[86]

In general, higher drug-loading capacities can be expected for hydrophilic nanogels than those normally observed for

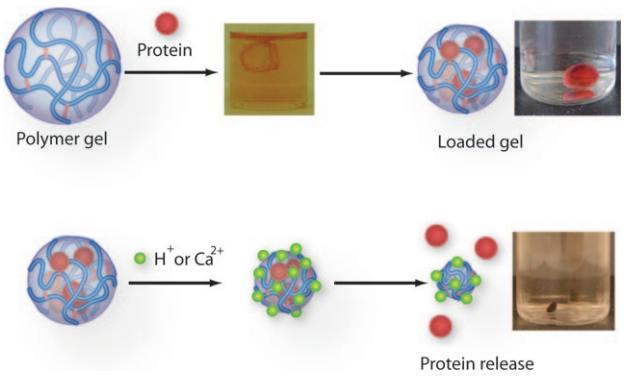

Figure 6. Loading and release of biomacromolecules in a cross-linked polymer hydrogel. A cross-linked gel comprised of neutral PEG and anionic PAA polymer chains is immersed in a solution of a cationic protein cytochrome c. The cytochrome c is spontaneously loaded into the gel through the formation of a complex between the protein and PAA chains. The gel collapses and acquires the red color of cytochrome c, while the external solution becomes clear. Acidification or addition of Ca^{2+} ions results in protein release as a result of either deprotonation of the carboxylic groups of the PAA (acidification) or competitive binding of the Ca^{2+} ions with the carboxylic groups of the PAA. In both cases the external solution acquires the red color of cytochrome c, while the gel further collapses.

other nanosized pharmaceutical carriers such as polymeric micelles, liposomes, and biodegradable nanoparticles. The main reason for this is that swollen nanogels are mainly comprised of water and, therefore, provide a larger cargo space for the incorporation of solutes, which is important in the case of low-molecular-mass drugs and, especially, biomacromolecules. Furthermore, high loading in nanogels can be achieved by self-assembly and under relatively mild conditions compared to other carriers. This property is very important for the preservation of the biological activity of labile drugs and biomacromolecules, such as proteins and polypeptides.

The biological agents can be released from nanogels through 1) simple diffusion, 2) degradation of the nanogel; 3) a shift in the pH value, 4) displacement by counterions present in the environment, or 5) transitions induced by an external energy source (Figure 7). Examples include diffusion-controlled release of doxorubicin from pluronic-based hydrogels.[87] A similar release mechanism has been employed in polymeric micelles which are already in clinical studies.[88] There is also increased interest in developing nanogels that can release biological agents in response to environmental signals at the disease site. As already discussed, a change in the pH value or the presence of a reducing environment can serve as chemical signals that trigger the release. For example, an acrylamide-based nanogel with acetal cross-links is stable at an extracellular pH value of 7.4, but degrades and releases entrapped protein at pH 5.0.[17] Similarly, the PEG-*cl*-PEI nanogels with disulfide cross-links rapidly degraded in the presence of a reducing agent.[28,29] Likewise, a poly-[oligo(ethylene oxide)-methyl methacrylate] nanogel with disulfide cross-links degraded in the presence of a glutathione tripeptide commonly found in cells.[21] The degradation of these nanogels was shown to trigger the release of the

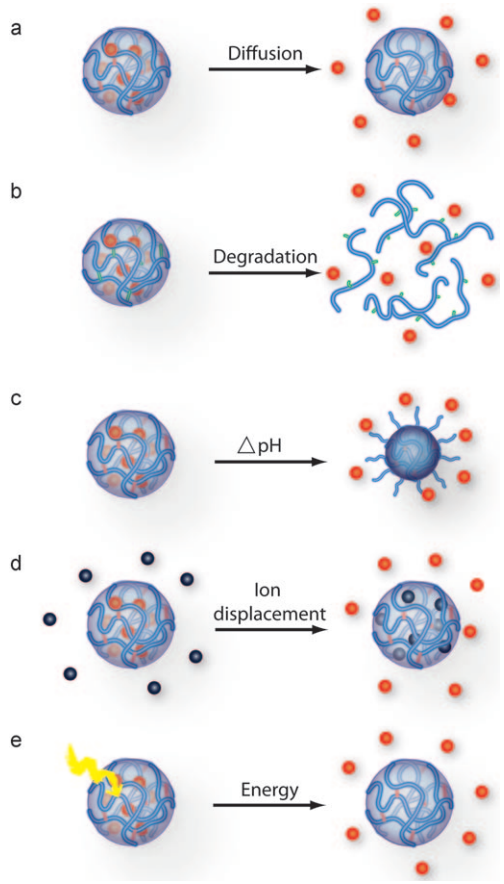

Figure 7. Drug release from nanogels. a) Diffusion of the drug from nanogels. b) Drug release through degradation of the biodegradable polymer chains or cross-links. c) A change in the pH value results in deionization of the polymer network and the release of the electrostatically bound drug. d) Multivalent low-molecular-mass cations or polyions of positive or negative charge can displace drugs having the same charge sign from electrostatic complexes with an ionic nanogel. e) Drug release can be induced by the application of external energy to the nanogels which induces degradation or structural transition of the nanogel polymer chains.

encapsulated low-molecular-mass solutes rhodamine 6G and doxorubicin. In another study, the dissolution of disulfide cross-linked HA nanogels and release of siRNA was induced by adding glutathione.[30] Clearly, the release kinetics can be fine-tuned in each case by altering the number of cross-links.

Polyelectrolyte hydrogels that incorporate biological agents through electrostatic bonds can also release biological agents in response to environmental changes. For example, pH-sensitive nanogels based on PAA can release an oppositely charged protein in tumor sites or endosomal compartments upon acidification.[10,73,89,90] A different mechanism was proposed for the release of nucleotide drugs from cationic PEG-*cl*-PEI nanogels.[91] In this case, negatively charged biomacromolecules bound to nanogels can be displaced by negatively charged cellular components. For example, the interaction of cationic nanogels with cellular membranes can trigger the release of anionic 5′-triphosphates of nucleoside analogues (NATP), as described in Section 6.[91]

In summary, the combination of the approaches presented in Figure 7 can provide a very useful means for the control of the drug-release characteristics of the nanogel carriers. In the case of regular non-cross-linked polymeric micelles, drug release to the external environment is controlled by two factors.[92] The first is the strength of the binding of the drug in the micelle core (whether hydrophobic or electrostatic) which is characterized by drug partitioning between the external environment and the micelle. The second is the binding of the polymer chains in the micelles to each other, as characterized by the cmc. Both factors are considered in terms of "thermodynamic" and "kinetic stability".[88,93] The small sizes of the micelle core and shell results in the diffusion of drugs from the micelles not usually being rate-limiting (at least for low-molecular-mass drugs). As a result, the thermodynamic and kinetic stabilities of drug–micelle constructs are interrelated, that is, the stronger the binding, the slower the release. As a rule, polymeric micelles are less stable than liposomes or nanoparticles made of degradable polymers, and hence the rates of release from micelles are faster. Nanogels provide the means to fill the gap between these different carrier types. For example, drug release can be decreased by cross-linking the polymer chains in the nanogels, and it can be adjusted and made responsive to environmental changes by introducing cleavable cross-links. Furthermore, this technology offers the possibility to control the drug-release profiles. In contrast to liposomes and insoluble nanoparticles, the hydrophilic nanogels swell as the drug is released, which should sustain the release of the drug from the inner layers of the nanogels as the amount released increases. This can be used to modify or eliminate batch release or even to achieve zero-order release kinetics of the drug from nanogels delivered to the disease site.[74,94]

6. Delivery of Small Therapeutic Agents Using Nanogels

Significant progress has been made in the application of nanogels to the delivery of small therapeutic agents. As already mentioned, retinoic acid was encapsulated into PEG-*cl*-PEI nanogels.[71] In a similar manner, valproic acid was formulated in PEG-*cl*-PEI nanogels.[69] In this case the transport of the nanogels with valproic acid across a blood brain barrier (BBB) model in vitro was investigated. The permeability of the nanogels loaded with valproic acid across bovine brain microvessel endothelial cells (BBMEC) monolayers was increased by at least 70 % compared to a free drug. This finding suggests that this formulation may be useful for the delivery of valproic acid to the brain.

In another study, *N*-hexylcarbamoyl-5-fluorouracil, an anticancer prodrug of 5-fluorouracil (5-FU), was encapsulated in poly(*N*-isopropylacrylamide)-*co*-poly(*N*-vinylpyrrolidone) (PNIPAAm/VP) nanogels coated with polysorbate 80.[95] Drug release from this carrier was pH- and temperature-dependent.[96] These nanogels were shown to accumulate in rabbit brains. In another study, the anti-leishmaniasis drug arjunglucoside I was also loaded into PNIPAAm/VP nanogels.[97] This formulation showed

enhanced therapeutic efficacy against parasites as well as reduced hepatotoxicity and nephrotoxicity compared to free drugs.

A promising application of nanogels involves the delivery of NATPs. These nucleoside-based anticancer and antiviral agents usually enter cells via specific nucleoside transporters and then undergo intracellular activation through 1) phosphorylation into nucleoside 5′-phosphates by intracellular nucleoside kinases, 2) formation of nucleoside 5′-diphosphates; 3) conversion of ribonucleotides into deoxyribonucleotides by nucleoside reductases, and finally 4) synthesis of NATP.[98] The latter are actual active molecules, which arrest DNA replication and transcription. The low efficiency of conversion of the nucleosides into NATPs has resulted in many prospective molecules being withdrawn at preclinical or clinical stages of drug development. However, by using PEG-*cl*-PEI nanogels as carriers it became possible to directly deliver NATPs into cancer cells.[83] Nanogel-encapsulated NATPs demonstrated enhanced cytotoxic activities in many cancer cell lines and inhibited tumor growth in the mammary carcinoma animal model.[99] Furthermore, an antiviral NATP, 5′-triphosphorylated ribavirin in a nanogel formulation, exhibited higher activity in MDCK cells infected with influenza A virus compared to a non-phosphorylated analogue.[29] Interestingly, this formulation also demonstrated a significantly reduced mitochondrial toxicity.

7. Biomacromolecules in Nanogels

There are several examples of the delivery of biomacromolecules in vitro or in vivo using nanogels.[1] Perhaps most remarkably, nanogels loaded with oligonucleotides were shown to cross cellular barriers. In particular, the incorporation of a phosphorothioate oligonucleotide into a cationic PEG-*cl*-PEI nanogel resulted in a drastic increase in the transcellular permeability of the oligonucleotide in polarized Caco-2 cell monolayers used as an in vitro model of gastrointestinal epithelium.[1] The permeability of cell monolayers with respect to ^{3}H-mannitol, a paracellular marker, was, however, not affected. This finding suggested that the oligonucleotide-loaded nanogel was transported across the cells, rather than passively diffused by a paracellular route. Furthermore, in contrast to a free oligonucleotide, which was essentially degraded in cells, the oligonucleotide in the nanogels was protected against degradation. This study thus indicates that nanogels are promising carriers for the oral delivery of oligonucleotides.

Moreover, another study evaluated the transport of a phosphorothioate oligonucleotide encapsulated in a PEG-*cl*-PEI nanogel across the blood–brain barrier (BBB).[64] This study showed that the oligonucleotide–nanogel complex was transported effectively across polarized BBMEC monolayers, which served an in vitro model of the BBB. Permeability was further increased when the surface of the nanogel was modified with bovine transferrin or insulin. These two proteins target specific receptors at the blood side of the brain epithelium and facilitated transport of the loaded nanogels across the BBB to the brain side. Importantly, as in the previous example, the oligonucleotide was transported by a transcellular pathway and remained intact and incorporated in the nanogels after release at the brain side. Biodistribution studies in a mouse model further demonstrated that accumulation of the oligonucleotide–nanogel complex in the brain 1 h after intravenous injection was increased nearly 15-fold compared to the free oligonucleotide. Overall, this study suggested that cationic nanogels have the potential to deliver oligonucleotides to the brain.

Similar to oligonucleotides, a plasmid DNA immobilized in cationic nanogels can be protected from enzymatic degradation by extracellular and intracellular nucleases. Furthermore, such nanogels can be used to deliver genes into a cell. For example, a transferrin-modified PEG-*cl*-PEI nanogel loaded with a plasmid DNA was shown to transfect cells in a serum-containing medium.[69] Interestingly, the dimensions of supercoiled DNA are comparable to those of nanogel networks, which in a swollen state have a hydrodynamic diameter between 100 and 200 nm. It is unlikely, therefore, that the plasmid DNA can percolate deep into the pores of the cross-linked nanogels. A flexible cationic network is probably capable of "wrapping around" supercoiled DNA following the initial binding of the DNA with the charged nanogel surface.

There is emerging interest in the pharmacological effects of polymer excipients and nanomaterials in combination with drugs.[100] In particular, synthetic polymers were shown to interact with some drug transport systems and activate selected cell-signaling pathways, and this was shown to alter pharmacological, genomic, and immune responses to biological agents.[79,80] Along similar lines, Fréchet and co-workers used acid-degradable nanogels for antigen presentation in vitro and vaccination in vivo.[17,19,101–103] They have shown that nanogels can be designed to generate immune responses when delivered to phagocytic cells of the immune system. For example, a plasmid DNA incorporated in nanogels with acid-labile cross-linkers induced secretion of cytokine IL-6 and immunostimulation of macrophages.[18] In another study, the incubation of these nanogels loaded with ovalbumin (as a model antigen) with dendritic cells derived from bone marrow resulted in enhanced presentation of ovalbumin-derived peptides.[101] It was also shown that adjuvant molecules such as CpG oligonucleotides and anti-interleukin-10 oligonucleotides can be co-delivered with the protein antigen for maximized cellular immune response.[103] Taken together, these studies suggest that nanogels may be useful as delivery and immune response modulating vehicles for the development of DNA and peptide vaccines.

8. Conclusions

In conclusion, nanogels form a distinct class of hydrophilic dispersed drug carriers with promising properties for encapsulating small biologically active agents and biomacromolecules. The advantages of these systems include their simplicity of formulation, high loading capacity, and the stability of the resulting dispersion. The swelling and collapse properties of the nanogels are unique and provide multiple benefits for

engineering optimal drug loading and release of drugs. Nanogel networks are responsive to external environmental factors, can undergo rapid volume changes, and allow for stimuli-controlled release of encapsulated biologically active compounds including charged or hydrophobic drugs and biopolymers.

Furthermore, nanogels can be chemically modified to incorporate various ligands for targeted drug delivery, triggered drug release, or preparation of composite materials. Preclinical studies suggest that nanogels can be used for the efficient delivery of biopharmaceuticals in cells as well as for increasing drug delivery across cellular barriers. It is clear that there is no universal delivery system that can address all the needs of current and future drug therapies. In this regard, the capabilities of nanogels as well as other classes of pharmaceutical carrier are not infinite, but for humans "infinite" often means beyond ones imagination.

We certainly hope that future application of nanogels as pharmaceutical carriers will exceed our expectations and believe that many capable scientists across the globe will contribute outstanding work to advance these novel carriers for practical use.

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