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Recent Progress of Polymeric Nanogels for Gene Delivery

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Abstract

With its nearly unrestricted possibilities, gene therapy attracts more and more significance in modern-day research. The only issue still seeming to hold back its clinical success is the actual effective delivery of genetic material. Nucleic acids are in general challenging to administer to their intracellular targets due to their unfavorable pharmaceutical characteristics. Polymeric nanogels present a promising delivery platform for oligonucleotide-based therapies, as the growing number of reports deliberated in this review represents. Within the scope of this article, recent progress in the employment of nanogels as gene delivery vectors is summarized and different examples of modified, stimuli-responsive, targeted and co-delivering nanogels are discussed in detail. Furthermore, major aspects of successful gene delivery are addressed and critically debated in regards to nanogels, giving insights into what progress has been made and which key issues still need to be further approached.

Keywords

Nanogels; Polymeric Carriers; Gene Delivery; Nucleic Acids; siRNA; pDNA

1 Introduction

Gene therapy describes the process of introducing foreign genomic material into specific host cells in order to gain a therapeutic benefit by correcting existing disfunctions or sustaining respective cells with new functions. [1] While at early stages, gene therapy mainly focused on rare genetic disorders, the concept of delivering nucleic acids, including plasmid DNAs, short interfering RNAs (siRNAs), as well as messenger RNAs (mRNAs), aiming to restore a specific gene function or to silence certain genes, is nowadays exploited for a great range of various diseases. Following the first human gene transfer in 1989 [2], the first gene therapy was applied in 1990. [3] Since the release of the human genome sequence in 2001 [4] and the discovery of the mechanism of RNA interference (RNAi) just a few years later [5], the opportunities of gene therapy vastly increased, as it became

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Competing Interests Statement

The authors have no competing interests to declare.

hypothetically possible to target and treat any chosen gene. Despite all progress, however, there are still several hurdles yet to overcome on the way to a successful translation of these findings into the clinical routine. It should be noted that after 20 years of research, only one RNAi-based drug has been approved by the FDA and EMA. [6]

The most challenging step towards effective gene delivery, in fact, appears to be the search for a suitable carrier system. As for most biotherapeutics, the transport of genetic material to their intracellular targets is demanding, due to their unfavorable biopharmaceutical properties. [7] Nucleic acids are not only heavily susceptible to enzymatic and chemical degradation and rapidly cleared upon systemic injection, but also generally hindered from crossing cellular membranes. It is therefore inevitable to package therapeutic DNA or RNA in appropriate delivery systems that protect their payload, facilitate cell internalization and guide its way towards the required intracellular target compartment: nucleus for DNA, or cytosol for siRNA and mRNA. Although viral vectors show high gene transfection efficiencies, their clinical utility remains very limited due to their potential immunogenicity and severe side effects. [8] Modern gene delivery approaches, therefore, mainly focus on nonviral vectors with a particular emphasis on polymeric carrier systems. Polymers can purposefully be designed for specific application needs regarding characteristics such as different molecular weights or charge densities and can be modified by coupling of targeting ligands or tailored to be reactive to certain physiological conditions. Furthermore, their production can rather easily be scaled-up to large quantities. [9]

One central aspect in successful drug delivery is the controlled release of the delivered therapeutic agent. The drug has to be available at the target region in a specific concentration within the therapeutic window in order to bring about its desired effect without causing any unwanted toxic reactions due to overdosing. Owing to their large surface area, accordingly designed nano-sized systems can offer finer temporal control over drug release rates than macro-sized vehicles. As opposed to bulky delivery systems, vehicles in the nano-scale can enter target cells with greater ease and are able to specifically attack diseases at their site of action as they can circulate in the body after injection. [10]

Polymeric nanogels are a special representative of nano-sized systems, consisting of nanoparticles composed of hydrogels which are in turn made of cross-linked polymer networks. Combining beneficial functions of dendritic systems with those of hydrogels such as large encapsulation cavities and the capability of swelling as well as responsiveness, these novel structures not only fill the size gap, but also present a functional link between common dendrimer or polymer scaffolds and macroscopic hydrogels. [11] As opposed to larger hydrogel particles, nanogels can easily be administered intravenously and deliver their payload to various target regions and cells. Further advantages of these promising drug delivery platforms comprise simple and efficient drug loading, physical stability of both carrier and incorporated drug, and a versatile design. As they form complexes with biomacromolecules such as proteins in suitable size dimensions, they not only ease the way for their delivery, but also help to maintain their biological activity by keeping them in the correct conformation, arousing special interest for biomedical applications. [10] Due to their characteristic properties such as softness and swelling behavior, nanogels are predestined to achieve controlled as well as responsive release at the target location [12]. The possibility to

trigger these soft delivery systems to alter their structure upon changes in parameters such as temperature, pH, or ionic strength in the environment facilitates both storage and administration of the therapeutic formulation compared to hard nanomedicines. Nanogels can therefore e.g. be applied in a low viscous form that transitions into a dense film or a high viscous depot form after administration. [13] The versatile architecture of nanogels enables the loading with various cargos featuring different physical properties while maintaining their gel-like behavior. Their high degree of porosity, owed to their weakly crosslinked polymer chains, even allows for efficient encapsulation of macromolecules, which often cannot be realized with conventional nanoparticles. [12]

Their stimuli-responsive nature makes nanogels particularly suitable for the treatment of cancer and inflammatory diseases, since those are commonly paired with acidic pH, generation of heat and ionic changes. [11] Nevertheless, nanogels are nowadays intensely investigated throughout a great variety of application fields, having the great benefit of being customizable for respective needs not just in terms of size and crosslinking density, but also surface modifications such as with specific targeting ligands. [7] While drug delivery represents the area with the greatest impact of nanogels, they have also emerged to be vastly applicable in other fields, particularly in the biomedical area, comprising imaging and diagnostic purposes [14], sensing [15], bioengineering [16] and the exploitation of responsive nanomaterials [11]. A tabular compilation of nanogels that have been formulated and investigated as gene delivery systems during the last five years can be found in Table 1.

2 Synthesis and Characterization of Nanogels

The synthesis of nanogels is mostly achieved by two major strategies that are illustrated in Figure 1: the use of polymer precursors or the heterogeneous polymerization of monomers. As amphiphilic copolymers are prone to self-assemble into nanoscaled structures in an aqueous environment, the former can be stabilized by utilizing different cross-linking methods, based on amines or disulfides, click chemistry, or are photo- or physically induced. The fabrication of nanogel networks by polymerization of monomers can proceed in an emulsion or inverse emulsion process, depending on the continuous phase. By incorporation of bifunctional monomers and initiation of polymerization in these heterogeneous colloidal systems, nanogels can be manufactured. [10] As opposed to this approach, in the initially homogenous dispersion and precipitation polymerization, all components are soluble in the solvent, allowing a synthesis in a single batch process. More detailed information concerning the synthesis of nanogels can be found nicely summarized in a review article by Asadian-Birjand et al. [11] Due to the mostly rather harsh conditions and the oftentimes required use of catalysts during the synthesis of nanogels [17], it is usually preferred to add the sensitive nucleic acid payload in the aftermath. In order to use polymeric nanogels as effective gene delivery vectors, it is generally necessary for them to possess or be modified to contain site-specific cationic entities [18]. In most cases, the siRNA, pDNA, or mRNA is then just added to the readily prepared nanogel at the desired N/P ratio (residual molar ratio of the amine groups of the nanogel to the phosphate groups of the nucleic acid) and mixed thoroughly. During a short incubation period, a polyion complex is then formed spontaneously via electrostatic interactions between the cationic nanogel and the negatively charged nucleic acid. It was recently shown that the size of nanogel particles is a crucial

factor influencing the gene knockdown potential of siRNA loaded systems. Two well-defined types of cationic nanohydrogel particles were synthesized using amphiphilic reactive ester block copolymers of pentafluorophenyl methacrylate (PFPMA) and tri(ethylene glycol)methyl ether methacrylate (MEO3MA) with similar compositions, but different molecular weights, resulting in differently sized particles after crosslinking. Only those particles with an average diameter of 40 nm, but not with 100 nm, induced moderate gene knockdown. As the smaller-sized ones were revealed to especially avoid acidic compartments and hence endolysosomal uptake pathways, it is suggested that these properties explain their greater knockdown potential. [19]

Moreover, the applied nanogel : nucleic acid ratio appears to play an important role. In a study aiming for gene knock-down in primary mouse osteoblasts, weight to weight ratios of nanogels : siRNA from 1:1 to 1:10 of quaternized dimethyl aminoethyl methacrylate (qDMAEMA) based nanogels were tested, revealing that two compositions (1:1 and 1:5) were particular favorable for the use of gene silencing. [20]

Shatsberg et al. prepared functionalized nanogels for microRNA (miRNA) delivery with a surfactant-free inverse nanoprecipitation method resulting in disulfide crosslinked redox-sensitive gels based on polyglycerol scaffolds that are degradable under intracellular reductive conditions. By attaching different amine-modified linkers to the polyglycerol moieties in the nanogel structures, they were able to vary and thereby investigate the interactions between the nanogels and the miRNA in more detail. In this way, they synthesized and characterized six potential nanocarriers, depicted in Figure 2, giving new insights into some important features for the design of oligonucleotide delivery systems by comprehensive comparison of the varying nanogels. Nanogels 3 and 4 (NG3 and NG4) showed particularly high efficiencies to complex miR-34a, a miRNA that targets genes playing a key role in the regulation of apoptosis and cell cycle arrest as well as inducing the inhibition of cell proliferation and migration. Both cationic nanogels were able to neutralize the negatively charged miRNA in a dose-related manner and showed higher cellular uptake than the less positively charged NG2, confirming the widely accepted hypothesis that cationic surface charge of nanoparticles aids their internalization process. Complexes of miR-34a with NG3 and NG4 were successfully taken up by U-87 MG cells and significantly increased the miR-34a levels after transfection. NG3 complexes, however, showed superior knockdown abilities *in vitro* as well as *in vivo*, inhibiting the proliferation of U-87 MG cells and significantly arresting the tumor growth in mice bearing human U-87 MG glioblastoma multiforme, respectively. A polyanion competition assay revealed a distinct difference in the stability of the miRNA complexes, showing a lower affinity of NG3 towards miR-34a that resulted in a higher capability to release the miRNA. The authors, therefore, concluded that the stability of nanogels has to be carefully weighed with their ability to liberate the encapsulated cargo upon successful delivery. [21]

3 Modification with natural components

3.1 Heparin

In an attempt to overcome known drawbacks of using the non-biodegradable polymer polyethylenimine (PEI) as a gene carrier system, including its relatively high cytotoxicity

and induction of aggregation of erythrocytes and hemolysis, the former was coupled to the natural polysaccharide heparin to form novel biodegradable cationic hydrogels. [22]

The transfection efficiency of heparin-PEI (HPEI) was found to be comparable to that of 25k PEI, while demonstrating improved blood and biocompatibility and decreased toxicity. While being stable *in vitro*, the nanogels were easily degradable through enzymolysis and hydrolysis into low molecular weight PEI and excreted through the urine *in vivo*. In several follow-up studies, the HPEI nanogels were tested for application in antitumor therapy. For instance, HSulf-1, a gene playing a key role in regulation of cell proliferation, tumorigenesis and angiogenesis, which is down-regulated in most examined tumor types, was successfully transfected and expressed in SKOV3 ovarian cancer cells by HPEI nanogels. [23] The observed reduction in tumor weight, angiogenesis and cell proliferation as well as the induction of tumor cell apoptosis could even be extended in a pursuant combination of the HSulf-1 HPEI complexes with the anticancer drug cisplatin. [24]

Moreover, heparin-Pluronic supramolecular nanogels were synthesized by coupling aminated Pluronic to heparin through amide linkages. This conjugate was loaded with basic fibroblast growth factor (bFGF), an inducer of neovascularization, by a direct dissolution method. The highly negatively charged heparin was shown to stabilize genetic material and growth factors such as bFGF by forming high-affinity complexes. After coating these with PEI, polyplexes were prepared with pDNA encoding vascular endothelial growth factor VEGF₁₆₅ and delivered to endothelial progenitor cells (EPCs), where they promoted endothelial cell differentiation and neovascularization in an ischemic limb model system. [25]

The shielding effect of heparin was utilized to successfully diminish the toxicity of a PEI-based nanogel comprising the cell penetrating peptide R8. This peptide is in turn able to enhance the cellular uptake of the vehicle containing a therapeutic plasmid, making the PEI-R8-heparin nanogel a promising gene delivery system. [26]

3.2 Surfactant

Although RNAi has great potential for application in the treatment of pulmonary diseases, the lack of stable, biocompatible carriers to overcome the various intra- and extracellular barriers still impedes clinical translation. To enhance the colloidal stability of siRNA-encapsulating nanogels and to prevent siRNA release in the presence of competing polyanions abundantly present in respiratory biofluids, such as lung surfactant and mucus, novel bioinspired hybrid nanogels were manufactured. siRNA-loaded dextran nanogels were combined with a pulmonary surfactant coating to build up a core-shell nanoarchitecture. Despite the fact that the surfactant shell considerably reduced the uptake of the obtained nanogels in lung cancer cells, the resulting lower intracellular doses did not hamper the gene silencing effect, indicating that pulmonary surfactant may play an important role in the processing of the nanogels inside the cells. To stimulate receptor-mediated endocytosis of the particles, folate was attached as a targeting ligand. Indeed, both uptake and gene knockdown were enhanced, eventuating in efficient silencing at nanomolar siRNA concentrations. [27]

Subsequently, surfactant-coated as well as uncoated nanogels were delivered to resident alveolar macrophages (rAM), critical contributors in lung inflammatory responses, via pharyngeal aspiration in BALB/c mice. While both achieved high levels of siRNA uptake in rAM, only the coated formulation significantly reduced gene expression on the protein level. Additionally, 70 % knockdown of target mRNA levels could be achieved with $\sim 1 \text{ mg kg}^{-1}$ siRNA doses, while only evoking mild acute pro-inflammatory cytokine and chemokine responses. [28]

3.3 Silica

An enhancement in stability and functionality of PEG-block-polycation/siRNA complexes was achieved by wrapping them with hydrated silica via polycondensation of soluble silicates onto their surface comprising a disulfide cross-linked core. This nanogelling process efficiently protected the polyplexes from counter polyanions under non-reducing conditions, while maintaining the environment-responsive disulfide cleavage leading to the release of the siRNA. Assumedly, a lower endosomal entrapment or lysosomal degradation of the siRNA resulted in an increased gene silencing effect in HeLa cells without arousing respective cytotoxicity. The authors hypothesized that deprotonated silanol groups and/or a modification of the intracellular trafficking could be responsible for a faster endosomal escape of the particles. [29]

4 Stimuli-responsive nanogels

Several nanogel types have been generated to change their assembly or architecture in response to certain stimuli. Variations in the chemical design enable them to respond to a variety of environmental factors, such as temperature, pH, ionic strength, reduction, and light. [30]

4.1 Temperature

Thermally responsive nanogels for gene delivery have widely been investigated as drug delivery systems, especially in the research for tumor therapy and inflammatory diseases, since they are able to accumulate and release their payload at the desired target site via structural changes evoked by temperature changes. [31]

Cao et al. integrated the thermo-sensitive polymer Poly(N-isopropylacrylamide) (PNIPAM) in the side chain of low molecular weight PEI via the conventional radical graft copolymerization to form an amphiphilic graft copolymer at a reaction temperature of 80 °C and encapsulated TRP53 gene, a tumor suppressor gene playing a central role in cell cycle regulation and programmed cell death. The resulting cationic thermo-responsive non-cytotoxic nanogel with a well-defined core-shell structure showed considerably higher transfection efficiency compared to Lipofectamine 2000 or PEI alone. Additionally, distinctly higher *in vivo* tumor accumulation and inhibition were achieved after i.v. administration to Balb/c nude mice compared with PEI. [32]

In an approach to enable the challenging cutaneous application of proteins, thermo-responsive PNIPAM-polyglycerol (PNIPAM-dPG)-based nanogels were synthesized with a thermal trigger point at 35 °C, congenial to the native thermal gradient of human skin. The

size of the ~200 nm protein loaded particles was instantly reduced by 20% at 35 °C, releasing 93% of the protein without any alterations in its structure or activity. Efficient intraepidermal protein delivery, especially in barrier deficient skin, was detected and transglutaminase 1 was successfully transported to respective knock-down models of human skin, restoring skin barrier function. [33]

Further thermo-sensitive hydrogels were constructed by PNIPAM-co-acrylic acid (PNIPAM-co-AAc) to generate self-assembled particles with a nanogel character. Binding of the carboxyl group on the outside of PNIPAM-co-AAc with the amine group of amine functional magnetic iron oxide nanoparticles was mediated by hydrophobic interactions. Fluorescent dye containing nanogels were then coated with cationic PEI and complexed with certain genes by the electrostatic formation of polyplexes. Efficient internalization of the nanogels by human mesenchymal stem cells (hMSCs) and expression of incorporated green fluorescent protein suggest a potential for respective gene delivery systems. [34]

4.2 pH

Similar to thermo-responsive carriers, pH-sensitive nanogels are well suited to deliver the incorporated payload to certain target areas with altered environmental conditions due to pathological processes taking place.

Based on two linear polymer precursors, disulfide-containing tetralysine (TetK) and oligoethylenimine (OEI), pH-dependent nanogel particles were formed by covalent *in situ* cross-linking with homobifunctional cross-linkers. The resulting nanogels were proven to increase in size as well as zeta-potential when encountering a decrease in environmental pH. Besides that, they were degraded in presence of glutathione at 10 mM, a concentration similar to the intracellular space. [30]

In another approach to generate both pH- and temperature-responsive delivery systems for the co-delivery of plasmid DNA and proteins, carbohydrate-based nanogels were synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization technique containing 2-lactobionamidoethyl methacrylamide (LAEMA) and methyl ethyl methacrylate (PEGMA). DNA-nanogel complexes were formed by the interaction of carbohydrate tails with DNA and stabilized with a linear cationic glycopolymers which further improved cellular uptake and gene expression *in vitro*. The acid-degradable profile of the nanogels enables a burst release of the encapsulated biomaterials in cell endosomes. [35]

Dimde et al. developed an elegant pH-sensitive nanogel on the basis of two macromolecular precursors, pH-reactive dendritic polyglycerol (dPG) and low molecular weight PEI-acrylamide, that were combined via thiol-Michael nanoprecipitation method. Owing to these mild conditions, lacking the need of any catalyst, the sensitive siRNA cargo could be encapsulated directly during the synthesis process minimizing siRNA loss or degradation. Resulting nanogels with pH-sensitive benzacetal-bonds containing GFP-siRNA demonstrated comparable gene silencing effects as unmodified PEI while showing significantly reduced cytotoxicity. [17]

4.3 Light

The swelling behavior of nanogels, caused by the difference in osmotic pressure in- and outside of the gel and alterable by charge or cross-linker density can be influenced by external stimuli to a great extent. Nanogels with unique photodegradation properties were developed using ethylene glycol diglycidyl ether (EGDE) as cross-linker, in conjugation with the polyamines spermine, protamine sulfate and PEI. These systems were then employed to condense different plasmids as well as anticancer drugs into nano-range particles encouraging their controlled release. Degradation of these nanogels upon UV light exposure occurs via the photo-oxidation of EGDE, leading to the removal of cross-links allowing the subsequent release of the constituent network polymer, resulting in changes in gel weight, mechanical properties, mesh size and porosity as well as swelling degree. The tunability of these systems harbors their potential for the controlled release of mono or dual delivery of biomolecules as well as for biosensing or –patterning technological purposes. [36]

5 Active Targeting

Under certain pathological circumstances, such as inflammation or hypoxia which are for example characteristic for tumors or infarcts, a phenomenon called ‘EPR’, enhanced permeability and retention, is hypothesized to take place at the disease area. The impairment of the protecting endothelial lining of the blood vessel walls by secreted factors such as kinin and vascular permeability factor leads to leaky vessels with larger cell gap sizes. This increased vascular permeability combined with vitiated lymphatic drainage can be exploited for a form of passive targeting, as it results in an increased accumulation of drug payloads in the desired regions while circumventing off-target sites, thereby minimizing side effects in healthy organs. [37] It is currently critically discussed if EPR has a viable impact on tumor targeting in patients, or if it is more relevant in artificially induced animal models of tumors. However, the pathological changes that cause experimental passive targeting are well described. [38]

As opposed to this passive targeting, it is also possible to attach specific ligands to the delivery systems in order to actively target certain diseased regions. This is often achieved by aiming for cell surface receptors which are overexpressed in the respective disease in order to enhance accumulation of the delivered agents in the tissues of interest. Since folic acid is a vital nutrient crucial for cells to biosynthesize nucleotides and maintain important cellular pathways [39], many human malignancies, especially aggressively growing cancers, are associated with elevated expressions of the folate receptor, making it an attractive candidate for actively targeted drug and gene delivery. [37]

In a recent attempt to generate targetable nanogels for siRNA delivery, a glycol chitosan nanogel was synthesized by chemically grafting hydrophobic chains onto a polysaccharide, and the obtained macromolecular micelles were decorated with folate using a PEG linker. An extra amount of PEG was added to overcome the slight decrease of solubility caused by folate grafting and, additionally, to reduce the opsonin adsorption and subsequent scavenging by the mononuclear phagocyte system in order to lengthen the system’s lifespan in blood. After incubating HeLa cells with both the targeted folate-decorated and non-

targeted nanogels, the latter were detected on the cell surface, while targeted gels were localized in the cytoplasm, proving the support of internalization by receptor-mediated endocytosis via folate. [40] In a subsequent study aimed to further characterize the uptake mechanisms and intracellular fate of folate-functionalized nanogels, specific siRNA sequences were used to selectively inhibit uptake-mediating proteins such as clathrin or caveolin and thereby attenuate the respective endocytic pathways. Nanogel uptake was shown to occur mainly via flotillin-1 and Cdc42-dependent endocytosis and a shown impairment by free folate suggests a competitive inhibition and shared internalization mechanism. Cdc42- and Pak-1 involvement, furthermore, strongly hint to the need of actin reorganization for nanogel uptake. [41]

6 Co-delivery and Add-on treatments

Several of the mentioned studies employed co-delivery of different substances in one transport system in order to strengthen the therapeutic effect of the delivered genetic material. While a certain emphasis is put on the dual delivery of nucleic acids with anticancer drugs such as doxorubicin [42], paclitaxel [43], or cisplatin [24], there are also approaches to co-deliver growth factors [25] or other proteins [35] alongside with DNA therapeutics.

Ding et al. recently published their findings on a self-assembling protamine-based nanogel co-delivering epigallocatechin-3-O-gallate (EGCG) with matching siRNA for sensitization to EGCG-involving chemotherapy in an approach to combine two promising strategies, co-delivery and selective tumor targeting. Their multicomponent carrier system was able to increase the cytotoxicity to a drug-resistant cell line by 15-fold compared to EGCG chemotherapy alone and demonstrated enhanced selectivity and tumor growth inhibition in respective xenograft tumor-bearing mice. [44]

A special type of co-delivery was used in an attempt to cope with one of the most challenging hurdles for polymer-based gene delivery: the issue of overcoming the endosomal entrapment of polyplexes. Therefore, a polysaccharide-based cationic nanogel composed of hexadecyl group-bearing cycloamylose was generated and complexed with plasmid DNA (pDNA) as well as the membrane phospholipid hydrolyzing enzyme phospholipase A₂ (PLA₂). Complexation with specific concentrations of PLA₂ were shown to enhance pDNA expression levels and resulted in similar hemolytic activity to that of native PLA₂, implicating a membrane disruption ability of the nanogel/PLA₂ complex when delivered into cells, triggering the subsequent release of pDNA from the endosome to the cytoplasm. [45]

Another approach to facilitate the cytosolic transport after delivery by nanogels was pursued by Joris et al. for siRNA. Here, it is thought to be of particular importance that the sensitive cargo is released to the cytoplasm prior to fusion of endosomes with lysosomes to prevent degradation. In an effort to circumvent this issue and even take advantage of the lysosomal accumulation, cells were treated with drugs provoking siRNA release from lysosomes to the cytosols, after having been transfected with nanogels. The group was able to show how a simple incubation of H1299 eGFP-expressing cells with the applied FDA-approved cationic

amphiphilic drugs (CADs) after siRNA treatment could significantly increase the induced gene silencing effect. CADs cause lysosomal phospholipidosis in the cancer cells resulting in the permeabilization of lysosomal membranes, as illustrated in Figure 3, aiding siRNA release without affecting cell viability. These findings might pave the way for intracellular depot forms allowing for controlled siRNA release via respective CAD treatments. [46]

7 *In vivo* studies

Several of the nanogels currently under investigation for their use as gene delivery systems were already examined *in vivo* in respective animal models, the majority thereof were considering siRNA or other types of therapeutic RNA.

A recent report describes cationic nanogel polymers prepared by atom transfer radical polymerization (ATRP) in inverse miniemulsion and loaded with siRNA. The use of quaternized dimethylaminoethyl methacrylate (Q-DMAEMA) as hydrophilic cationic moiety enabled siRNA binding without facing the problem of polymer aggregation. Moreover, the poly (ethylene oxide) (PEO) arms partially masked surface charges, enhancing biocompatibility and preventing enzymatic siRNA degradation through steric hindrance. 2'-O-methylation of siRNA was proven to maintain the polyplex integrity in presence of RNase A without compromising gene knockdown efficiency. After successful reduction of GAPDH enzyme activity by nanogel mediated siRNA treatment, significant inhibition of *in vivo* GFP expression via sequence-specific knockdown was confirmed in wild type mice. [47]

Intra-tumor delivery of siRNA was aimed for with self-assembled nanogels of cholesterol-bearing cycloamylose with a spermine group to deliver vascular endothelial growth factor (VEGF)-specific siRNA. The nanogel complexes were taken up by renal cell carcinoma (RCC) cells through endocytosis resulting in efficient knockdown, and intra-tumor injections were able to significantly suppress neovascularization and growth of RCC in mice. [48]

Another study tested the delivery of self-amplifying replicon RNA (RepRNA) in biodegradable, chitosan-based nanogel-alginate and demonstrated RepRNA delivery to dendritic cells. Accumulation in vesicular structures with patterns typifying cytosolic release promoted RepRNA translation *in vitro* as well as *in vivo* after vaccination of Balb/c mice and New Zealand white rabbits. [49]

A further nanogel for siRNA delivery was manufactured on the base of fully water-soluble DNA-grafted polycaprolactone brushes, that were used to further assemble a crosslinked nanogel via functional nucleic acid hybridization. After being intravenously administered to MDA-MB-231 tumor-bearing mice, the delivery system exhibited favorable physiological stability as well as a prolonged blood half-life and an increased accumulation at the tumor site compared to lipofectamine as positive control. siRNA specific for polo-like kinase 1 (PLK1), the chosen oncogenic target over-expressed in many tumor cells, resulted in the highest knockdown effect and, consequently, in the most effective tumor inhibition when delivered inside of the fabricated nanogel. [50]

Li et al. designed a reduction-sensitive nanogel by introducing thiolated low molecular weight PEI (1.8 kDa) into a biodegradable dextrin backbone resulting in a bioreduction-rupturing siRNA delivery system with a switch on/off controlled release. The dynamic covalent bond crosslinked nanogel is susceptible to high cytosolic concentrations of glutathione, leading to rupture of the bioreducible crosslinks and degradation of the nanocarriers into its base materials PEI and dextrin, followed by a burst release of the incorporated siRNA. The nanogel exhibited equally high downregulation capabilities on the protein expression level as 25 kDa PEI *in vitro* and even superior tumor suppression rates in 4T1-luc tumor cell bearing BALB/C mice. At the same time, the developed system showed lower cytotoxicity and negligibly low hemotoxicity in healthy mice, evading recognition and clearance by the reticuloendothelial system. Biodistribution of the nanogel comprising Cy5-siRNA was, moreover, tested in tumor bearing mice after injection into the tail vein. The highest fluorescence intensity signal was obtained at the tumor site 12 h post-injection and the preferred accumulation in the tumor tissue was confirmed by examination of harvested single organs, as shown in Figure 4. The authors concluded that the longer blood circulation time, as opposed to naked siRNA that was rapidly cleared by liver and kidney, as well as the suitable size of the designed nanoparticles were decisive characteristics leading to the effective tumoral enrichment. [51]

On a related note, chimeric antigen receptor (CAR) T cell therapy as a kind of gene therapy with immense relevance is worth mentioning here as an example of the diverse application field of nanogels. In a recent approach, nanogels were used to selectively deliver large quantities of supporting protein drugs onto T cells and release their payload in a particular responsive way, namely upon T cell receptor (TCR) activation. Therefore, surface-conjugated nanogels were designed reacting to the increased redox activity of activated T cells in contrast to naïve ones and carrying an IL-15 super-agonist (IL-15Sa). In contrast to most conventional delivery systems, these nanogels operate as so-called “backpacks” that are not expected to be internalized by the cells, but in fact to bind to the cell surface in order to sustain stimulation. *In vitro*, T cells treated with TCR-responsive nanogels expanded 16-fold more in tumors than those supported with systemic cytokine injections, and *in vivo*, nanogel backpacked CAR T cells eradicated tumors in four of five mice, while responses were only marginally improved with equivalent systemic doses of free IL-15Sa. [52]

8 Conclusion

Altogether, the ideal carrier system for successful gene delivery has to meet several specific requirements. First of all, the genetic material has to be efficiently encapsulated in a stable complex that at best endures circulation in the body. Nanogels certainly fulfill this demand, as their hydrophilicity contributes to high loading capacities for hydrophilic biotherapeutics and their tailorable size and crosslinking density allow for adjusting their pore sizes to various loaded molecules. Thus, nanogels can stably encapsulate their cargo during the synthesis. It is, however, essential to ensure that drug molecules are not chemically modified during this process. [53] Nucleic acids, as strongly charged biomolecules, can also be loaded post-synthesis into an oppositely charged nanogel via electrostatic interaction. Once loaded, nanogel networks generally protect their payload well from degradation, as enzymes are not

able to penetrate into the particles. For special stability needs, the surface properties can easily be adjusted, for example via PEGylation.

The next step would be to transport the therapeutic molecule to the desired target region and, if applicable, to specific target cells. As discussed before, nanogel systems are both adjustable to benefit from passive targeting effects and to be customized by the linkage of certain targeting ligands to actively aid pointed drug delivery. By these means, high drug concentrations can be achieved in diseased areas, while healthy tissue is not affected, and side effects can significantly be reduced.

A critical point after reaching the target location is the exhaustive release of the carried drug so that it can bring about its effect. As important as a stable complex of delivery system and payload is for transporting intact therapeutics, it has to be stated that the affinity can also be too strong, compromising release and, therefore, therapeutic effect. Nanogels just like nano-systems in general have the advantage of providing comparably fine control over release profiles [10] and feasibility for both sustained or burst release. Most common nanogels release their payload by hydrolytic degradation of their gel network, resulting in a sustained release leading to relatively low drug concentrations in- and outside of cells. As nucleic acids need to reach their site of action in the intracellular room, respective nanogel carriers should be taken up by cells and degrade upon the altered physiological conditions therein. Several bio- and stimuli-responsive nanogels have successfully been designed for the use as gene delivery systems as described in this review. Improvement of therapeutic efficacy as reflected in parameters such as enhanced transfection efficiency for reduction-sensitive nanogels as opposed to non-reducible representatives, implicates the intracellular cleavage of disulfide bonds. However, no direct evidence for this phenomenon has been found so far, leaving the exact intracellular fate of these nanogels uncertain. [7] The low pH of lysosomes is commonly used to reduce respective carrier systems releasing their payload in a controlled way. For nucleic acids, however, it is particularly important that they are able to escape from these acidic compartments in order not to be degraded. Some approaches to cope with this dilemma have been evaluated in this article, nevertheless, more efforts have to be made to accurately comprehend and exploit these mechanisms to the fullest extent.

A further aspect not to be underappreciated is the biocompatibility of a drug delivery system, as this eventually determines whether it can be applied as a therapeutic. To achieve non-toxic carriers, they can either be straightaway manufactured from well-tolerated materials, or they have to be built upon substances that are transformed into non-toxic products via metabolization. Most modern nanogels currently designed for therapeutic purposes are already constructed from some of the many available biodegradable polymers. Even though sparsely relevant for the scientific level, but nonetheless crucial for ultimate drug approval and industrial production is the ease of synthesis, scale-up and purification of a drug transport system. Polymeric nanogels provide an attractive option in this matter as well, as their manufacturing is commonly straightforward and can easily be scaled up to larger quantities.

The highlighted favorable characteristics and discussed examples of promising nanogel formulations underline the great potential of this carrier system for drug delivery in general,

and for the application as gene delivery vectors in particular. [54] Despite the considerable progress that has been made over the last years of intense research, there are, however, still some aspects that need closer assessment on the way towards clinical translation. Taking into account that toxicity and immunogenicity rank among the most important criteria for drug evaluation, one crucial factor for easing this translation especially in regard to nucleic acid delivery is a comprehensive investigation of the immune-compatibility of respective systems. Both nanotechnology-based formulations in general and biological products such as therapeutic nucleic acids in detail bear the risk of unintended immune-mediated adverse effects. [54] Although this area is substance of intensive research and several immunological targets such as cell surface or endosome Toll-like receptors and cytosolic sensors have already been identified [55], there is still an unmet need to further examine underlying molecular mechanisms in order to produce safe nanogel formulations. Guo et al. recently compared numerous different RNA nanoparticle formulations and demonstrated that their immune response is not only highly dependent on size and shape of the carrier system, but also the RNA sequence itself has influence on possible immunostimulations. [55] Their results suggest that immunogenicity of respective formulations is tunable and can be used to manufacture delivery systems with specifically designed immune responses as needed in order to achieve a minimal response for safe therapeutics or a strong response for cancer or vaccine adjuvants.

One of the main hurdles specifically related to nanogel-based delivery systems is that currently only 5-10 % of injected doses effectively reach the target location, while the largest share is still gathered by clearing organs such as kidney, liver and spleen. [56] The molecular weight of copolymeric nanogels lying above the renal threshold (~ 40 kDa), they are not excretable via the kidneys and might tend to accumulation, making it inevitable to accurately examine metabolism and elimination of the carrier system before planning for long-term clinical application. [12] Several parameters such as size, shape, composition and surface properties influence tissue distribution and clearance of nanogels and have to be carefully balanced in order to achieve successful delivery to the desired areas. PEGylation, or coating with polysarcosine as a newly arising alternative [57], can be a way to prevent adsorption to plasma proteins and subsequent uptake by liver and spleen and at the same time provide shielding of undesired charges, as a rather neutral surface charge has been shown to prolong the circulation time of gel particles. [12] This improvement, however, has to be carefully weighed with maintaining the stimuli-responsiveness of the nanogel, oftentimes being reliant on charged groups. Another nanogel-related drawback is the continuing heterogeneity of respective formulations. Despite advances in nanoscale fabrication allowing for finer particle size distribution control, exact reproducibility of particle size and stoichiometry of nanogels remains difficult to achieve. [12]

Furthermore, some questions not satisfyingly acknowledged yet, such as the exact intracellular fate of both carrier material and nucleic acid cargo or the accurate balance between stability and release behavior of built complexes, still leave room for further improvements. More detailed investigations of the pharmacodynamics and -kinetics of nanogels as well as their interactions with their encapsulated payload would be an important step towards application in the clinical routine. Rationally designed nanogels taking all

mentioned aspects into account then offer an auspicious base for a variety of biomedical applications, one of particular interest being the usage as a versatile gene delivery platform.

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publications of special interest (*)

publications of outstanding interest (**)

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1. Precursor technique

2. Emulsion technique

Figure 1.

Typical nanogel synthesis techniques: The precursor method vs. the emulsion method.

(Adapted from [10])

Figure 2.

Schematic representation of the polyglycerol-based nanogels with different amine-bearing moieties. (Reproduced with permission from [21])

Figure 3.
CAD-mediated inhibition of Acid sphingomyelinase causes lysosomal lipid accumulation and lysosome membrane permeabilization. (Reproduced with permission from [46])

Figure 4.

In vivo distribution of Cy5 after i.v. injection of the Cy5 siRNA loaded nanogel into 4T1-luc tumor-bearing mice. (Reproduced with permission from [51])

Nanogels as Gene Delivery Systems published during the last five years.

Table 1

Year of Publication	Carrier Material	Genetic Payload	Target Cells/Organism	Therapeutic Aim/Disease	Special Features	Ref.
2018	Epigallocatechin-gallate, protamine	siRNA	MDA-MB-231 cells and -tumor-bearing mice	Drug-resistant triple-negative breast cancer	Targeting ligands: hyaluronic acid, cell-penetrating peptide	[44]
2018	DNA-grafted polycaprolactone	siRNA	HeLa cells, U2OS cells, MDA-MB-231 tumor-bearing mice	Cancer		[50]
2018	PEI, R8	pDNA	HCT-116 cells, BALB/c mice	Abdominal metastatic colon carcinoma	Heparin modification	[26]
2018	Thiolated PEI, dextrin	siRNA	4T1-luc cells and -tumor-bearing mice	Cancer	Reduction-sensitive	[51]
2017	Dextran	siRNA	H1299 cells		Add-on treatments for lysosomal escape	[46]
2017	Dendritic polyglycerol, PEI	siRNA	HeLa cells		PH-sensitive	[17]
2016	Polyglycerol, varying amines	miRNA	U-87 cells, U-87 MG GBM-bearing SCID mice	Glioblastoma multiforme		[21]
2016	PGMA, lipotic acid	pDNA, siRNA	Hepatoma cells		PH-responsive	[58]
2016	PEI, heparinized pluronic 127	pDNA	Mesenchymal stem cells		Quantum dots complexes	[59]
2015	Methacrylates	siRNA	MC3T3 E1.4 cells, wild type mice			[47]
2015	Methacrylates	siRNA	Murine osteoblasts	Trauma-induced heterotopic ossification		[20]
2015	Dextran	siRNA	BALB/c mice	Inflammatory pulmonary disorders	Surfactant shell	[28]
2015	PEI	pDNA	SKOV3 cells, BALB/c mice	Ovarian cancer	Heparin modification	[60]
2015	Glycol chitosan	siRNA	HeLa cells		Folate receptor targeting	[41]
2015	PEI, Cellulose	pDNA	Various stem cells			[61]
2015	Dextran	siRNA	H1299 cells, A549 cells	Lung cancer	Folate receptor targeting, surfactant coating	[27]
2015	PNIPAM-g-PEI	pDNA	BALB/c mice	Gastric tumors	Thermo-responsive	[32]
2015	EGDE	pDNA	Human fibroblasts	Cancer	Photo-responsive, co-delivery	[36]
2015	Methacrylates	siRNA	Murine calcarial preosteoblasts	Heterotopic ossification		[62]
2014	PFPMA, MEO3MA, spermine	siRNA	HeLa cells			[19]
2014	Cycloamilose, spermine	siRNA	ACHN cells, 786-O cells, tumor-bearing mice	Cancer		[48]
2014	Chitosan, alginate	repRNA	Dendritic cells			[49]