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Nanogels for intracellular delivery of biotherapeutics

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ABSTRACT

Many biomolecules, such as proteins and genes, are presently used as therapeutics. However, their delivery to target sites inside cells is challenging because of their large molecular size, difficulties to pass cellular membranes and their susceptibility for enzymatic and chemical degradation. Nanogels, three-dimensional networks of hydrophilic polymers, are attractive carrier systems for these biotherapeutics because they protect the biologicals against degradation and, importantly, facilitate cell internalization. Furthermore, the development of responsive nanogel delivery systems has resulted in particles that release their payloads due to a certain physiological trigger inside cells, such as in the cytosol or endocytic compartments. This paper reviews and discusses the use of nanogels, with special emphasis on biologically responsive systems, for intracellular delivery of biotherapeutics.

1. Introduction

Many biotherapeutics (e.g. proteins and nucleic acids) have their targets inside the cells [1–4]. However, delivery of biotherapeutics to these intracellular targets is challenging due to their unfavorable biopharmaceutical properties (hydrophilic molecules with a high molecular weight), which make them prone to both enzymatic and chemical degradation and prevent them to cross cellular membranes by Fickian diffusion [5–7]. Nanoparticle delivery systems have been shown to be effective in protecting drugs from degradation, overcoming biological barriers, and controlling the rate and duration of drug release [7–13]. Moreover, nano-sized particles can after e.g. intravenous administration accumulate in sites of high vascular permeability (sites of inflammation in e.g. tumors) via the enhanced permeability and retention (EPR) effect [13–15], and nanoparticles can also be rendered cell-specific by coupling of targeting ligands to their surface [16,17]. So far, various types of nanoparticle systems have been developed and applied for (targeted) drug delivery, among which polymer based nanoparticles, micelles, liposomes, as well as inorganic particles [18–24].

Hydrogels are crosslinked networks of hydrophilic polymers that retain a large content of water and can be used for loading and release of biotherapeutics because of this feature [25–27]. Since their discovery and application in the biomedical field, macroscopic systems of hydrogels have been developed and investigated for the design of tissue engineering scaffolds and for local delivery of biotherapeutics [28–31] Nanogels are nano-sized hydrogel particles, which in contrast to macroscopic hydrogel particles, can be injected in the circulation to reach target tissues and deliver their payloads locally and also intracellularly [32–37]. The hydrophilicity of nanogels contributes to some of their desirable features including biocompatibility and high loading capacity for hydrophilic biotherapeutics, and their network protects the encapsulated molecules against degradation because enzymes cannot penetrate into the particles [34–38]. Importantly, the characteristics of nanogels can be tailored by altering their size, crosslink density, and surface properties (PEGylation and surface decoration with targeting ligands) [36,37,39]. However, it is difficult to load and retain molecules with a size that is smaller than the pore meshes in nanogels because the loaded molecules will be released from the particles during their preparation. This can be solved by increasing the crosslink density of nanogels to stably entrap their payloads during gel formation. However, once the biotherapeutics are loaded in hydrogel particles during preparation, this might result in chemical modification of the loaded molecules [40–43]. In other alternative methods, strongly charged biotherapeutics, such as nucleic acids, can be post-loaded into oppositely charged nanogels and stably immobilized by strong electrostatic interaction under physiological conditions [44–48]. For both approaches, the entrapped biotherapeutics can subsequently be released by hydrolytic degradation of the gel network [46–50]. However, this sustained release in turn will result in low concentrations of the released biotherapeutics for prolonged times in the extracellular as well as intracellular environment, which is particularly not wanted for drugs that have their sites of action inside cells. Fast intracellular release of therapeutics can be established by the design of nanogels that are taken up by cells and subsequently degrade rapidly in a triggered manner because of physiological differences between the intracellular environment and the extracellular space. Particularly the low pH of the endo/lysosomes as well the low reduction potential in cells have been exploited to develop nanogels that release their payload in a triggered manner, as discussed in the next sections of this review.

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2. The needs and challenges for intracellular delivery of biotherapeutics

Over the last decades, biotherapeutics have evolved as attractive agents for the treatment of various diseases [1,3,51,52]. Pharmaceutical peptides and proteins as well as nucleic acid based drugs are developed to interfere with key pathways of the target cells to treat both chronic and acute pathologies [1,3,53]. Besides, vaccination with specific antigens provides immunological protection and treatment against different types of cancer and infectious diseases [54,55]. Many peptides and proteins, including antibodies, exert their effect by interactions with cell surface receptors [1,56]. However, a significant number of peptides and proteins have their therapeutic actions inside cells, e.g. in the cytoplasm and specific cellular compartments [2,53,57]. Various forms of RNA based drug (siRNA, mRNA, and miRNA) need to be delivered into the cytoplasm where the cellular translation machinery is located, while pDNA must also cross the nuclear membrane to enable expression of the target genes [3]. In the case of vaccine delivery to induce antigen specific humoral or cellular immune responses, the antigen needs to be translocated in lysosomes or the cytosol of antigen presenting cells (APCs), where it is processed and presented to T cells [58–60].

Biotherapeutics in their free form have some unfavorable pharmaceutical properties. Firstly, these complex molecules are often rapidly eliminated from the circulation by renal filtration (for biotherapeutics ~< 60 kD) or by scavenger cells in the liver (for larger biotherapeutics) and/or inactivated by enzymatic degradation. Secondly, they do not spontaneously pass biological barriers such as lipid membranes of cells. For these reasons, appropriate delivery systems of biotherapeutics are essential to prevent their fast degradation and renal clearance, and to render their intracellular delivery possible. Therefore, in recent years various nano-sized delivery carriers have been developed for encapsulation of biotherapeutics to increase their stability, improve their efficacy by assisting their intracellular delivery to reach to intracellular target sites [5,35,61]. Besides that biotherapeutics need to be retained by the carriers until they reach their target sites, intracellular delivery of these biomolecules with nano-carriers is another key step. These nano-carriers can enter cells from the extracellular space by cell uptake processes including endocytosis and phagocytosis to result in their localization of these particles in endo/lysosomes [62,63]. To reach the aimed intracellular target sites in the cytoplasm or nucleus, the particles and/or the released payload have to undergo endo/lysosomal escape [64–66].

3. Effect of the particle size and surface chemistry on cell internalization

The internalization of nanoparticles and their endocytic processes are impacted by their size and surface chemistry [62,63,67,68]. Larger particles (>1 μm) are taken up by phagocytosis, while the uptake of particles with size between 500 nm to 1 μm occurs essentially via micropinocytosis [67,68]. Particles of about 100 nm are taken up by clathrin mediated endocytosis, while cavelae-mediated endocytosis takes place when the particle size is between 50 and 80 nm [62,67,69]. It has further been shown in many studies that nanosized particles are beneficial to enter the cells rapidly and the preferred size for drug delivery is smaller than 100 nm [62,63,67,70,71]. Further, nanogels with size between 20 and 350 nm all showed more or less internalization by different cells [47,72–78].

On the other hand, many studies suggest that the size of particles may not be that important compared to other factors for cell internalization. In many studies it has been shown that nanoparticles with a positive surface charge bind to the negatively charged cytomembrane via electrostatic interaction, which subsequently results in a rapid entry of the cells through adsorptive endocytosis [30,67,79–85]. However, it should be noted that positively charged nanogels generally speaking are more cytotoxic than neutral or negatively charged particles. These latter particles might interact with cells with hydrophobic domains present on their surfaces [62,67,86–89]. Cellular uptake of nanogels can be promoted by the introduction of targeting ligands on their surface which bind to receptors expressed on certain cells. Hyaluronic acid is often used as a component of nanogels because it can target the CD44 receptor that is overexpressed in many cancer cell lines [90,91]. Furthermore, the surface of nanogels can be modified using antibodies, polypeptides, aptamers and other targeting groups for specific binding with receptor specific cells [92–95].

4. Biologically responsive nanogels as delivery systems

As pointed out in the previous sections, biotherapeutics can be stably encapsulated either in highly crosslinked nanogels or by strong electrostatic interactions with nanogels to minimize their premature leakage. Such nanogels mostly slowly release the encapsulated biomolecules due to hydrolytic degradation of (crosslinks in) the polymer network. However, this sustained release may also lead to too low concentrations of the biotherapeutics at their site of action. Therefore, in recent years, nanogels have been designed with crosslinks that can be broken by external stimuli such as temperature, light, and ultrasound, or by biological triggers, such as differences in pH and/or reduction potential that might result in rapid swelling and/or degradation which in turn is associated with release of the payload [35,36,96,97]. For nanogels that respond to external stimuli, highly functionalized equipment is required to provide a focused trigger after the nanogels reach their targets, which is not always feasible. Therefore, in the following subsections, the emphasis is on the triggered release of biotherapeutics from responsive nanogels by biological stimuli.

4.1. Reduction responsive nanogels

The intracellular environment is characterized by a reducing environment which is due to the fact that the glutathione (GSH) levels in the cytosol and nucleus (approximately 2–10 mM) are hundred-fold higher than that in the extracellular fluids (approximately 2–20 μM) [98]. This substantial difference in GSH concentration can be exploited as a potential stimulus for cytosolic release of biotherapeutics from internalized carrier systems. Particularly disulfide linkages are readily cleavable in reducing environments and converted to thiols [99], which can be exploited for the design of intracellular degradable nanogels. However, as mentioned in Section 2, nanogels and nanoparticles in general enter cells mostly via endocytic pathways [39,100–102]. Therefore, these reduction responsive nanogels are likely to be entrapped in endo/lysosomes in which the GSH concentration is much lower than in the cytosol [103,104]. This means that the nanoparticles need to escape from the endo/lysosomes to access GSH [64–66]. It should be noted that endocytic compartments also provide reducing environments for disulfide reduction by other means [105]. To mention, it has been reported that redox enzymes expressed on cell surfaces or secreted by cells, such as protein disulfide isomerase (PDI), are transported into endosomes during the invagination process [106]. However, PDIs loses their catalytic activity at low pH of the late endo/lysosomes [105]. Thus, the activity of PDI is likely restricted to the early endosomes. The redox potential in endocytic compartments is mainly modulated by a specific reducing enzyme called gamma interferon-inducible lysosomal thiol reductase (GILT), which has its optimal enzymatic activity at a low pH (4.5–5.5) [107]. Furthermore, the reductive activity of GILT has been reported to be maintained by cysteine and GSH [108–110]. Taken together, disulfide crosslinks may also be reduced in the endocytic compartments.

Biotherapeutics can be reversibly immobilized in nanogels via reduction sensitive disulfide bonds exploiting mainly two approaches. Firstly, therapeutics can be covalently conjugated via disulfide linkages to nanogel networks and in this way burst release of the conjugated molecules is avoided [111–113]. The release requires a reductive trigger

in the cells to cleave the link between the carrier and therapeutic molecules. For example, DeSimone et al. [111] synthesized siRNA coupled via a degradable disulfide linkage to a polymerizable acrylate (Fig. 1A). Subsequently, the derivatized siRNA was copolymerized with PEG dimethacrylate, PEG acrylate and 2-aminoethyl methacrylate to prepare cationic nanogels in which siRNA was covalently incorporated via a disulfide linkage. Triggered release of siRNA was indeed observed in a reducing environment while the therapeutic retained in the nanogel particles under physiological conditions. Furthermore, dose-dependent silencing of luciferase expression was elicited for the Hela cells incubated with disulfide-conjugated siRNA nanogels, while the control nanogels loaded with free/non-degradable-conjugated siRNA did not show significant gene silencing effects (Fig. 1B). It should be mentioned that for this strategy, the biotherapeutics lacking free thiol groups need to be chemically modified with functional groups, which is not always feasible because the conjugation reaction might lead to lower biological activity or even loss of function [99,114].

In another approach, biotherapeutics are physically entrapped in disulfide-crosslinked nanogel networks during the preparation. The release of the encapsulated molecules occurs due to the reductive response to break the structure of the nanogels and allowing subsequent release of the payload [47,48,85,115–117]. Physical entrapment of the biotherapeutics adds versatility to a reduction responsive nanogel system because a variety of different molecules can be entrapped into the same nanogel particles. Reduction responsive nanogels containing disulfide linkages in their crosslinks keep their payloads stably encapsulated in the extracellular space provided that the hydrodynamic size of the entrapped compounds is greater than the mesh sizes of the hydrogel network. The release of the encapsulated molecules can subsequently occur after internalization of the nanogels and reduction of the disulfide crosslinks which results in an increased hydrogel mesh size or complete disintegration of the nanogel structure allowing diffusion of the payloads into the intracellular space. Park et al. [118] reported that DNA/thiol-functionalized six-arm branched PEG complexes were crosslinked through the formation of disulfide linkages between the thiol groups, resulting in stable DNA/PEG nanogels (Fig. 2). Because the hydrodynamic size of DNA was greater than the meshes of the crosslinked PEG nanogels, DNA release only occurred in presence of GSH due to the cleavage of disulfide crosslinks. Moreover, the transfected cells exhibited appreciable green fluorescent protein (GFP) expression once incubated with the DNA-loaded reducible PEG nanogels, but the GFP transfection efficiency was lower than that of commercially available transfection agents (Lipofectamine or polyethylenimine (PEI)-based formulations). This might be due to entrapment of the DNA/PEG nanogels in the endocytic compartments preventing DNA translocation into the nucleus. Beside physical entrapment of biotherapeutics in strongly disulfide-crosslinked nanogels, another approach to stably immobilize actives, particularly long chain nucleic acids with highly negative charges, is by strong electrostatic interaction in cationic disulfide-crosslinked nanogels. Under reductive conditions, cleavage of disulfide linkages resulted in breakdown of the nanogels and release of the payloads [47,48,119]. Hollinger et al. [120] prepared cationic nanogels with disulfide crosslinks for the delivery of two types of siRNA (against GAPDH or GFP) for gene silencing. Nanogels were prepared by copolymerizing dimethyl aminoethyl methacrylate, oligo(ethylene oxide) methacrylate, and a water soluble disulfide methacrylate crosslinker using a poly(ethylene glycol 2-bromoisobutyrate) initiator via electron transfer atom transfer radical polymerization. SiRNA was encapsulated in these cationic nanogels by electrostatic interaction with a high efficiency. The expressing of GAPDH was inhibited by reduction responsive nanogels-mediated GAPDH siRNA delivery to MC3T3 cells. Further, it was demonstrated that these GFP-siRNA loaded nanogels facilitated the knockdown of GFP in a GFP expression mouse model after intramuscular administration. Our group reported on decationized disulfide-crosslinked nanogels for intracellular gene delivery [121–123]. Cationic polyplexes were prepared by the transient presence cationic groups coupled to the polymer backbone to allow electrostatic driven condensation with pDNA. After condensation, cationic nanogels were formed by disulfide crosslinking of polymer chains in which pDNA was thus entrapped. Finally, the labile cationic groups were removed by hydrolysis at pH 9, yielding neutral nanogels with a core of disulfide crosslinked poly(hydroxypropyl methacrylamide) and a shell of poly(ethylene glycol). pDNA was stably entrapped in the disulfide crosslinked core of decationized nanogels under physiological conditions, and released from the nanogels triggered by intracellular reducing environment due to cleavage of the disulfide crosslinks. Furthermore, forced introduction of the nanogels into the cytosol of HeLa cells by electroporation resulted in a high level of gene expression similar as naked pDNA, demonstrating intracellular disassemble of the nanogel and release of entrapped pDNA. These decationized nanogels exhibited excellent cytocompatibility, an increased circulation time and higher tumor accumulation when compared to their cationic precursors.

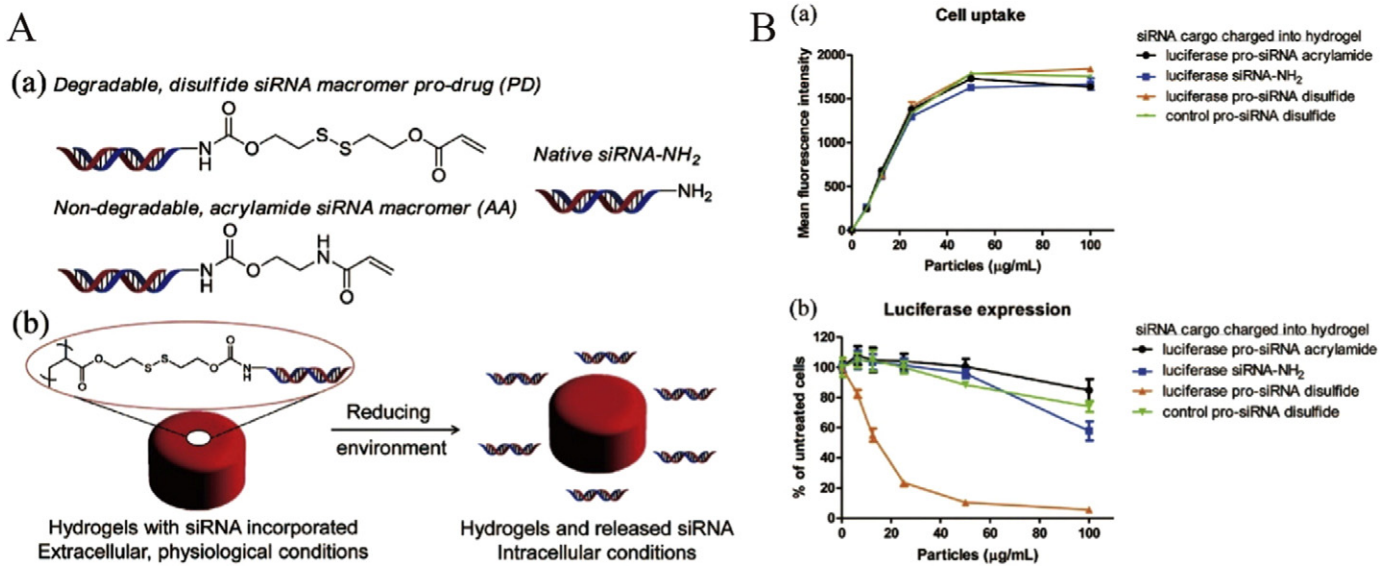

Fig. 1. (A) Structures of degradable and nondegradable siRNA macromers as well as native siRNA (a) and illustration of nanogels with disulfide-conjugated siRNA behavior under physiological and intracellular conditions (b). (B) Cellular uptake (a) and luciferase expression (b) in HeLa/luc cells after incubation with nanogels loaded with various modified siRNA. Reproduced with permission from ref [111].

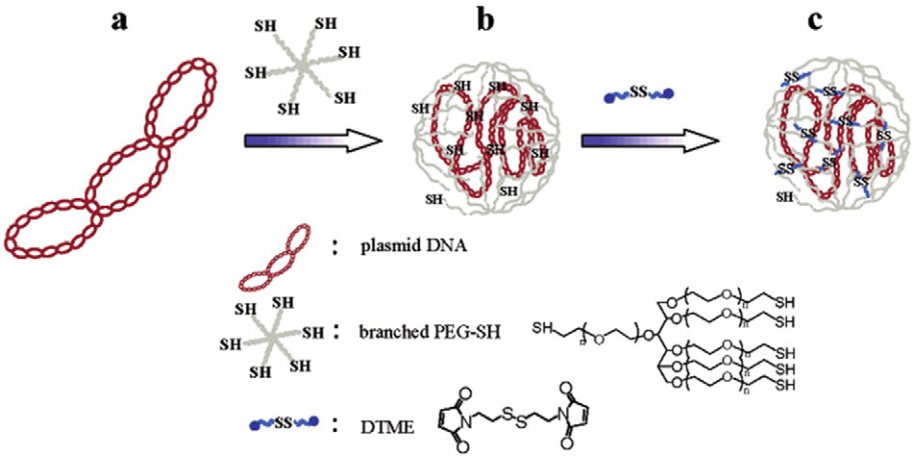

Fig. 2. Preparation of disulfide-crosslinked DNA/PEG nanogels. Reproduced with permission from ref [118].

Furthermore, histological analysis of tumors sections showed that decationized nanogels were able to induced transgene expression in vivo.

Most of reported reduction responsive nanogels increased the efficacy of the loaded biotherapeutics likely due to the relative higher amount of biomolecules that are delivered and released into cells compared to

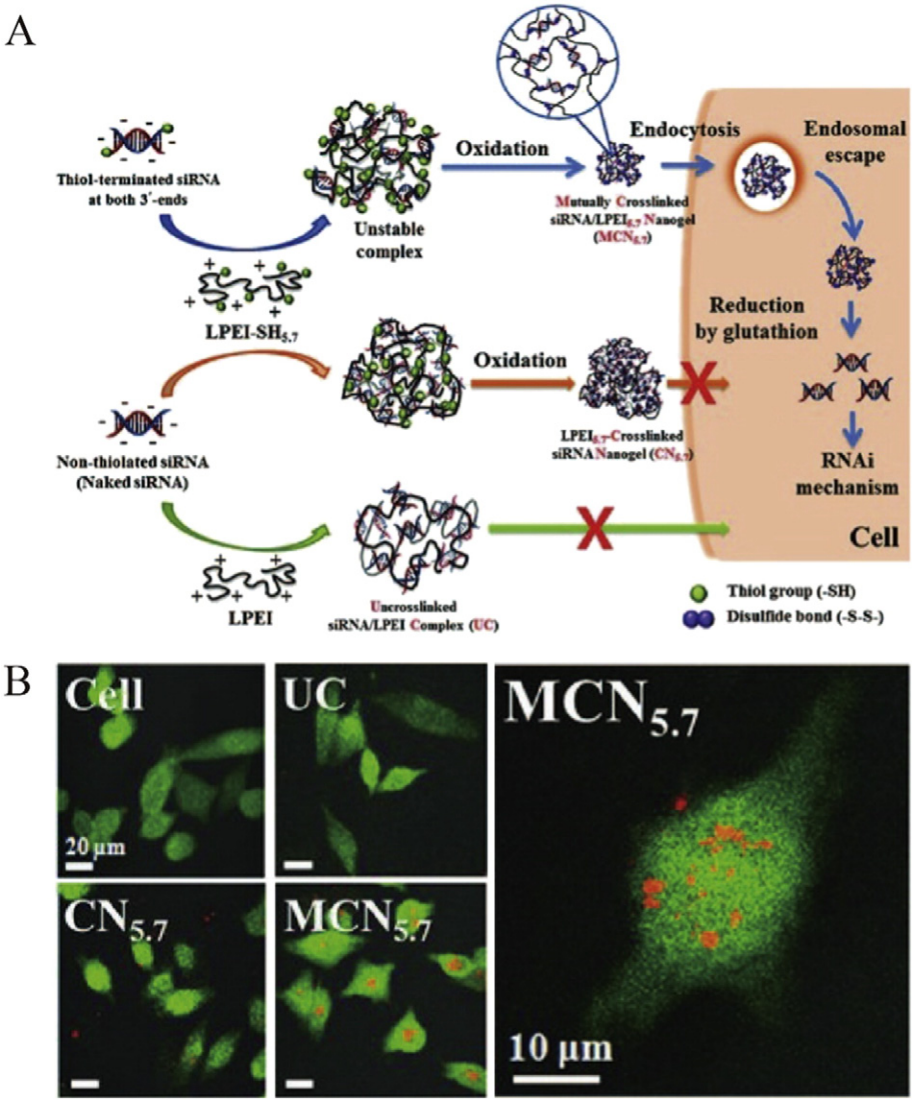

Fig. 3. (A) Schematic illustration of the preparation and intracellular processing of siRNA/LPEI nanogels (MCN), naked siRNA loaded LPEI nanogels (CN) and uncrosslinked siRNA/LPEI complexes (UC), respectively. (B) Confocal microscopy images of MDA-MB-435-GFP cells after the cellular uptake of Cy5-labeled carriers. High magnification confocal image of the cell treated with MCN. Reproduced with permission from ref [112].

those from their non-reducible counterparts. Although the observed effects with the reduction sensitive formulations are conclusive, at present no convincing paper has been published in which the intracellular trafficking and fate of disulfide containing carriers and/or their payloads were visualized, probably because of limitations of confocal imaging technologies. Nam et al. [112] developed an approach in which thiol-terminated siRNA was grafted onto thiol-functionalized linear PEI (LPEI) via disulfide bonds to form stable siRNA/PEI nanogels (referred to as MCN, Fig. 3A). As control, unmodified siRNA loaded LPEI nanogels (referred to as CN) only with the crosslinking between LPEI chains were used, and uncrosslinked siRNA/LPEI polyplex (referred to as UC) using naked siRNA and unmodified LPEI were also prepared. Confocal images of Cy5-labeled LPEI carriers incubated with MDA-MB-435-GFP cells showed that MCN was internalized to a greater extent than the other two controls (CN and UC, Fig. 3B). Furthermore, the red fluorescent dots observed in the cytoplasm suggested that a considerable number of the internalized particles were entrapped in the lyso/endosomes. Nevertheless, MCN showed excellent gene silencing activity as compared to CN and UC. In another approach, Dai et al. [119] coupled two amine groups of L-cystine with acetaldehyde to form disulfide bond-linked double Schiff-bases, aldehyde-L-cystine, which possesses autofluorescence based on the $n \rightarrow \pi^*$ electron transition (—N = C—). Subsequently, the two carboxyl groups of aldehyde-L-cystine can be further used to crosslinked branched PEIs via amine coupling reaction to generate cationic nanogels with disulfide crosslinks. These cationic nanogels showed high pDNA loading due to strong electrostatic interactions. The release of pDNA occurred upon degradation of the nanogels via cleavage of the disulfide bonds in the reductive intracellular environment. Meanwhile, cleavage of disulfide bonds also led to loss of autofluorescence of aldehyde-L-cystines, which enabled tracking the intracellular degradation of the nanogels. Confocal images of HeLa cells exposed to these pDNA loaded nanogels during the first 6 h of incubation showed that fluorescent signals increased within endo/lysosomal membrane, indicating cellular uptake and internalization of the nanogels by endocytosis. The decrease of fluorescence was observed after 6 h likely because disulfide bonds were cleaved and subsequently aldehyde-L-cystines lost their autofluorescence, indicating degradation of the nanogels. However, due to the loss of fluorescence after degradation, no direct evidence of cytosolic release can be obtained. To prove that reductive degradation of nanogels contributed to pDNA transfection in CHO cells, a GSH inhibitor (duroquinone) was used to deplete GSH during the cell transfection process and it was observed that transfection efficiency decreases by ~50% in the presence of this GSH inhibitor. This observation suggests that the reduction responsiveness of these nanogels indeed plays an important role in the transfection process.

The release of loaded biotherapeutics from reduction sensitive nanogels and their biological effects do not only depend on the design of the carriers, but is also dependent on the reducing potential of the intracellular environment, which differs for various cell types. Park et al. [124] synthesized thiolated heparin-pluronic firstly by coupling carboxylated pluronic (F127) to the hydroxyl groups of heparin. Subsequently, amino groups of cystamine were conjugated to carboxyl groups in heparin and the disulfide bonds of cystamine were cleaved to form thiol groups. RNase A is an enzyme that can hydrolyze single stranded RNA without sequence specificity in the cytosol and the nucleus and thereby inducing cytotoxic effect. However, this enzyme is not able to pass cellular membranes by diffusion. RNase A has a high pI and therefore binds at neutral pH to thiolated heparin-pluronic via electrostatic interaction to yield nanogels. Subsequently, stable nanogels were obtained by oxidation of the thiol groups of thiolated heparin-pluronic to form disulfide crosslinks. The release of RNase A from these disulfide crosslinked nanogels was much slower than that from non-crosslinked nanogels. A sustained release of ~40% RNase A from disulfide-crosslinked nanogels was observed during 20 h in a non-reducing environment, while ~80% was released under reductive conditions in the presence of 10 mM GSH during same time period. However, the cytotoxicity results showed no improved effect of RNase A loaded disulfide-crosslinked heparin-pluronic nanogels after incubation with NIH3T3 cells compared to drug-free nanogels. Nevertheless, in another paper [125], the same authors reported that the cytotoxicity of RNase A loaded non-crosslinked heparin-pluronic nanogels was significantly increased as compared to free RNase A. It should be noted that the cytotoxicity study of RNase A loaded disulfide-crosslinked heparin-pluronic nanogels was performed on NIH3T3 cells. The NIH3T3 cell line is a fibroblast cell line that is known for its non-reductive endo/lysosomal compartments with relatively low levels of intracellular GSH [110,126,127], which might explain the low effects observed in this study. Some tumor cell lines have been shown upregulated expression of GILT, which increases the reducing potential of lysosomes [128,129]. Zhong et al. [130] developed reduction sensitive degradable nanogels based on poly(ethylene glycol)-b-poly(2-(hydroxyethyl) methacrylate-co-acryloyl carbonate) block copolymers and tested them on HeLa cells. These copolymers formed disulfide-crosslinked nanogels in the presence of cystamines via ring-opening reaction with pendant cyclic carbonate groups (Fig. 4). Cytochrome C is a membrane-impermeable protein, which initiates the caspase mediated apoptosis cascade in the cytoplasm that results in programmed cell death [131]. Cytochrome C was encapsulated in the disulfide crosslinked network during formation of nanogels with high loading efficiency. It was shown that ~30% of the loaded cytochrome C was released in 22 h in non-reducing environment, while ~95% was released in same time period under reductive conditions in the presence of 10 mM DTT. Cytochrome C encapsulated in these reduction sensitive nanogels were more cytotoxic than cytochrome C loaded in reduction-insensitive control nanogels as well as free cytochrome C after incubation with HeLa cells.

In addition to the delivery of nucleic acid based drugs and therapeutic proteins as discussed so far, nanogels have also been used for the delivery of protein and peptide antigens in antigen presenting cells (APCs) for vaccination purposes. GILT is constitutively expressed in APCs and plays an important role in exogenous antigen processing and presentation via the endocytic pathway by cleavage of the disulfide bonds for protein unfolding [132–134]. In addition, GILT is considered to facilitate transfer of disulfide containing antigens into the cytosol, thereby enhancing their cross-presentation in the MHC (major histocompatibility complex) class I pathway for cellular immune response [135,136]. Ma et al. [137] developed bioreducible cationic nanogels by the electrostatic interaction of negatively charged alginate with branched PEI, followed by crosslinking with a disulfide linker, 3,3′-dithiobis(sulfosuccinimidyl propionate). A model protein antigen, ovalbumin (OVA), was post-loaded in these nanogels by electrostatic interactions. The stability of OVA encapsulation in these nanogels in reducing and non-reducing conditions was not studied. However, confocal images showed that more OVA from reduction sensitive nanogel formulation were processed by dendritic cells (DCs) as compared to from the non-reducible counterparts. Furthermore, the percentage of DCs that have detectable antigen fragments in the cytosol was ~2 fold higher than those incubated with non-reducible nanogels. Moreover, compared with non-reducible nanogels, the bioreducible nanogels enhanced both MHC class I and II antigen presentation in vitro and in vivo. Our group developed cationic dextran based nanogels containing thiol-reactive groups [113]. Thiolated OVA was absorbed in these particles exploiting electrostatic interactions between the negatively charged protein and the positively charged network and subsequently covalently linked via disulfide bonds. The release of OVA only occurred in reducing environments in the presence of 2.5 mM GSH. Furthermore, MHC class I antigen presentation was substantially enhanced by intracellular delivery of disulfide conjugated OVA as compared nanogels that were only physically loaded with the same protein.

4.2. pH responsive nanogels

After endocytosis of carriers by cells, an acidification process causes a decrease in pH values to as low as 5.5 or even 4.5 in endosomal and/or

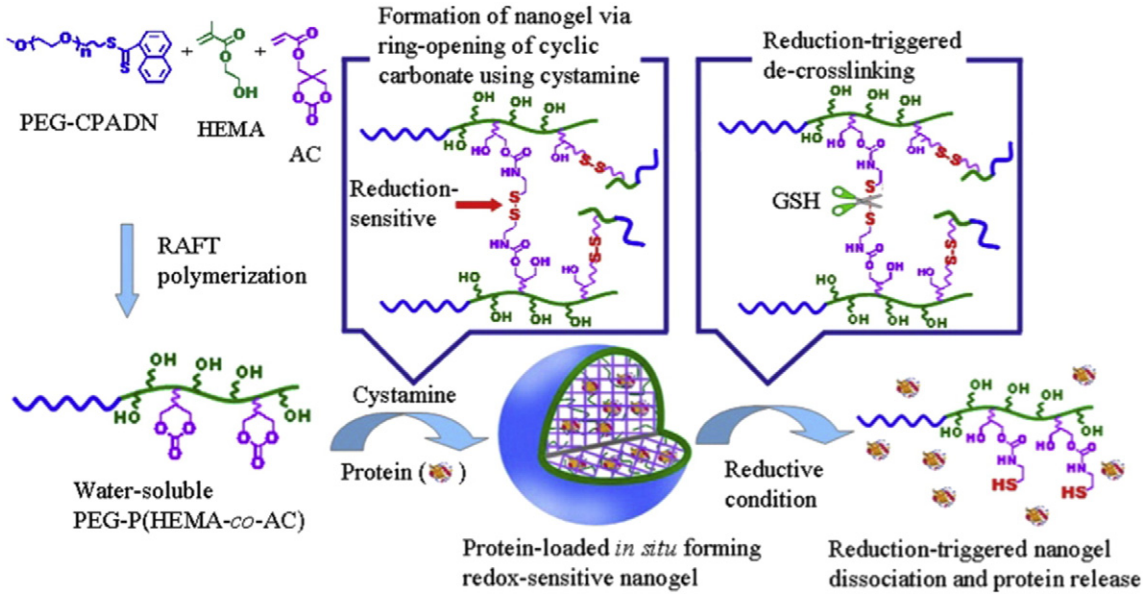

Fig. 4. Illustration of in situ forming reduction-sensitive nanogels for loading and triggered release of proteins. Reproduced with permission from ref [130].

lysosomal compartments, respectively [138,139]. This relatively low pH in endo/lysosomes can be exploited to design nanogels for intracellular delivery of biotherapeutics. One strategy for development of pH responsive nanogels is to use materials having acid-labile functional groups within the polymer backbone or crosslinks [139–141]. The triggered degradation of these nanogels is based on cleavage of acid-labile bonds within the nanogel networks upon lowering the pH. The release of encapsulated biomolecules in turn results from the rapid degradation of nanogels at low pH. Therefore, pH sensitive nanogels can retain the loaded biotherapeutics in physiological conditions, and release their payloads in acidic cellular compartments after their internalization [142–148]. Thayumanavan et al. [146] prepared pH degradable nanogels by copolymerizing tetraethylene glycol methacrylate with a crosslinker containing β-thiopropionate, which is cleavable at low pH. Acid α-glucosidase, an enzyme which is essential for the conversion of glycogen to glucose in lysosomes and which has its highest activity at low pH, was loaded in the nanogels during gel formation. It is hypothesized by the authors that the enzyme would be less available to the substrate when encapsulated, and therefore would have a lower activity. No enzymatic activity was detected when exposing the non-pH-degradable control nanogels to pH 5 buffer. However, enzymatic activity was observed after incubation of the degradable nanogels for 30 min with the same buffer, indicating release of the enzyme due to nanogel degradation. Lu et al. [144] reported a pH triggerable delivery system based on single protein nanogels. Polymerizable vinyl groups (5 to 20 per protein) were covalently coupled to Caspase-3, an essential protein involved in apoptosis by cleaving cellular proteins involved in DNA repair and cell structure. Subsequently, the derivatized protein was loaded in a pH degradable crosslinked polymer shell by copolymerizing it with acrylamide, 2-dimethylaminoethyl methacrylate and an acid cleavable glycerol dimethacrylate crosslinker. These Caspase-3-nanogels were broken down and the Caspase-3 was released after cellular internalization in HeLa cells. Moreover, the labeled nanogels showed colocalization with early endosomes and lysosome after 30 min, with gradual release to the cytosol, which suggested endo/lysosomal escape. These Caspase-3-nanogels showed significantly higher cytotoxicity than their non-degradable counterparts after incubation with HeLa cells, confirming that the protein was indeed released in the cells.

Another frequently applied strategy for the design of pH responsive nanogels concerns the use polymer with functional groups that ionize at low pH, such as amines [139,149,150]. Given the pH difference that exists between the extracellular environment/cytosol on the one hand and endo/lysosomal vesicles on the other hand, polymers with a pK_a between 5.0 and 8.0 show changes in their physicochemical properties. Protonation of these groups in the intracellular acidic compartments can cause swelling or disassembly of the nanogels, which leads to triggered release of the encapsulated biomolecules [139,140]. Most importantly, protonation of the polymers/nanogels not only provides a trigger for the release of their payloads at low pH, but can also facilitate escape from the endo/lysosomal compartments by the so-called proton sponge effect [64]. The proton sponge mechanism relies on the buffering effect of polymers undergoing protonation that consumes protons, which in turn induces an influx of protons and counter- ions into the endo/lysosomal compartments to maintain their desired pH. Subsequently, the high ion concentration in the endo/lysosomes causes water inflow from the cytosol, which eventually leads to osmotic swelling and rupture of the endo/lysosomal membrane and thereby releasing the entrapped components into the cytoplasm [66]. PEI is frequently used for gene delivery because of its proton sponge effect upon protonation of the amine groups present in its structure [151–153]. Park et al. [154] synthesized catechol grafted branched PEI for siRNA delivery. These polymers self-assembled in acidic and neutral aqueous solutions, and subsequent self-crosslinking under basic conditions by Michael addition between quinones of the oxidized catechol group and amines of PEI occurred to yield cationic PEI nanogels. SiRNA silencing GFP expression was loaded in the nanogels and stable siRNA/nanogel complexes were formed by electrostatic interactions. The hydrodynamic diameter of the nanogels gradually increased with decreasing pH, suggesting that the amine groups were protonated causing absorption of significant amount of water in the gel structure at reduced pH values. Although endosomal escape was not clearly seen by confocal imaging, the siRNA/nanogel complexes exhibited enhanced cellular uptake and promoted gene silencing efficiency when incubated with GFP over-expressing MDAMB-435 cells. Besides PEI, other polyamines have shown to be susceptible for the proton sponge effect as well. Tasciotti et al. [155] described a one-pot synthesis method to produce nanogels based on free radical co-polymerization of the cationic monomer 2-(diethylamino)ethyl methacrylate and the monomeric silica coupling agent vinyltrimethoxysilane in the presence of polyethylene glycol methacrylate and triethylene glycol dimethacrylate linkers (Fig. 5A). Nanogels with a crosslinked 2-(diethylamino)ethyl methacrylate hydrogel shell around silica nanoparticle cores were obtained, and siRNA was loaded in the cationic hydrogel shell via electrostatic interactions. When the pH changed from 7 to 5, the tertiary amine groups of the

poly (2-dimethylamino) ethyl methacrylate shell were protonated, which resulted in swelling and in a ~16-fold increase in hydrodynamic volume (diameter increased from 100 to 250 nm). Further, the ability of these nanogels to escape endo/lysosomal compartments was evaluated using a human breast cancer cell line (MDA-MB-231) by transmission electron microscopy (TEM). TEM images (Fig. 5B) showed that untreated and non-responsive nanogels treated cells maintained distinguishable vesicles with compact borders, while cells incubated with the pH responsive nanogels showed subcellular structures with an irregular shape and non-continuous borders. Moreover, the pH responsive nanogels were scattered throughout the cytoplasm, indicating that these nanogels indeed escaped from endo/lysosomal vesicles. Furthermore, CXCR4 siRNA delivered by these pH sensitive nanogels in MDA-MB-231 cells showed reduced protein expression of CXCR4 with an efficacy comparable to that of a commercial HiPerFect transfection reagent. Moreover, mice that received intravenously injected siRNA-loaded pH sensitive nanogels showed a reduction of CXCR4 expression at the tumor site as compared to mice treated with free siRNA and non-responsive nanogels.

In other studies, pH-dependent charge-reversal nanogels were designed to maintain a negative surface charge to increase their stability under physiological conditions (pH 7.4), and subsequently reverse their surface charge to enhance cell uptake at the tumor site due to decrease of pH value [156,157]. For example, Liu et al. [157] synthesized pH responsive charge conversional chitosan-agmatine conjugates (Fig. 6A). Chitosan was reacted with dimethylmaleic anhydride to convert its primary amines into amides with a carboxylic functional group. This amide could be hydrolyzed quickly at a slightly acidic pH value (<pH 6.8) to result in the re-formation of the primary amines and thus charge reversal. The modified chitosan was subsequently coupled to agmatine. Vascular endothelial growth factor (VEGF) is a protein that is essential for tumor growth, progression, and metastasis [158]. The chitosan-agmatine conjugates condensed VEGF-suppressing siRNA into the cationic agmatine core to form stable nanogels with an acid responsive charge-reversal anionic shell. Chitosan/VEGF-siRNA complexes were prepared as a control and had the same particle size as VEGF-siRNA chitosan-agmatine nanogels (around 150 nm). These nanogels were stable in the presence of serum at physiological pH

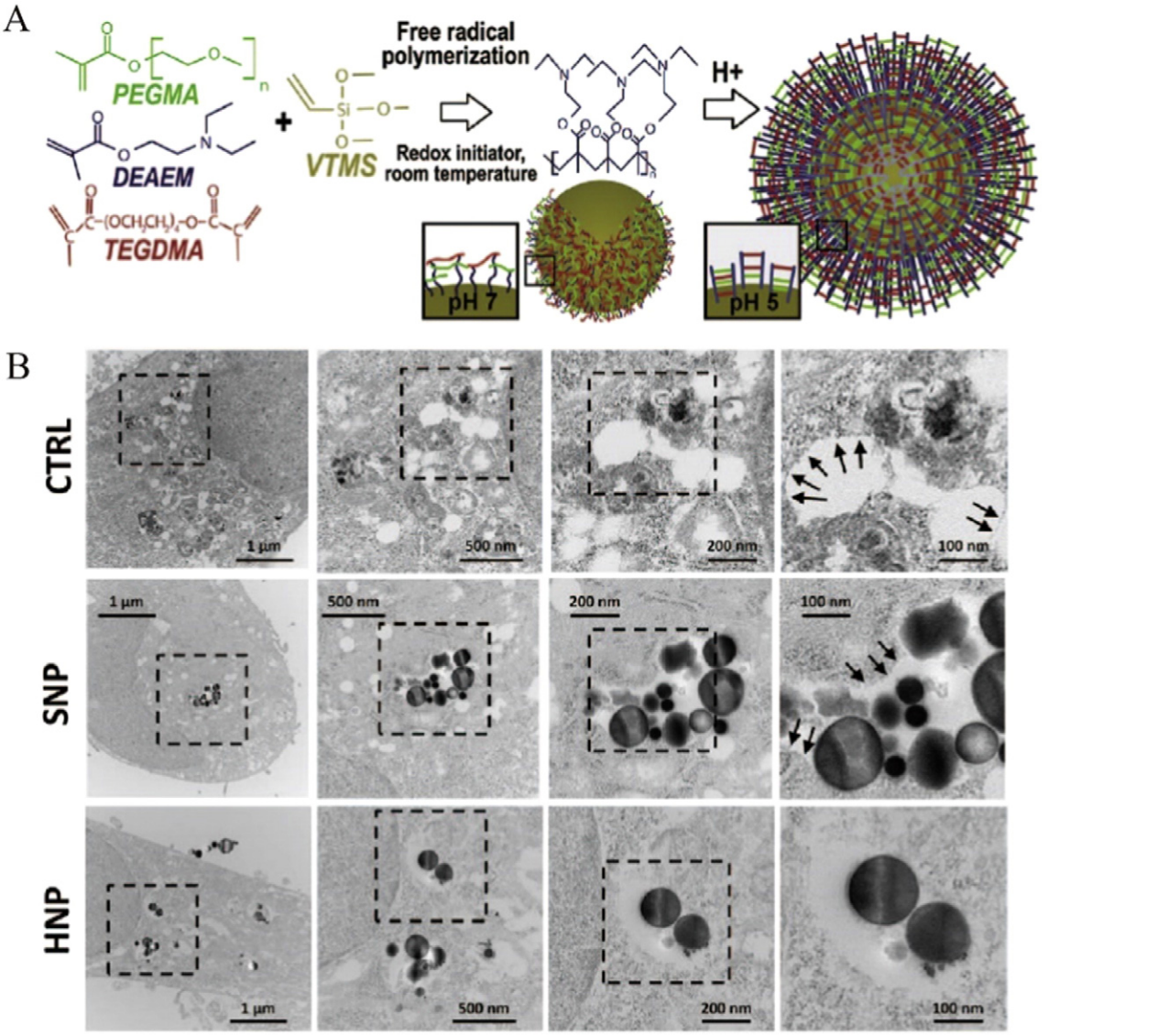

Fig. 5. (A) Schematic representation of the preparation of pH responsive nanogels based on free radical polymerization of the cationic monomer 2-(diethylamino)ethyl methacrylate (DEAEM) and the monomeric silica precursor vinyltrimethoxysilane (VTMS) in the presence of polyethylene glycol methacrylate (PEGMA) and triethylene glycol dimethacrylate (TEGDMA) linkers. (B) TEM images of MDA-MB-231 cells 3 h after treatment with pH responsive nanogels (HNP), non-responsive nanogels (SNP) and untreated (CTRL). The black arrows show the border of endo/lysosomes. Reproduced with permission from ref [155].

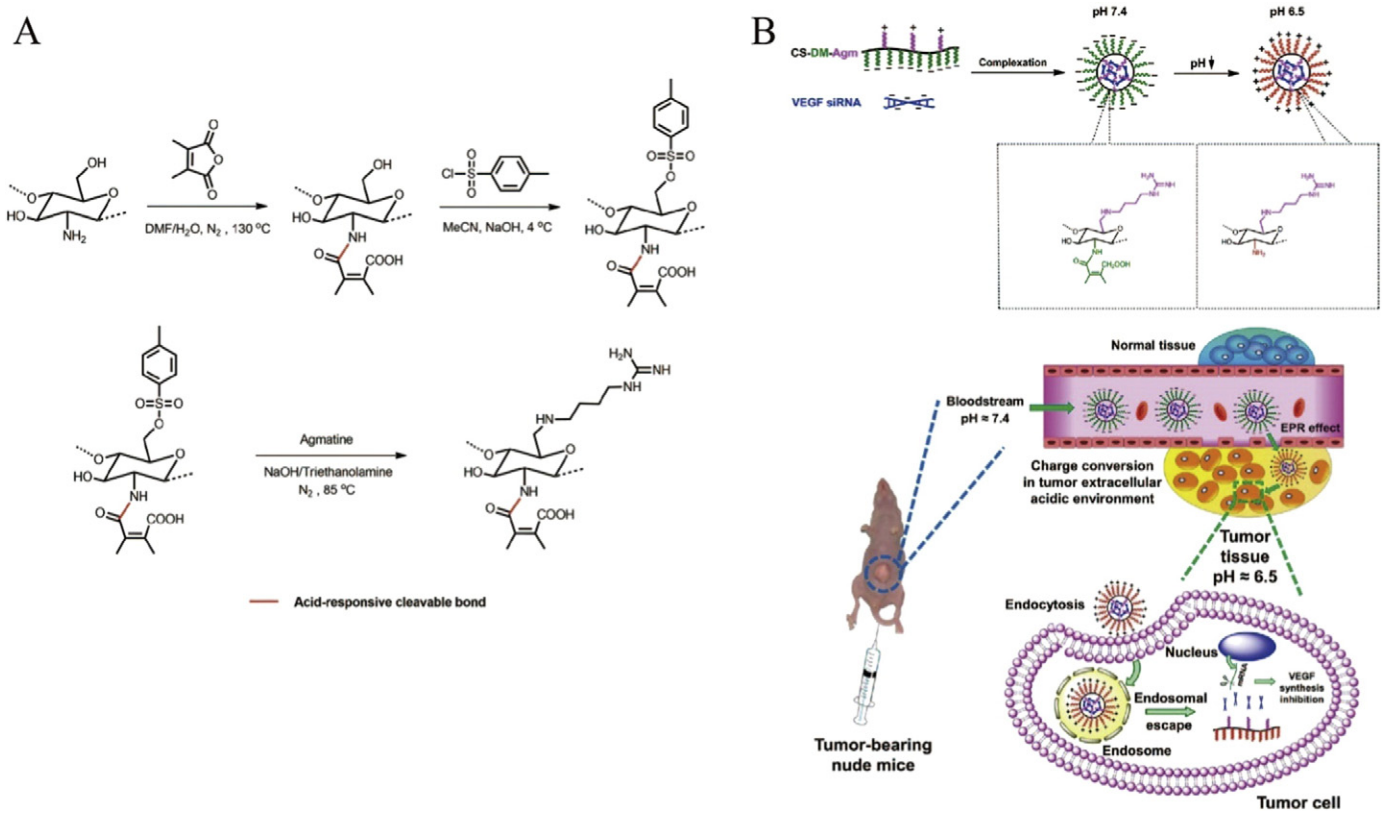

Fig. 6. (A) Synthetic procedure of chitosan-agmatine conjugates. (B) Schematic illustration of the formation of VEGF-siRNA complexes by the pH responsive charge conversional chitosan-agmatine conjugates (CS-DM-Agm), the change of surface charge property in response to the tumor acidity, and the hypothesized mechanism for tumor suppression of the siRNA complexes. Reproduced with permission from ref [157].

(7.4) owing to their negatively charged surface, whereas chitosan/VEGF-siRNA nanogels rapidly aggregated in the presence of serum proteins. The zeta potential of the nanogels reversed rapidly within 30 min from −12 to +9 mV when the pH was decreased from 7.4 to 6.5. VEGF-suppressing siRNA loaded charge-conversional nanogels were administered to Hela tumor-bearing nude mice by intravenous administration to investigate their therapeutic effects (Fig. 6B). It was shown that these siRNA loaded charge-conversional nanogels effectively suppressed VEGF expression and microvessel growth, and also inhibited tumor cell proliferation as compared to chitosan/VEGF-siRNA complexes. This better efficacy is likely because the initial negative surface charge prolonged the blood circulation kinetics and as consequence a higher number of particles deposited in the tumor due to the EPR effect. Moreover, the exposed cationic amine groups after charge reversal at slightly acidic tumor extracellular environment provided the nanogels a higher affinity for the negatively charged cell membrane and facilitated cellular uptake and endo/lysosomal escape via the proton sponge effect.

Besides nucleic acid based therapies, there is also a growing interest in development of pH sensitive nanocarriers that can enhance cytoplasmic entry of antigenic peptides and proteins in APCs. To improve the efficacy of vaccines against tumors, the antigen specific cellular immune response is considered to be crucial [159–161]. The delivery of antigens to the cytosol of APCs is essential, in order to facilitate antigen processing and loading into MHC class I molecules to generate a $CD8^+$ T cell response [162,163]. Irvine et al. [164] developed pH responsive nanogels for endosomal escape and cytosolic delivery of an antigen (OVA). The nanogels were synthesized by a two-stage polymerization reaction to yield particles with a pH sensitive crosslinked core containing poly(2-dimethylamino)ethyl methacrylate) and a pH insensitive hydrophilic shell layer of poly(2-aminoethyl methacrylate) (Fig. 7A). OVA were electrostatic adsorbed to the cationic surfaces of the core-shell particles. These nanogels swelled abruptly due to protonation of tertiary amines in the core between pH 7.0 and 6.8, corresponding to a ~22-fold volume change. Calcein, a membrane-impermeable fluorophore, was used to monitor the integrity of endosomes/phagosomes following particle uptake. DC2.4 cells were co-incubated with labeled pH sensitive nanogels, calcein and lysotracker Red DND-99 for 1 h to label endo/lysosomal compartments. The confocal images (Fig. 7B) revealed that a significant fraction of the internalized pH sensitive nanogels did not colocalize with endo/lysosomal vesicles while calcein fluorescence was observed throughout the cytosol and nucleus. However, cells treated with non-responsive nanogels exhibited a punctuate distribution of the nanogels and calcein fluorescence, which both colocalized with lysotracker. These observations suggest that the pH sensitive nanogels indeed facilitate endo/lysosomes escape. To obtain more direct evidence of endo/lysosomal escape, TEM images of cells treated with nanogels were taken (Fig. 7B). These images showed that pH-insensitive nanogels were localized within membrane-bound compartments, while pH sensitive nanogels were observed both within membrane-bound vesicles as well as within the cytosol. Ma et al. [165] reported that pH sensitive galactosyl dextran-retinal (GDR) nanogels enhanced MHC class I antigen cross presentation of OVA and anticancer immunity. Amphiphilic pH sensitive GDR was synthesized by conjugating all-trans retinal (a metabolite of vitamin A) to dextran through a pH sensitive hydrazone bond, followed by galactosylation of dextran. Upon dissolving in water in the presence of OVA as a model antigen, GDR self-assembled into pH sensitive nanogels loaded with OVA. The cleavage of the hydrazone bond at pH 5 led to release of retinal from the GDR nanogels, which in turn caused disassembly of the nanogels and release of OVA. GDR nanogels were shown to promote antigen uptake after incubation with DCs for 2 h as compared to free antigen, and the labeled antigen did not colocalize with lysotracker. The authors hypothesized that the cleavage of hydrazone bonds in the endo/lysozymes consumed a

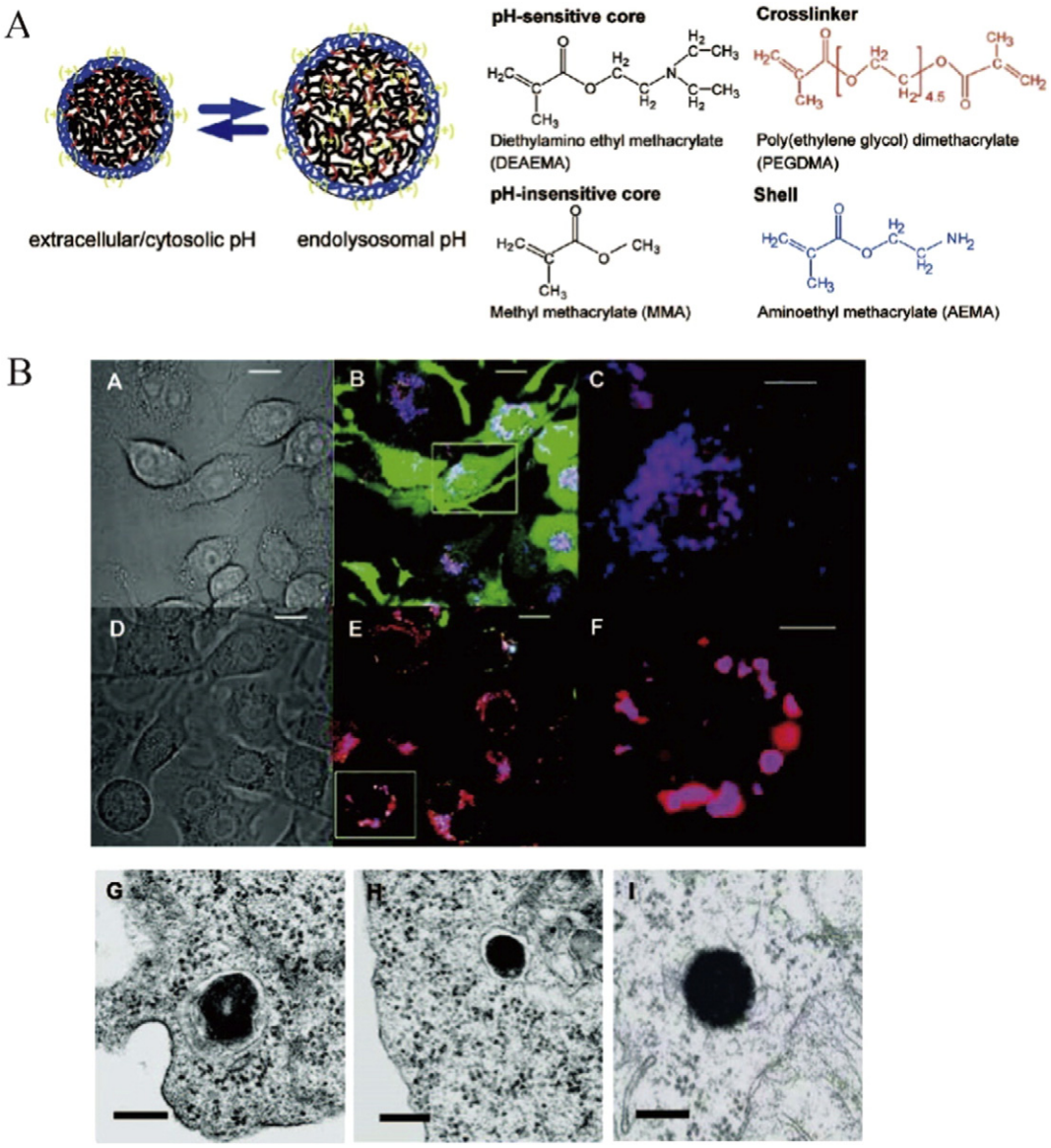

Fig. 7. (A) Schematic structure and chemical composition of pH responsive and non-responsive nanogels based on two-stage polymerization reaction to yield particles with either a pH sensitive crosslinked core containing poly(2-dimethylamino)ethyl methacrylate) or a pH insensitive core containing poly(methyl methacrylate), with a pH insensitive shell layer of poly(2-aminoethyl methacrylate). (B) Endosomal escape of pH responsive nanogels. DC2.4 cells were co-incubated with LysoTracker Red DND-99 (red), calcein (green), and either pH responsive (A–C) or non-responsive (D–F) nanogels (blue). TEM images of cell sections with non-responsive nanogel in membrane-bound compartments (G) and pH responsive nanogels either in membrane-bound compartments (H) or in the cell cytosol without a clear binding membrane structure (I). Reproduced with permission from ref [164].

considerable amount of protons leading to lysosomal escape; however, no evidence is presented. Antigen loaded pH sensitive GDR nanogels enhanced both MHC I and II antigen presentation in vitro and evoked stronger anticancer immune responses in vivo than the free antigen.

4.3. Enzyme responsive nanogels

An increased expression of a number of certain enzymes (e.g. proteolytic enzymes, hyaluronidase, lipase, matrix metalloproteinases and plasmin) is often observed under pathological conditions, such as cancer and sites of inflammation [166–170]. Enzyme responsive nanogels can take advantage of the altered expression of local enzymes to develop enzyme-triggerable drug delivery systems. Most reported enzyme-mediated nanogel delivery systems respond to enzymes in the extracellular environment, such as matrix metalloproteinases and plasmin [171–174]. Besides, it is also possible to deliver bioactive molecules into cells using enzymes present in intracellular compartments, such as lysosomal enzymes [175,176]. Tang et al. [177] described protein-containing nanogels that are degradable upon the digestion by furin, an endoprotease present in various intracellular locations. To prepare the protein-containing nanogels, monomers acrylamide and positively charged *N*-(3-aminopropyl) methacrylamide and a peptide crosslinker were first physically adsorbed onto the surface of the target anionic protein, which included enhanced green fluorescence protein, caspase-3, bovine serum albumin, or the transcription factor Klf4, in this study. This was followed by in situ free radical interfacial polymerization to form the polymeric shell and to assemble nanogels on the protein. The size increased from ~5 nm for empty nanogels to ~10 nm for the protein-loaded nanogels. The peptide crosslinker can be specifically recognized and cleaved by furin, which leads to degradation of the nanogels and subsequent release of the entrapped protein. These nanogels showed increased uptake and intracellular release as compared to the free proteins and non-degradable nanogels in different cell lines, including CHO, HeLa and MEF cells. Furthermore, cell death was observed in

HeLa cells incubated with furin-degradable caspase-3-containing nanogels, while cells exposed to free caspase-3 and with non-degradable nanogels exhibited minimal apoptotic death, confirming the increased uptake and intracellular release of the protein by furin-degradable nanogels.

5. Conclusions

Current knowledge provides us the insight that biotherapeutic molecules require not only delivery to the site of diseases but often also inside target cells, or even into specific subcellular compartments. There is no doubt that nanogels are suitable carriers for biomolecules that can protect their payloads from premature degradation and facilitate cellular internalization. Furthermore, it is clear from the papers summarized and discussed in this review that many biologically responsive nanogels significantly enhance the therapeutic effect of biomolecules by their delivery and release in relatively high doses intracellularly. For reduction sensitive nanogel systems, many studies have provided indirect evidence that disulfide bonds are cleaved and encapsulated therapeutic molecules are released intracellularly, as shown by significantly enhanced therapeutic efficacy, e.g. cytotoxicity, transfection efficiency, and antigen presentation, etc., in comparison to their non-reducible counterparts. Nevertheless, it should be noted that little is known about the exact intracellular fate of these reduction responsive nanogels, especially within endo/lysosomal compartments, due to the lack of direct evidence of breaking of disulfide bonds. The use of pH responsive nanogels allows endo/lysosomal release of biotherapeutics at low pH and facilitates endo/lysosomal escape for their cytosolic release. Although the effect and mechanism of endo/lysosomal escape is often explained by the proton sponge effect of the pH sensitive materials, detailed understanding is still lacking. Therefore, the further development of responsive delivery systems requires better comprehension of their intracellular trafficking and fate. Moreover, the need for targeting biotherapeutics to specific sites in vivo is expected to lead to new design requirements for nanogel delivery systems. With rational design, responsive nanogels are expected to advance biotherapeutics based therapies.

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