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Functional Nanogels for Biomedical Applications

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Abstract: This review addresses current and future perspectives of nanogel technology for nanomedicine. The synthetic methodologies and material properties of nanogels prepared by chemical meanings are discussed in detail, and examples that illustrate the different methodologies are presented. Applications in the fields of drug and gene delivery, smart imaging modalities, responsive materials, and multivalency as a therapeutic approach highlight the enormous potential of the functional nanogels as novel polymeric platforms for biomedicine.

Keywords: Biomedicine, drug delivery, functional polymer, imaging, multivalency, nanocarrier, nanogel, polymer therapeutics, responsive polymer.

1. INTRODUCTION

The continuous need for new polymeric entities for biomedical application calls for a careful investigation of some dimensional aspects and topological features of polymers. In this context, several methodologies have been reported for the synthesis of nanogels as novel hydrogels with sizes in the nanometer scale [1]. These nanosized cross-linked polymers have emerged as polymeric soft nanoparticles that fill the size gap between dendrimers/polymers, with diameters up to 10-20 nm, and macroscopic hydrogels [2]. Nanogels are certainly of interest for the biomedical research community because they have similar dimensions as biomacromolecules like proteins, viruses, receptor clustering in cells, etc. which widen the range of applications for these polymeric materials.

Following the megamer and microgel approaches by Tomalia [3] and Antonietti [4], respectively, nanogels have emerged as an interesting class of polymeric scaffolds due to their controllable size, shape, and functionality. The nanogel structure is based on a novel polymeric architecture that combines some functional advantages of dendritic systems with hydrogel ones like swellability, the opportunity to introduce multiple responsive modalities, and the presence of big voids for encapsulation. Therefore they have found their way into the biomedical field through applications as drug delivery systems and lately have also shown potential in fields like diagnostics, antivirals, and embolic therapies [5].

The nanogel internal structure emulates the 'tectodendrimer' concept described by Tomalia [3, 6], in which dendritic polymers are used to construct nanoarchitectures that possess a higher complexity and dimension. It is expected, therefore, that inherent properties of dendrimers like controlled shape, size, functionality, etc., will be imparted to the nanogel structure to enhance their potential in the biomedical field.

Several polymerization techniques have been described for the preparation of nanogels with relatively low molecular weight dispersities. In most of these cases, the reactions are performed in confined volume reaction vessels as nanodroplets in miniemulsions, nano-imprinting, etc. [2]. Recently, several review articles describing polymerization techniques and the use of nanogels in biomedical applications have extensively discussed the potential of such novel macromolecular architectures [5, 7-9]. Along with the use of nanogels as soft materials, several methodologies have combined them with metal nanoparticles for the preparation of remotely triggered nanodevices [10-13]. In this review the discussion is focused on techniques for nanogel synthesis and on its potential biomedical applications to highlight the latest research performances in this promising field.

2. SYNTHETIC METHODOLOGY

Nanogels can be described as aqueous dispersions of hydrogel particles in the nanometer range formed by physically or chemically cross-linked polymer network chains [5, 8]. For the successful synthesis of nanogel systems with low dispersities, three aspects are of crucial importance: (1) control over the size of the particles, (2) nature and chemical composition of the polymer network, and (3) the cross-linking strategy within the polymer chains. These parameters exert significant influence on the stability of the particles and determine their suitability for biomedical applications [14, 15]. While chemically cross-linked nanogels draw upon a broad spectrum of organic chemistry including cyclo-addition [16], conventional [17] and controlled radical [18] chemistry, the synthesis of physically cross-linked nanogels solely depends on non-covalent interactions between polymer network chains [8]. Due to their relatively weak field strength, physical interactions with hydrogen bonds, Van der Waals forces, and electrostatic and hydrophobic interactions critically affect the stability of gels and cause poor size control during synthesis [8]. To this end, chemically cross-linked nanogels are an attractive approach from the synthetic reproducibility and stability stand point and are therefore the main focus of this review.

Among the vast array of synthetic strategies available for the fabrication of chemically cross-linked nanogels, free radical cross-linking copolymerization of monovinylic or acrylic monomers with di- or multifunctional comonomers as cross-linkers remains the simplest and the most popular approach [19]. Such synthetic pathways require a minimum of specific equipment and only need conditions that avoid the formation of long range networks which could potentially lead to macroscopic gelation. A common method relies on the control of the distance between growing polymer chains that can be increased by working, for instance, in highly diluted solutions [20]. Thus, the risk of intermolecular cross-linking is reduced and the probability of intramolecular cross-linking is so increased that macroscopic gelation can be prevented. Stopping the polymerization at low conversion ratios before the gel point is reached or using low amounts of monomer or high amounts of cross-linker, however, also enables the formation of branched polymers [19-22].

An efficient way to control the size is to use heterogeneous polymerization processes like (inverse) emulsion, (inverse) miniemulsion, and (inverse) microemulsion polymerization, or precipitation and dispersion polymerization [23, 24]. As a result, the reactions

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can be templated by limiting the cross-linking process to occur within the particle which facilitates the formation of nano- or micro-particles with an internal structure comparable to macroscopic networks [4, 25]. (Table **1**) summarizes the advantages and limitations of chemical techniques commonly used for nanogel synthesis.

2.1. Emulsion Polymerizations

Emulsions are dispersions of two or more liquid phases that are immiscible. One liquid, the so-called dispersed phase, is dispersed in the other liquid, referred as the continuous phase. For the synthesis of nanogels the choice of the phase system in emulsion processes strongly depends on the nature of the monomers. The preparation of nanogels which are composed of a high amount of hydrophilic monomers is generally performed in inverse emulsions. In such cases, the hydrophilic monomers dissolved in water droplets are dispersed in a continuous oil phase (W/O emulsion). In contrast, hydrophobic monomers can be polymerized in oil/water emulsion (O/W emulsion). Here the polymerization directly affects the structure and the size of the gel particles. Contrary to homogenous polymerization, the monomers in emulsion processes are not soluble but dispersed with the aid of a surfactant in the continuous phase [17].

The most popular methodologies used for the preparation of nanogels are the mini- and microemulsion polymerizations. Miniemulsion generally indicates a method that allows the preparation of small droplets in a continuous phase by applying high shear stress such as ultrasonication or high-pressure homogenization [26]. Under high shear, macrodroplets are broken and closely distributed into nanodroplets with defined sizes between 50 and 500 nm as shown in Fig. (**1**) [27]. These nanodroplets may degrade by Ostwald ripening (aggregation and precipitation of the droplets) or coalescence (phase separation). Therefore the use of a surfactant and co-stabilizer is necessary which stabilizes the droplets whereby the amount used in miniemulsion mainly influences the size of the droplets [28]. While surfactants are soluble in the continuous phase and form micelles which surround the dispersed droplets to prevent coalescence, co-stabilizers are highly soluble in the dispersed phase but insoluble in the continuous phase. The co-stabilizer creates an osmotic pressure inside the droplets that counteracts the Laplace pressure responsible for diffusional degradation (Ostwald ripening). In O/W miniemulsion, for instance, long-chain alkanes and alcohols act as an ultra-hydrophobic co-stabilizer. Ionic compounds such as salts or sugars, so-called lipophobes, on the other hand, act as co-stabilizers in inverse miniemulsions (W/O systems). Typically used surfactants are emulsifiers like sodium dodecyl sulfate (SDS) and quarternary ammonium salts. Previous studies by Landfester *et al.* [29] have also elucidated that the use of non-ionic amphiphilic block copolymers, like poly(ethylene-*co*-butylene)-*block*-poly(ethylene oxide), as surfactants seemed to be very efficient for inverse miniemulsion. Due to their sterically demanding nature, they provided maximal steric stabilization, which is the predominant mechanism in inverse emulsions [27].

The main difference between mini- and microemulsion processes is the initial size of the dispersed phase. Microemulsions allow the formation of initial monomer droplets of diameters between 10 and 150 nm [30] without the need for high shear conditions generally used in the formation of miniemulsions. High shear stress generates miniemulsions which are kinetically stable but thermodynamically unstable in a time-dependent pattern. Over time, they will begin to separate again into two phases. In microemulsions, a large amount of surfactants is used which causes an ultra-low free energy

Table 1. Summary of Common Chemical Nanogel Synthesis Methodologies

Synthetic Strategy	Key Step	Pro	Contra	References
Miniemulsion Polymerization	Nanodroplet formation due to high shear stress	Broad range of cross-linking chemistry. Narrow size distributions for diameters in the range 50 - 500 nm. Allows *in situ* encapsulation.	Non-commercial surfactant often required. Special equipment necessary (ultra-sonic device). Difficulties with scaling up of synthesis.	[27, 28]
Microemulsion Polymerization	Nanodroplet formation due to surfactant and phases composition	Very small systems tuneable: 10 - 150 nm. No shear stress necessary.	High surfactant concentration needed. Co-surfactant necessary.	[30]
Precipitation Polymerization	Nanogel properties determined by the critical chain length of propagating polymer	Simplicity: single batch process, no surfactant required. Tuneable sizes as a function of monomer concentration in the range 100 – 600 nm.	Only suitable for chemical compositions which are able to undergo phase separation.	[62]
Dispersion Polymerization	Choice of dispersant determines reaction process	Simplicity: single batch process. Broad size range tuneable as a function of monomer and dispersant concentration: 0.1- 15 μm. Suitable for core/shell systems synthesis.	Preferred for vinylic and acrylic functionalized monomers.	[40, 42]
Micelles cross-linking	Micelle formation	Simplicity of self-assembly micelle formation.	Only amphiphilic building blocks can be used. Difficult to tune materials properties (size, elasticity, shape).	[20, 52]
Micromolding Technique	Non-wetting stamps	Various shapes, sizes, and monomer compositions possible. Easy scaling up. Allows in situ encapsulation.	Requires clean room facilities and lithographic devices. Surface modification of molds necessary to improve harvesting.	[55, 60, 61]

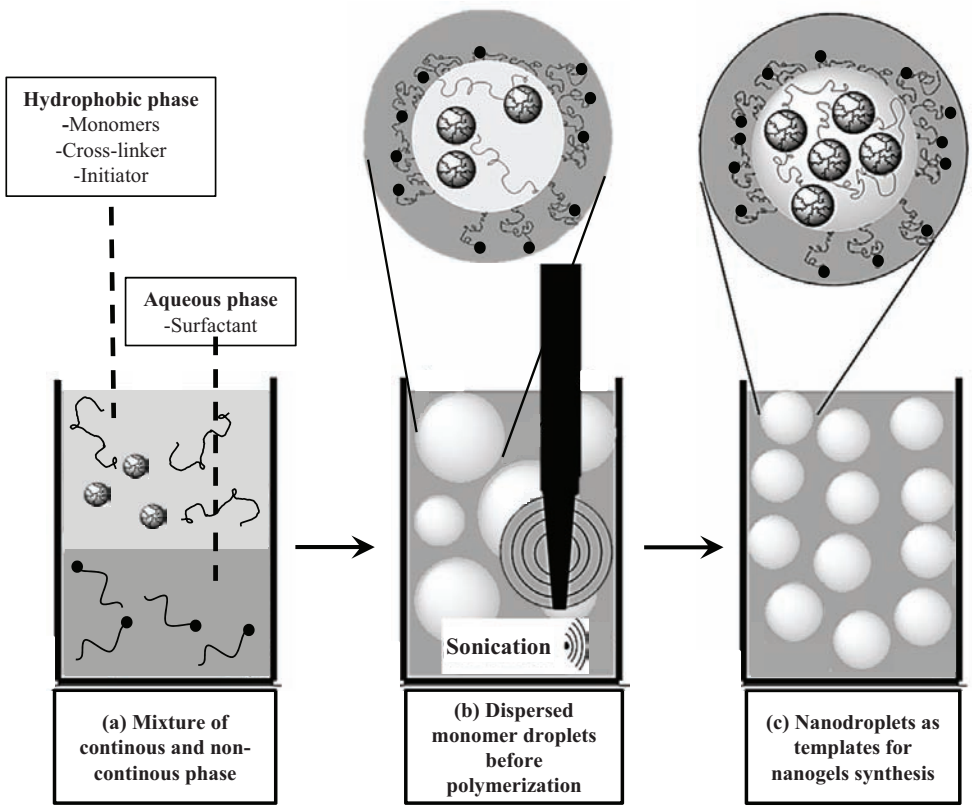

Fig. (1). Synthesis of nanogels via miniemulsion technique. (**a**) Initial two phases mixture. (**b**) Formation of small droplets in a continuous phase by applying high shear stress, such as ultrasonication. (**c**) Monodisperse nanosized 50-500 nm template for nanogel synthesis.

per unit of interfacial area between the two phases and therefore generates thermodynamically stable emulsion. This stable emulsion forms upon simple mixing of the components. Moreover, the ability to form microemulsions mainly depends on the composition of the continuous and dispersed phases, as well as on the amount of surfactant [31]. Typical non-ionic surfactants used in microemulsion polymerization range in their hydrophilic–lipophilic balance (HLB) scale between 3 and 18 [32-34]. In addition, co-surfactants, which lower the interfacial tension of the interface, are often essential [35, 36].

The mechanisms for both micro- and miniemulsion polymerizations are similar. In contrast to standard emulsion polymerization, where the initiation starts in the continuous phase and finally migrates into the micelles due to diffusion processes, the polymerization of miniemulsion proceeds inside the droplet. Here, the initiator, as well as the monomer, are only soluble in the dispersed phase. Each dispersed droplet (also denoted as 'nanoreactor') is ideally converted into a polymer particle with a similar size.

As an example, Haag and coworkers prepared nanogels based on dendritic polyglycerol (dPG) using the nanoreactor template, whereas cross-linking was achieved by a 'click'-type Huisgen alkyne/azide cycloaddition reaction [16]. It is noteworthy that due to the confinement of space, no copper was needed for this thermal [3+2] cycloaddition at only 80 °C. Both hydrophilic and hydrophobic dendritic-based nanoparticles could therefore be prepared by direct and inverse miniemulsion process, yielding nanogels with particles sizes between 25 and 90 nm. This represents the first example described in literature in which the concept of megamer and tectodendrimer were followed to construct dendritic nanogels. More recently, a new concept was developed in which functional dPG nanogels were synthesized by an acid catalyzed polyaddition of glycerol to trisglycidyl glycerol ether utilizing the inverse miniemulsion technique where the polar reactants were dispersed in non-polar cyclohexane [37]. A poly(ethylene-*co*-butylene)-*block*-poly(ethylene oxide) surfactant was used as a stabilizer and a small amount of DMSO was employed to prevent Ostwald ripening. Multifunctional alcohols were used as monomers and di- and triepoxides as cross-linking agents [38]. The properties of these nanogels, i.e., size, degree of branching, viscosity, and swelling behavior, could be controlled by varying degree of cross-linking.

In an attempt to extend the length-scale of the dPG gels and their potential applications, Seiffert, Haag, and coworkers have developed a technique to prepare polyglycerol-based particles on different length scales by extending the size of dendritic polyglycerols (3 nm) to nanogels (32 nm) and microgels (140 and 220 mm) [39]. They have used a miniemulsion templating system for the preparation of nanogels and a microfluidic templating system for developing microgels, which was accomplished by a free-radical polymerization of dendritic polyglyceroldecaacrylate and polyethylene glycol-diacrylate, as illustrated in Fig. (**2**). The employed reaction conditions allowed the encapsulation of living yeast cells within the microgels.

2.2. Dispersion/Precipitation Polymerization

The advantage of dispersion and precipitation polymerization lies in the simplicity of the reaction which allows synthesis of nano- to micro-sized low-dispersed particles in only a single batch process. Contrary to emulsion polymerization, dispersion or precipitation polymerization is initially homogenous with all the components being soluble in the solvent. In the progress of polymerization process the growing chains are no longer soluble after reaching a critical length so that precipitation occurs. The main difference between precipitation and dispersion polymerization is the presence of a colloidal stabilizer in the latter case that generally leads to bigger particles [40, 41]. Depending on the reaction conditions, the

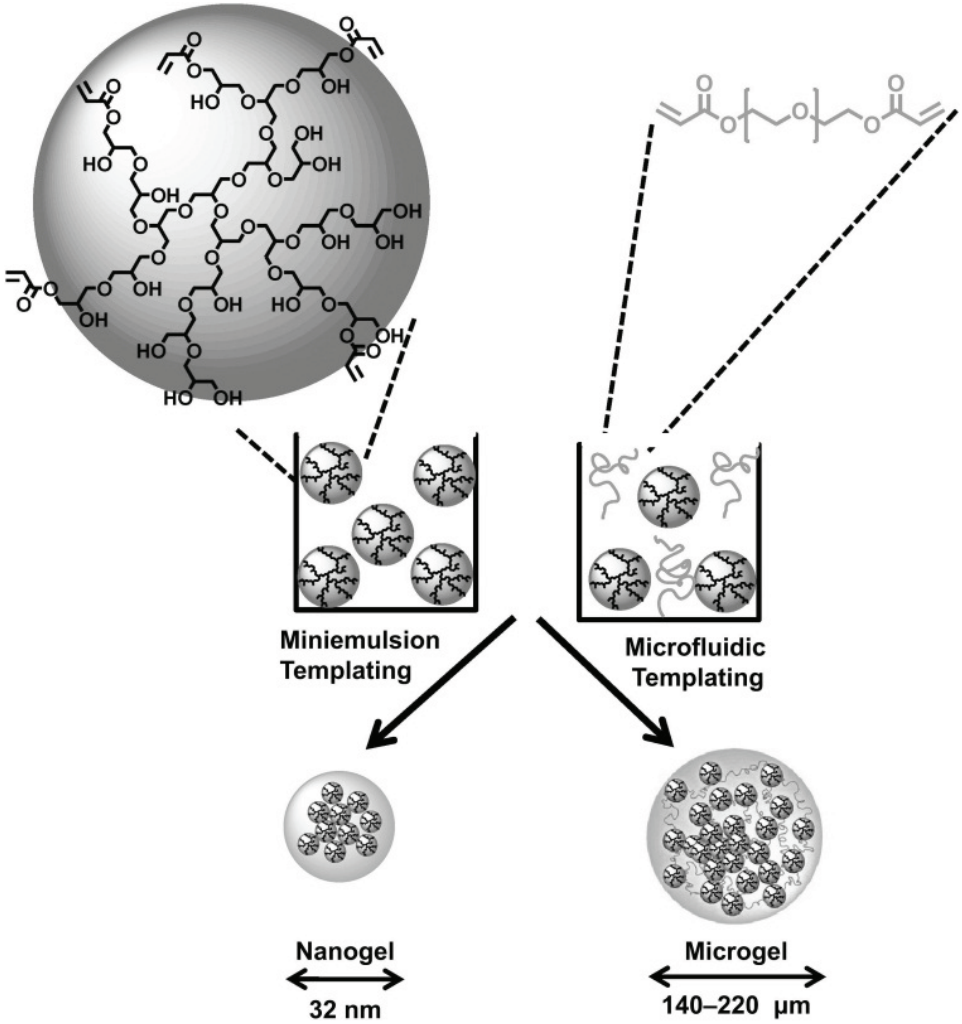

Fig. (2). Schematic representation of dPG–PEG gels obtained by free-radical polymerization of dendritic polyglyceroldecaacrylate and polyethylene glycoldiacrylate in nano- or micrometer-sized droplets. Reprinted with permission from ref. [39]. Copyrights (2011), Elsevier.

size of the resulting particles in precipitation polymerization varies between 100 and 600 nm [40]. Dispersion polymerizations often yield narrower particle size distributions than precipitation polymerizations and have particle sizes within the range of 0.1 - 15 μm [42]. Typical colloidal stabilizers are surfactants, which sterically inhibit the aggregation of nuclei during the polymerization and therefore stabilize the colloidal dispersion of the growing particles. Thermoresponsive nanogels, however, are often synthesized by dispersion polymerization at temperatures above the Lower Critical Solution Temperature (LCST) so that the forming gel particles undergo phase transition during the synthesis, yielding nanogels with narrower size distributions.

As an example, the fabrication of thermoresponsive dPG-based nanogels has recently been developed by Calderón and coworkers, in an attempt to develop stimuli-responsive materials based on dendritic polyglycerols [43]. In their work, the precipitation polymerization method was used to cross-link *N*-isopropylacrylamide (NIPAm) and dendritic PG to yield nanogels with sizes between 50 and 200 nm. The incorporation of dPG as cross-linking agent enhanced the water solubility of the nanogels, improved their biocompatibility profile, and allowed a fine tuning of the thermoresponsive nature regarding the size of the nanogels in solutions Fig. (**3**). The concept represents a variant of the 'tectodendrimer' idea from Tomalia, following a multimerization of the dendritic units connected by thermoresponsive polymers.

2.3. Cross-Linked Micelles

Cross-linked micelles are prepared by utilizing the controlled self-assembly behavior of amphiphilic block copolymers whereby they tend to aggregate on the nanometer scale Fig. (**4**). To improve the concentration-dependent stability of these nanoaggregates, chemical cross-linking is generally carried out within the polymer chains to generate stable nanogels. Since the aggregation is performed in water, this nanogel preparation proceeds under mild conditions which enable the entrapment of labile, therapeutically relevant agents into the gel network. The aggregation behavior, however, of amphiphilic block copolymers is difficult to manipulate and makes the control of nanogel material properties, like variation of size, shape, and elasticity, difficult. Structurally, these aggregates can be broadly classified as either core cross-linked [44-50] or shell cross-linked micelles [51-53]. Thayumanavan and coworkers prepared random copolymers that contained oligo(ethylene glycol) (OEG) and pyridyldisulfide (PDS) units as side-chain functionalities [48]. These copolymers self-assembled in aqueous solution and the PDS cores were cross-linked by the addition of a catalytic amount of dithiotreitol. Interestingly, the authors showed that encapsulated guests increased their retention time within the micelle structure with increasing cross-linking density.

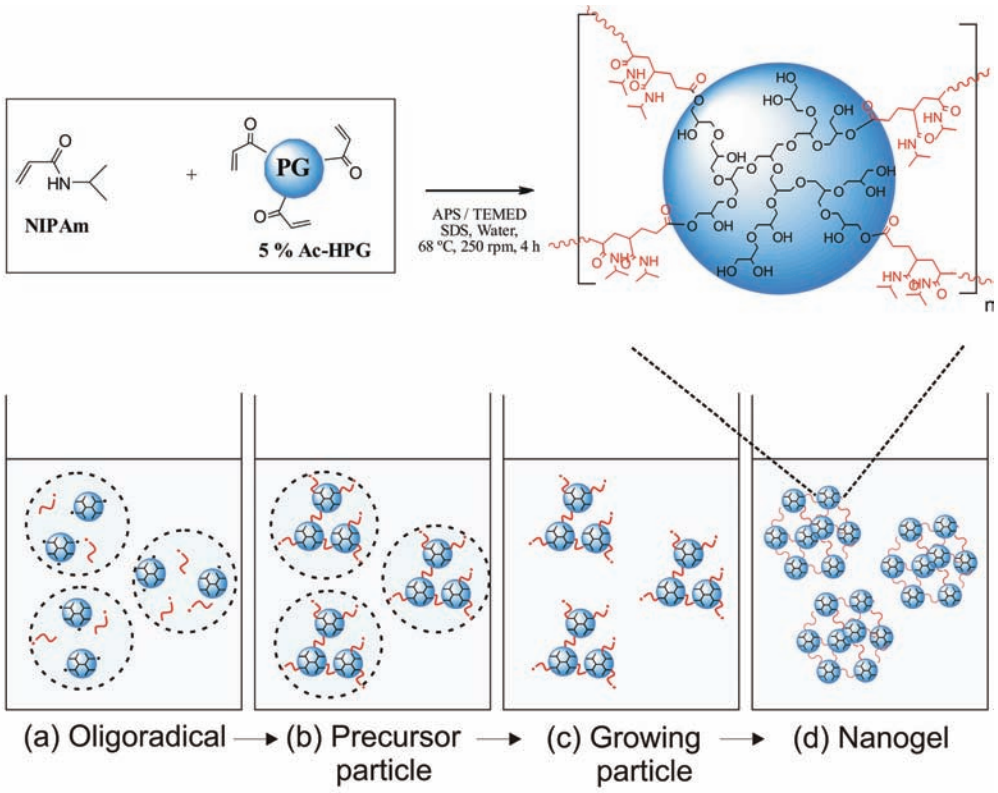

Fig. (3). Synthesis of thermoresponsive polyglycerol-based nanogels. The nanogels are formed by nucleation events that have been triggered by a radical initiation with partially acrylated dPG as cross-linking points and PNIPAm as growing chains. The precursor particles were stabilized by SDS below the critical micelle concentration at 68 °C [above the LCST of PNIPAm]. Dashes mark coagulation of oligoradicals and precursor particles (a, b) until sterically-stabilized particles form (c). After this point, new nuclei are not any longer formed and nanogel formation is obtained due to coagulation and diffuse capture of oligoradicals and precursor particles until all of the monomer is consumed (d). Reprinted with permission from ref. [43]. Copyrights (2011), RSC Publishing.

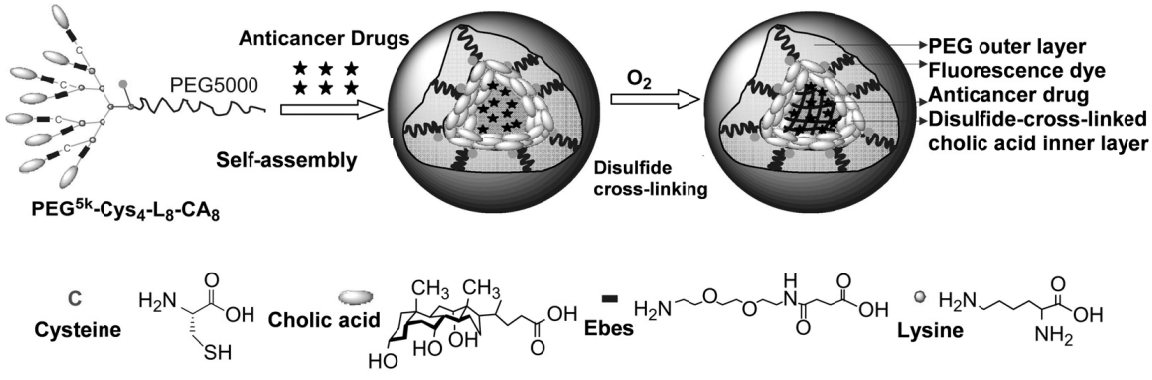

Fig. (4). Schematic representation of the disulfide cross-linked micelles formed by oxidization of thiolated telodendrimer PEG_{5k}-Cys_4-L_8-CA_8 after self-assembly. Reprinted with permission from ref. [52]. Copyrights (2012), Elsevier.

Lam and coworkers prepared amphiphilic, linear-dendritic polymers (telodendrimers), which were functionalized by a cystein moiety. After the telodendrimer micelles self-assembled in aqueous solution, they were cross-linked by oxidative disulfide formation Fig. (**4**). The authors could show that the cross-linked micelles increased micellar stability, decreased drug leaching and had a prolonged *in vivo* circulation time compared to their non-cross-linked counterparts [52].

2.4. Micromolding Techniques

Unique synthetic pathways to generate nanogels with control over manifold shapes Fig. (**5**), sizes, and different compositions can be obtained through so-called 'micromolding' methods. This type of methodology does not require harsh reaction conditions like high temperature, high shear, organic solvents, and light exposure. A popular example of micromolding methodology is the PRINT (Particle Replication In Non-wetting Templates) technique [54-56].

In this example, the initial precursor monomer mixture is applied on a non-wetting substrate. The mold with the specific pattern of interest mechanically stamps the initial mixture into the form. Due to external triggers such as light or temperature the chemical cross-linking proceeds inside the form. Finally, the fluorinated [58, 59] nature of the substrate and the mold facilitate the harvesting of the generated gels Fig. (**6**), as a consequence of the non-adhesive property of fluorinated surfaces similar to Teflon.

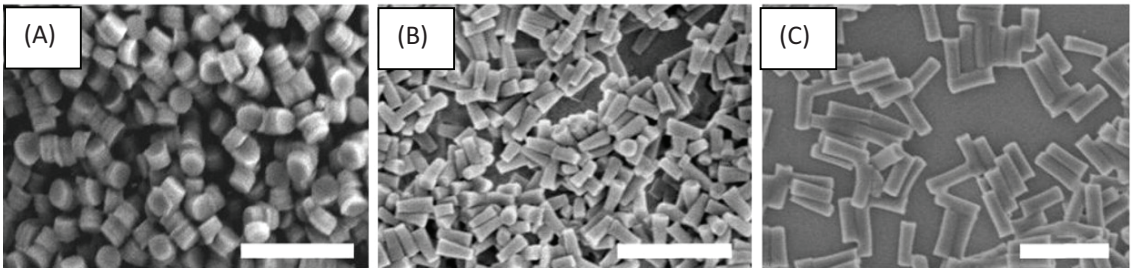

Fig. (5). Scanning electron micrographs of the series of cylindrical nanoparticles [diameter = 200 nm, height = 200 nm (**A**); diameter = 100 nm, height = 300 nm (**B**); diameter = 150 nm, height = 450 nm (**C**)]. Scale bars: 20 μm. Adapted from ref. [57]. Copyright (2008), The National Academy of Sciences of the USA.

Several therapeutics, diagnostics, proteins, and even living cells have been embedded inside the gel using the PRINT technique [55, 60, 61]. The great advantage of this method is a large-scale fabrication capacity and low material limitations. Sizes between 10 nm and 200 μm [56] have been generated with excellent low dispersities and satisfactory yields. This technique, however, is limited by the harvesting process and the special equipment which is necessary to install such devices.

2.5. Gelation in Liposomal Templates

Liposomes are typically composed of phospholipids, cholesterol, and other surfactants that entrap a polar solvent core, which is separated from the surrounding polar solvent by a lipid bilayer. Such solvent cores can serve as a nanoreactor in which monomers are cross-linked to build up nanogels. Liposomes are formed by the hydration of thin lipid films with an aqueous solution of monomers and initiator. Under strong stirring these films self-close and form monomer loaded liposomes. Combined with membrane extrusion, the liposomal diameter can be controlled with the pore size of the membrane [63]. In comparison to emulsion polymerization, the liposomal approach can be performed without the need of organic solvents. Nanogels based on poly(acrylamide), PNIPAm, and poly(NIPAm-*co*-1-vinylimidazole) were synthesized by radical polymerization in liposomes [64, 65]. This concept was extended by De Smedt and coworkers to dextran nanogels [63] and poly(ethylene glycol) (PEG) nanogels [66].

3. BIOMEDICAL APPLICATIONS OF FUNCTIONAL NANOGELS

Nanogel technology has already established itself as a robust platform for creation of functional materials with optimal size and multifunctionality for different fields of applications [1]. Nanogels have shown many interesting intrinsic properties, such as high water-content, soft nature, biocompatibility, nanometer size, and excellent water dispersibility. These properties, together with nanogels ability to incorporate active pharmaceutical ingredients, peptides, magnetic nanoparticles, and dyes, open many opportunities for their biomedical utilization. However, rapid clearance of circulating gel particles during systemic delivery is a critical issue for *in vivo* application of these systems. It has thus become critical to understand the factors affecting the biodistribution and blood circulation half-life of such delivery vehicles. The factors which can influence nanogels blood residence time and organ specific accumulation include interactions with biological barriers and tunable nanoparticle parameters, such as hydrophilicity, size, core properties, surface modifications (PEGylation and surface charge), targeting ligand functionalization, shape, and elasticity [67]. All these parameters have been shown to substantially affect the biodistribution and bioavailability parameter of circulating nanoparticles by reducing the level of nonspecific uptake, delaying opsonization, and increasing the extent of tissue specific accumulation. Fortunately, due to the variety of preparative methodologies [68] and the relatively facile modification of the physico-chemical properties, most of these issues can be addressed. For example, nanoparticles surfaces can be easily modified with classical organic transformations in order to increase blood circulation [69] or the nanogels size can be varied to modify the cellular uptake [70]. Recently, research based on functional nanogel technology has gained pace for the development of new systems for biomedical applications ranging from drug delivery or cellular sensing to probes for diagnostics. In this section, we attempt to demonstrate different representative examples for major directions in applications of nanogels with a special focus on recent works related to drug delivery for cancer therapy, cell sensing, and diagnostics.

3.1. Responsive Nanogels

In the last decade, considerable efforts have been focused on the design of smart drug delivery systems which undergo physical or chemical modifications in response to external environmental stimuli [15, 71, 72]. These nanodevices are especially appealing for applications in drug and gene delivery, since they behave not only as mere nanocarriers but play an important role in the controlled delivery of their payloads. In particular, nanogels with the ability to respond to typical biological stimuli (such as small changes in temperature, pH, or the presence of certain biomolecules) or to external triggers (such as light irradiation or the presence of magnetic fields) have been described in the literature and are summarized in (Table **2**) [5, 7, 15, 71, 73, 74]. Among the most interesting different types of stimuli for the biomedical field are those that are intrinsically characteristic of a certain tissue or disease. In this context, nanogels that selectively accumulate in tumors and release their cargos at the slightly acidic pH of solid tumors (~ 6.0) and/or at the more acidic endosome/lysosome cellular compartments (pH ~ 5.0-5.5) are important for applications in intracellular drug delivery and cancer therapy [75, 76]. Nowadays, the main strategy towards pH-sensitive nanogels has been to incorporate polymers containing protonable groups (such as amines or carboxylic acids). This way, a change in the ionization state by varying the pH can alter the size of the polymers, to render nanogels with a pH-responsive swelling and collapsing behavior that has been exploited for the controlled release of genes, proteins, and cytotoxic drugs. As an illustration, Nagasaki and coworkers designed core-shell type PEGylated nanogels constructed from a cross-linked core of poly[2-(N,N-diethylamino)ethyl methacrylate] (PDEAMA) and tethered PEG chains. These nanogels were equipped with a reversible swelling mechanism in response to pH and could be employed for the controlled release of doxorubicin (DOX) in endosomal compartments Fig. (**7**) [77]. DOX-loaded nanogels were assessed in a drug resistant HuH-7 tumor cell line, which showed an enhanced antitumor activity compared to the free drug. Fluorescence microscopy revealed a cellular internalization through the endocytic pathway, leading to protonation of amino groups and swelling of the PDEAMA core at acidic pH of the endosome/lysosome, which finally resulted in the release of DOX.

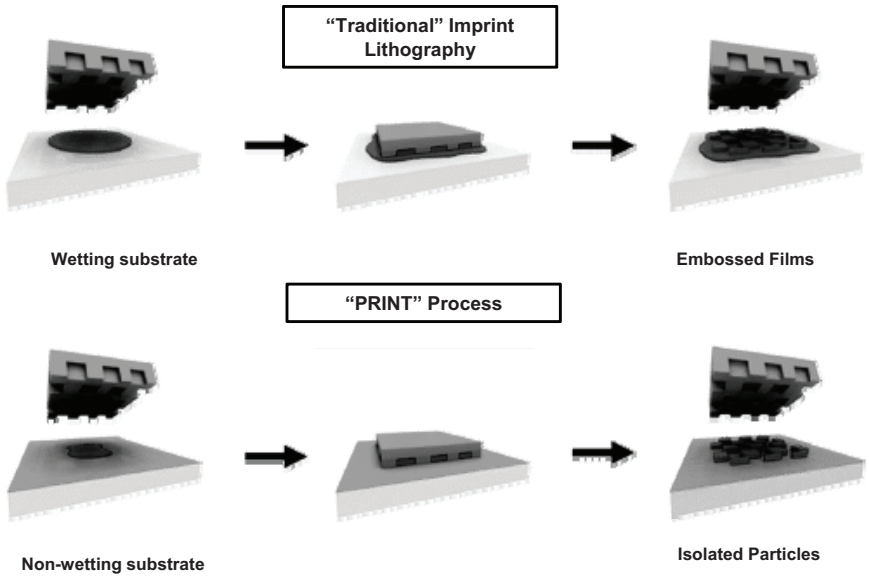

Fig. (6). Illustration of the PRINT process compared to traditional imprint lithography in which the affinity of the liquid precursor for the surface results in a scum layer. In PRINT, the non-wetting nature of fluorinated materials and surfaces (shown in dark gray) confines the liquid precursor inside the features of the mold, allowing for the generation of isolated particles. Reprinted (adapted) with permission from ref. [54]. Copyright (2012), American Chemical Society.

Table 2. Representative Responsive Nanogel Systems for Biomedical Applications

Responsive Modality	Chemical Functionality	Application	References
pH	Functionalization of polymer network chains with weak acidic or basic side groups	Delivery at site of action by drop in pH. Delivery due to external stimuli as electric field.	[75-78, 80-82, 114]
Temperature	N-alkyl substituted molecules (NIPAm) or OEG like monomers	Controlled release by external trigger. Permanent and temporary peripheral embolization. Intracellular temperature sensor.	[84, 85, 88, 115, 116]
Magnetic Field	Coating magnetic responsive particles like iron oxide	Magnetic intracellular hyperthermia. Contrast enhancement agents in magnetic resonance imaging.	[97-100]
Light	Photo-responsive groups such as imine or diazo functionalities within the polymer network. Coating of light responsive particles like gold nanorods	Controlled release by near infrared light. Contrast agent in alternative tomography method. (MSOT – Multispectral optoacoustic tomography).	[11, 93-96]
Redox / Bio-molecules (Peptides, Enzymes)	Sensitive cross-linkers like disulfides (glutathion), acetals (pH), esters (enzymes), etc.	Controlled release by degradation of environment responsive nanogel.	[75, 91, 92, 117-119]
Multi-Responsive	Combination of more than one responsive functionality	Theranostic approaches with degradable character.	[7, 103]

Kumacheva and coworkers prepared pH-responsive nanogels from the chitosan derivative N-[(2-hydroxy-3-trimethylammonium) propyl]chitosan chloride and sodium tripolyphosphate [78]. The negatively charged cytotoxic drug methotrexate (MTX) was incorporated within the nanogels through electrostatic interactions at pH 7.4. *In vitro* MTX release studies showed significant enhanced drug release when lowering the pH from 7.4 to 5. This fact has been explained as a result of the protonation of the carboxylic acid groups of MTX and the enhanced repulsion between the protonated amino groups of polymer molecules, which led to an increased pore size in the swollen nanogel network favoring the diffusion of MTX. Nanogels were further functionalized with apo-transferrin to ensure their entrance into cells through receptor-mediated endocytosis and, when incubated with HeLa cells, showed a significant increase in cell mortality compared with the free drug and non-bioconjugated MTX loaded nanogels. This enhanced cytotoxic activity suggested an effective cellular uptake and a pH-dependent drug release in the acidic receptor-mediated endocytic vesicles.

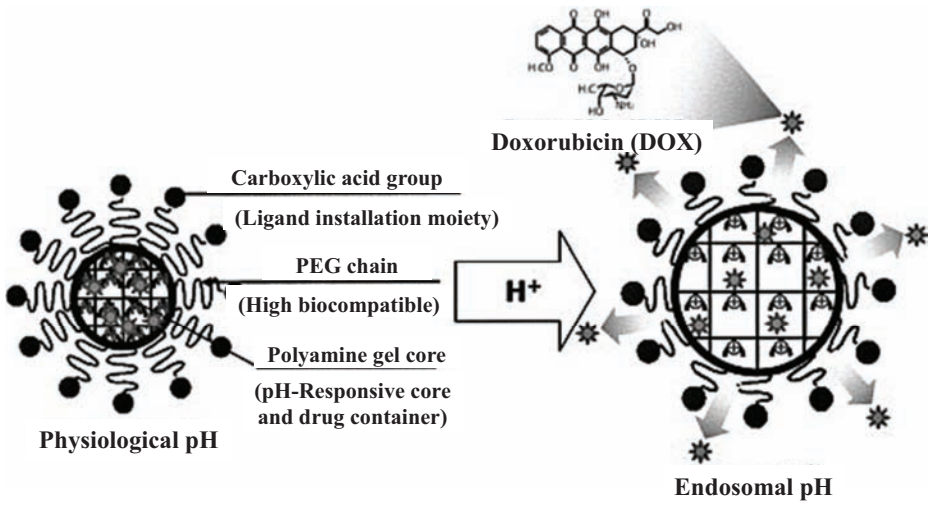

Fig. (7). Schematic representation of pH-induced DOX release from PEGylated PDEAMA nanogels. Reproduced with permission from ref. [77]. Copyright (2007), Elsevier.

More recently, Guo and coworkers employed chitosan (CS) and ethylenediaminetetraacetic acid (EDTA) for the preparation of biocompatible nanogels that presented reversible surface charge induced by external pH [79]. Such nanogels presented a positive z-potential below pH 4.8, due to protonation of the residual free amino groups in the CS chains, and negative z-potential above pH 5.2, that was attributed to deprotonation of carboxyl groups of the pendent EDTA moieties in the CS chains. Significantly, the integrity of the nanogels was maintained in the entire pH range. In this case, the ability to reversibly switch surface upon pH change was exploited both for the encapsulation and release of the anticancer drug camptothecin (CPT). By taking advantage of the switch properties exhibited by the nanogel, water-soluble lactone-ring-opened CPT could be encapsulated at basic pH, and then converted into the active form of the drug by acidifying the pH of the medium. In addition, the CPT-loaded nanogel showed a continuous release profile *in vitro* over 24 h.

Following a similar approach, Wang and coworkers developed pH-responsive charge-conversional nanogels to stimulate tumor-cell uptake and DOX delivery [80]. Thus, an amino-functionalized nanogel based on poly(2-aminoethyl methacrylate hydrochloride) (PAMA) was modified with 2,3-dimethylmaleic anhydride (DMMA), yielding a negatively charged PAMA–DMMA nanogel. The resultant amide bond was relatively stable at neutral and basic pH values, but degraded promptly under slightly acidic conditions. These nanogels were designed to transform their net charge from negative into positive at the slightly acidic tumor extracellular environment. At physiological pH, positively charged DOX could be loaded into the negatively charged nanogel with an extremely high drug-loading efficiency, however, at acidic pH, the degradation of DMMA on the nanogel facilitated an accelerated rate of DOX release. In addition, the charge-conversional character of the PAMA–DMMA nanogel at pH 6.8 enhanced the repulsive force between positively charged DOX and the resultant positively charged nanogel, contributing to the increase in total release. This charge conversion approach resulted in nanogels with enhanced cellular uptake, both *in vitro* and *in vivo*, and promoted cargo release, leading to a remarkably improved cytotoxic efficiency.

In an elegant contribution, Bae and coworkers exploited the strategy of pH-sensitiveness to prepare virus-mimetic nanogels (VM-nanogel) with the capacity to "infect" cells in a receptor-dependent manner, effectively kill tumor cells, and migrate to neighboring cells mimicking viral properties [81]. The VM-nanogel consisted of a hydrophobic polymer core made of poly(*L*-histidine-*co*-phenylalanine) (poly(His-*co*-Phe)) loaded with DOX, and a double hydrophilic shell. PEG formed the inner shell, and one PEG end was linked to bovine serum albumin (BSA), which formed a capsid-like outer shell. The BSA shell was conjugated with folate ligands for specific recognition of the folate receptor (FR), which is overexpressed in many tumors. At acidic pH values (6.8-6.4) the nanogel core swelled due to the protonation of His residues. However, at pH 7.4, the nanogels shrank showing a reversible swell-shrinking (or swelling-deswelling) behavior Fig. (**8**). Further studies in live human ovarian carcinoma A2780 cells demonstrated that VM-nanogels were able to disrupt the endosomal membranes due to the proton buffering effect of polyHis and the swelling of the nanogels at endosomal pH. Thus, VM-nanogels were transferred to the cytosol where they recovered their original size. In addition, it was found that the pH-induced reversible swelling/deswelling behavior of the core was closely related to the release of DOX, which was significant at the acidic endosomal pH but much lower at the pH of the cytosol. Finally, it was observed that the presence of DOX in the cells induced apoptosis, allowing the nanogels to be internalized by neighboring cells.

Complementary to the above-mentioned systems, pH-sensitive nanogels that exploit acidic environments to unload their contents have also been prepared by utilizing pH-sensitive linkers that are introduced into nanogels during polymerization. In this context, in a series of relevant contributions, Fréchet and coworkers prepared degradable acrylamide-based nanogels using highly acid-sensitive acetal cross-linkers for protein, antigen, and DNA delivery [82]. Acetal groups are stable under physiological conditions but cleavable under acidic intracellular environments, which results in degradation of the nanogel and facilitate the release of the payload [83, 84].

Besides pH, thermoresponsive nanocarriers can be used for various biomedical applications including drug delivery and tissue engineering [83]. In this sense, various bioresponsive micro- and nanogels have been developed for drug delivery based on thermoresponsive polymers. These nanogels swell and collapse significantly in an aqueous environment at temperatures below and above the LCST, respectively, which allows aqueous loading of drugs, and modulates drug release in response to temperature changes. PNIPAm is perhaps the best known thermoresponsive polymer. In particular, PNIPAm nanogels present a swelling/deswelling behavior in water upon reaching the LCST, which occurs around (independently of polymer chain length and concentration) 31 °C. Copolymerization of NIPAm with different types of monomers or post-polymerization modifications on PNIPAm nanogels results in nanogels with more versatile properties and may significantly shift

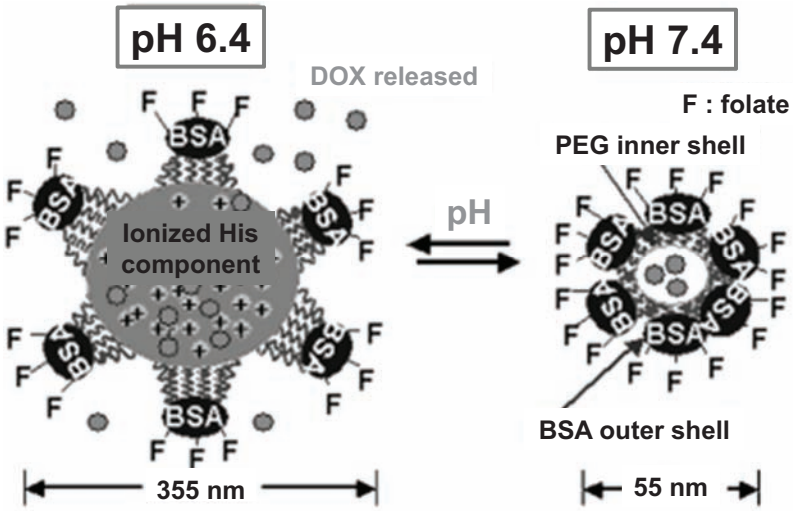

Fig. (8). Structure of the virus-mimetic-nanogel. Reprinted with permission from ref. [81]. Copyright (2008), John Wiley & Sons, Inc.

the LCST, allowing the employment of PNIPAm-based nanogels over a wide temperature range [84-87]. Recently, Yan and coworkers prepared *in situ* gellable thermoresponsive nanogels from poly(NIPAm-*co*-acrylamide) and studied their *in vivo* gelling behavior and the release of two model drugs [88]. In this case, the thermoresponsiveness of the nanogels allowed the absorption of the drugs into the swollen nanogels by diffusion at lower temperature, and their subsequent release from the shrunken nanogel at body temperature. As described in the above paragraphs, PNIPAm has also been employed by Calderón and coworkers for the preparation of thermosensitive nanogels based on biocompatible dendritic polyglycerol [43]. Besides PNIPAm, other polymers, such as the triblock copolymer Pluronic, have been incorporated in recent years within nanogels to provide them with temperature-responsive properties [89, 90].

Complementary to pH and temperature, which are the most common stimuli in biological systems, the use of redox labile disulfide bonds as biodegradable units is a promising way to achieve post-endocytic nanogel destabilization, due to a large intra/extracellular redox gradient in most cells. Haag and coworkers recently successfully prepared biodegradable polyglycerol-based nanogels with disulfides integrated into the structure that could be degraded into small oligomeric subunits in reducing environments [91]. Other biosignals such as the presence of certain enzymes or selected molecules can also be exploited to induce a controlled response in nanogels. Glucose has been employed, for instance, as a stimuli for glucose-responsive PNIPAm-based nanogels for triggered insulin release [92]. Finally, nanogels designed to respond to external triggers such as light [11, 93-96] or the presence of a magnetic field [97-100] have also been employed for targeted drug delivery and imaging.

More recently, multiresponsive nanogels have emerged as a new promising platform for biomedical applications [101, 102]. Li and coworkers prepared multiresponsive nanogels by miniemulsion copolymerization of monomethyl oligo(ethylene glycol) acrylate (OEGA) and an ortho ester-containing acrylic monomer, 2-(5,5-dimethyl-1,3-dioxan-2-yloxy) ethyl acrylate (DMDEA), with bis(2-acryloyloxyethyl) disulfide (BADS) as a crosslinker [103]. Such nanogels presented a triple pH/thermo/redox responsiveness, and their efficacy as a carrier of hydrophobic anti-cancer drugs was analyzed [103]. The nanogels showed good stability against incubation at pH 7.4, but displayed accelerated drug release behavior *in vitro* by a cooperative effect of both acid-triggered hydrolysis and dithiothreitol-induced degradation Fig. (**9**).

3.2. Nanogels for Active Targeting

Nanogels are potentially very beneficial for application in anticancer treatment procedures through targeting modalities, including the ability to load and transport bioactive molecules to the major sites of metastasis, such as lungs, liver and lymph nodes, as well as to target specific cell populations within these organs [73, 104]. As an example, in a strategy reported by Zhang and coworkers, galactose-decorated pH-responsive nanogels were prepared for hepatoma-targeted delivery of oridonin (ORI, a potential anticancer agent) [105]. The release behavior of ORI from these nanogels was pH-dependent and faster under mildly acidic conditions. In addition, the ORI-loaded nanogels exhibited a higher antitumor activity than drug-loaded nanogels without galactosylation.

In a recent report, Bronich and coworkers presented the preparation of folate-decorated nanogels for targeted therapy of ovarian cancer [45]. Folic acid (FA) is a prominent targeting moiety capable for specific interaction with cells expressing the FR [106]. Such receptors are overexpressed in human tumors compared to normal tissues [107]. In a previous work, Lyon's group used the folate mediated cancer cell targeting strategy to internalize nanogels, which showed that the cellular uptake of the nanogel conjugates bearing FA was 10 times higher than those without folate functionalization [108].

Likewise, hyaluronic acid (HA) is another highly efficient targeting molecule that can bind to the CD44 receptor, which is overexpressed in many types of cancer cells [109]. Kim and coworkers presented the preparation of nanogels by self-assembly of PEG and HA-ceramide for targeted delivery of DOX [110]. Greater uptake of DOX from these nanoparticles was observed in the CD44 receptor overexpressed SCC7 cell line compared to CD44-negative cell line, NIH3T3. Cytotoxicity tests in NIH3T3 and SCC7 cells suggested that the nanoparticles can be used as a drug delivery vehicle without serious side effects.

The application of RNA interference to treat diseases is an important, yet challenging concept in modern medicine. In particular, small interfering RNA (siRNA) has shown tremendous promise in the treatment of cancer. However, siRNA shows poor pharmacological properties, which presents a major hurdle for effective disease treatment especially through intravenous delivery routes [14]. The application of nanogels as carriers for siRNA drug delivery has been recently started to investigate their efficiency for delivery of therapeutic neucleotides. The incorporation of siRNA within the nanogels cavities appears to be a robust strategy to stabilize and deliver siRNA fragments inside cells. Fig. (**10**) shows the basic

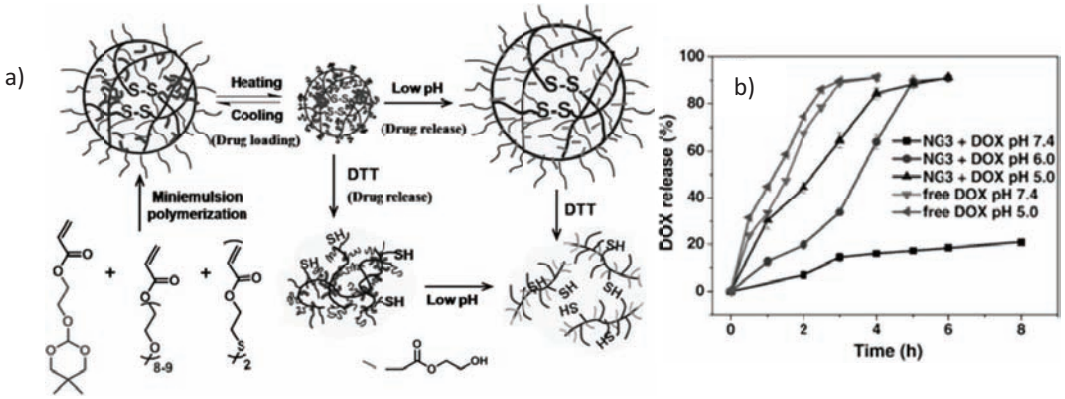

Fig. (9). (**a**) Synthesis and stimuli-responsive properties of the nanogels. (**b**) In vitro cumulative release of DOX from nanogels in aqueous solutions of different pHs. Temperature: 37 °C; buffer concentration: 50 mM. Adapted with permission of ref. [103]. Copyright (2011), Elsevier.

principle of this kind of nanosystem. Lyon and coworkers described the synthesis of core/shell nanogels with surface-localized peptides that specifically target ovarian carcinoma cell lines. A preliminary investigation of gene silencing illustrates that nanogel-mediated delivery of siRNA targeted to the epidermal growth factor (EGF) receptor results in a knockdown of that receptor [111].

De Smedt and coworkers analyzed the potential for siRNA delivery of biodegradable cationic dextran nanogels prepared by inverse emulsion photopolymerization [112]. The nanogel was able to entrap siRNA with a high loading capacity, based on electrostatic interactions. As the degradation kinetics of the nanogel could easily be tailored, these particles showed potential for intracellular controlled release of short interfering RNA. In a recent work, traceable multifunctional nanocarriers for cancer-targeted co-delivery of siRNA and DOX were constructed from degradable poly(ethylene oxide)-*block*-poly(ε-caprolactone) (PEO-*b*-PCL) block copolymers with functional groups on both blocks [113]. The nanocarriers integrate multiple functions in one system, including the capability to accommodate a combination of therapeutic entities with different physicochemical properties (i.e., siRNA and DOX), passive and active cancer targeting, cell membrane translocation, and pH-triggered drug release. This system was used to improve the efficacy of DOX in multidrug-resistant MDA-MB-435 human tumor models that overexpressed P-glycoprotein (P-gp), by simultaneous intracellular delivery of DOX and siRNA against P-gp expression. Such example highlights the unique potential of nanogels as nanotransporters of biomacromolecules with sizes higher than 5-10 nm.

3.3. Nanogels in Cellular Sensing and Diagnostic

Celullar events are marked by small changes in intra-cellular and intra-organelle temperature, pH, oxygen level, and free-radical status. Therefore, the ability to sense the minuscule change of these parameters within living cells in response for specific biochemical changes can be of substantial importance in understanding the phenomena that contribute to an early detection of diseases or monitoring of therapeutic efficiency of a given pharmacological entity [121]. In such context, the development of appropriate biocompatible temperature/pH sensing nanoprobes that have minimal interactions with the cellular constituents would allow one to measure accurate values of temperature or pH associated with different cellular changes [122]. If specifically designed, these sensing probes could allow the distinction between pathological and non-pathological cells. Stimuli-responsive nanogels are excellent candidates for the design of early detection and disease monitoring probes because of their dye-modifiability, biocompatibility, and cellular uptake profile. As a result, Uchiyama and coworkers developed a fluorescent nanogel thermometer for intracellular thermometry through which a temperature difference of less than 0.5 °C could be distinguished without any interference from precipitation or interaction with cellular components [123]. Nagasaki and coworkers presented the preparation of a smart nanoprobe based on fluorescence-quenching PEGylated nanogels containing gold nanoparticles for monitoring the response to cancer therapy [124]. The nanoprobe showed very little fluorescence in the absence of activated caspase-3 (normal cells) while pronounced fluorescence signals were observed in apoptotic cells. This behavior is mostly due to the cleavage of the peptide by activated caspase-3 present in the cancer cells, which results in the release of fluorescence molecules increasing fluorescence emission.

More recently, Wolfbeis and coworkers presented the preparation of a new nanogel for ratiometric fluorescent sensing of intracellular pH values [125]. The nanogel probe was prepared from a biocompatible polyurethane polymer that was made pH sensitive by loading it with the pH indicator bromothymol blue. Furthermore, it was rendered fluorescent by addition of two standard fluorophores, coumarin 6 (C6, green fluorescence) and nile red (NR, red fluorescence), that underwent efficient fluorescence resonance energy transfer (FRET) inside the nanogel as shown in Fig. (**11**). The principle behind this probe action depends on the measurement of total fluorescence emitted, which is modified by absorption spectrum of bromothymol blue at different pHs after photoexitation of C6 at 440 nm. Lim and coworkers showed the synthesis of a high performance magneto-fluorescent polyelectrolyte nanocomposite as magnetic resonance/near-infrared MR/NIR multimodal cellular imaging nanoprobe [126]. Poly(γ-glutamic acid) was used for the convenient phase transfer of $MnFe_2O_4$ nanoparticles dispersed in organic solvents into aqueous solutions and facilitated further ionic gelation with poly-*L*-lysine. The positively charged outer surfaces were assembled with other negatively charged NIR emitting fluorescent nanocrystals and enabled the highly efficient delivery of the magneto-fluorescent polyelectrolyte nanocomposites into cancer cells. The new material is expected to be used as a highly efficient MR/NIR dual-modality imaging nanoprobe in the detection of cancer cells and monitoring of therapeutic cells *in vivo*. In another recent work, Chen and coworkers presented the preparation of a new PNIPAm-based fluorescent nanothermometer with a ratiometric readout [127]. (Table **3**) summarized the current area of research with nanogel technology pertaining to polymer therapeutics and diagnostics.

3.4. Nanogels in Multivalent Applications

The important role of nanogel dimension in biological interactions was recently highlighted in a systematic analysis of multivalent glycoarchitectures based on dendritic polyglycerol nanogels in

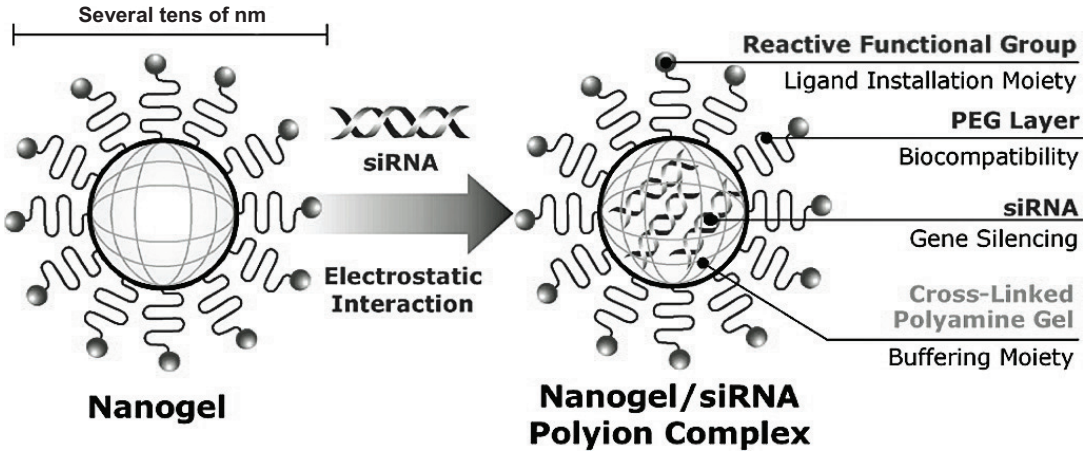

Fig. (10). Schematic illustration of the nanogel/siRNA polyion complex nanodevice. Reprinted with permission from ref [120]. Copyright (2009) American Chemical Society.

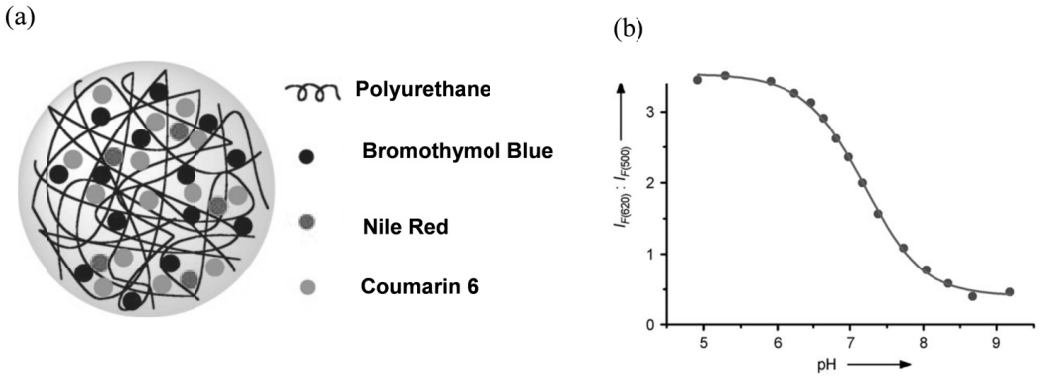

Fig. (11). (**a**) Model of the ratiometric pH sensing nanogel. (**b**) pH calibration plot of the ratiometric nanogels. The experimental data were calculated from the ratio of the fluorescence intensities at 620 nm and 500 nm. Adapted with permission from ref. [128]. Copyright (2010), John Wiley and Sons.

the inhibition of the influenza virus [128]. In this study, particle sizes were varied with the degree of functionalization to match the corresponding virus size and receptor multiplicity in order to achieve maximum binding efficiency. It was shown that the inhibitory activities of the polymeric glycoconjugates drastically increased with the nanoparticle size. Comparing the inhibition of binding and fusion to influenza virus, dPG nanogels with 50 nm of diameter was 7 x 10^3 fold more effective than dPG with a diameter of 3 nm at comparable sugar concentrations. Moreover, it was demonstrated that the nanogel reduced viral activity by up to 80%. This emphasizes the importance of matching sizes and multiplicity for biological surface interactions, which is achieved by the particles dimensionality of the dPG nanogels as shown in Fig. (**12**) [1].

3.5. Nanogels in Clinical Trials

In spite of the vast surge of publications describing innovative methodologies to develop novel nanogels with potential application in cancer therapies, only a few physically cross-linked nanogels have been translated into clinical trials [129]. Although these kind of nanogels do not fall within the scope of this review, it is worth mentioning some examples currently under clinical trials. One of these physically formed nanogels is the experimental product BIND-014, a prostate-specific membrane antigen (PSMA) targeted nanoparticle. This nanogel carries docetaxel within a matrix of polylactic acid enveloped within a coating of PEG embedded with PSMA targeting ligands [130]. BIND-014 allows the gradual release of docetaxel upon degradation of the polylactic acid, whereby the PEG coating and the targeting ligands restrict the cytotoxic effect to PSMA-expressing cells. Another interesting example that has been evaluated under clinical trials comprises nanogels prepared from cholesteryl pollulanes. These nanogels have shown efficient responses in patients vaccinated with proteins complexed within the nanogel structure [131, 132].

4. CONCLUSIONS

Although emulsion and template-based polymerization techniques have been well-documented, their application in nanogels synthesis has recently attracted much attention. The dimensionality, material properties, and tunable biocompatibility of nanogels make them particularly interesting for application in the fields of analytics and biomedicine. New robust synthetic methodologies have afforded a great variety of nanogels in which functionality and multiple responsive modalities can be included within their gel scaffolds. This is particularly important for the field of nanomedicine, where novel materials bearing smart properties are constantly in demand.

In this report, recent examples have been compiled and reviewed that highlight the enormous potential of nanogels for a whole spectrum of biomedical applications encompassing the fields of drug delivery, responsive nanomaterial, and diagnostic and imaging supplements. However, a substantial number of unmet issues regarding their pharmacodynamics, metabolism, and pharmacokinetics still need to be assessed before nanogels can fully make the transition from bench to bedside. Although nanogel technology is

Table 3. Examples of Nanogels Technologies Used for Biomedical Applications

Medical Area	Nanogel Composition	Description / Strategy	Application	Reference
Cancer Therapeutics	Poly(OEG-*co*-DMDEA)	Ester hydrolysis in acid medium / degradable cross-linker	DOX release in the weakly acidic or reductive environments	[103]
	CS-g-PNIPAm	Target moiety and pH structural changes	Target release of ORI under mildly acidic conditions	[105]
	PEO-b-PMA	Folate decorated nanogel	Tumor-specific delivery of cisplatin and DOX for receptor overexpression	[45]
	HA Ceramide-PEG	Hylauronic-ceramide conjugated with PEG	DOX delivered to the tumor site by active targeting via HA and CD44 receptor interaction	[110]
	PEO-*b*-PCL	Target moiety	DOX and siRNA release in tumor that overexpress P-glycoprotein	[113]
	PLA Matrix-*g*-PEG	Target moiety /degradation of PLA	Docetaxel release in prostate tumor (BIND-014 clinical)	[120]
	Cholesteryl-pululansx	Degradation / complex formation	Release of protein from Pullulans-protein complex	[131, 133]
Cell Sensing and Diagnostic	PNIPAm	Combination of thermoresponsive polymer with a fluorescent dye	Cell thermometer	[123]
	Polyurethane	Combination of the polymer with a pH sensitive dyes and two fluorescent dyes	Cell pHmeter	[125]
	Polyamine Containing Gold Nanoparticles	Nanogel with fluorescein isothiocyanate (FITC)-labeled DEVD peptides	Monitoring cancer therapies	[124]

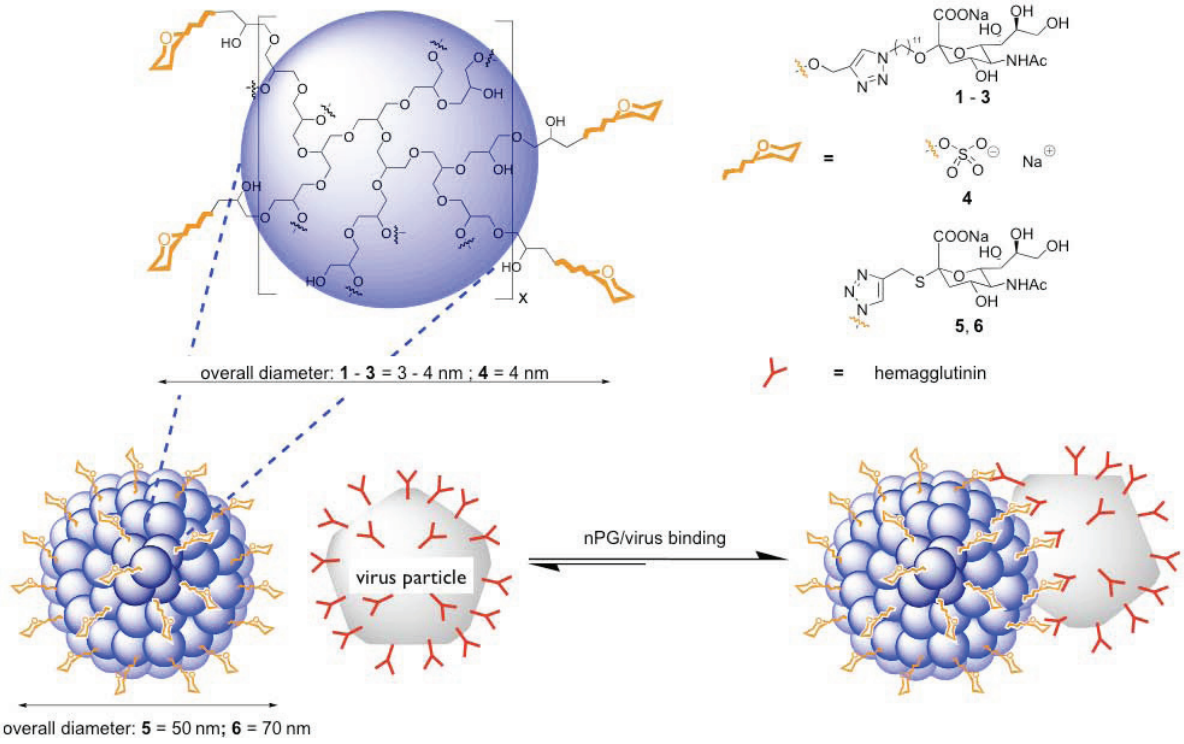

Fig. (12). Schematic representation of sialic acid-conjugated dPG-based nanoparticles that match influenza virus size and receptor multiplicity. Adapted with permission from ref. [128]. Copyright (2011), John Wiley & Sons, Inc.

still in its infancy in this regard, it has made considerable progress towards a regular and wide-spread application in the fields of polymer theranostics and biomedicine.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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