doi:10.1246/cl.2012.202
Published on the web February 25, 2012

Highlight Review

Self-assembled Nanogel Engineering for Advanced Biomedical Technology

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(Received January 25, 2012; CL-120064)

Abstract

Nanogels are polymer nanoparticles with three-dimensional networks. Recently, various nanogels have been designed, with a particular focus on biomedical applications. In this review, we describe recent progress in the synthesis of functional nanogels by self-assembly of associating polymers and nanogel engineering for advanced biomedical technology including regenerative medicine and drug delivery systems.

♦ Introduction

Living systems, for example biomembranes which are self-organized assemblies of proteins and lipids, reveal sophisticated biological functions such as signal transduction, energy production, and cellular communication. The concept of supramolecular assembly of biocomponents such as lipids, proteins, nucleic acids, and polysaccharides is central to bottom-up design of advanced biomaterials. For example, molecular organization based on supramolecular assembly enables fabrication of not only materials with well-controllable molecular orientation and arrangement but mechanically controlled nanomaterials and nanosystems[1] or self-assembled micro- and nanoshells for drug delivery applications.[2]

Recently, we have proposed nanogel engineering, which involves design of self-assembled nanogel and the construction of functional hierarchical gels or interfaces through their bottom-up assembly of nanogels as building units. The bottom-up nanogel engineering provides a new paradigm for development of hydrogel biomaterials with well-organized three-dimensional structures, multiple functions, sensitivity to a range of different stimuli, and programmed responses that can be controlled temporally and spatially.

Nanogels are nanometer-sized hydrogel nanoparticles (<100 nm) with three-dimensional networks of crosslinked polymer chains. They have attracted growing interest over the last several years due to their potential for biomedical applications, such as drug delivery system (DDS) and bioimaging.[3] Usual polymer nanoparticles, such as nanospheres, have a densely packed polymer inside core structure. In contrast, nanogels are able to stably trap bioactive compounds such as drugs, proteins, and DNA/RNA inside their nanospace with polymer networks. Moreover, nanogels show a rapid response to microenvironmental factors such as temperature and pH because of their nanoscale dimensions. These properties are useful for the controlled release of bioactive compounds.

Nanogels have been prepared using various methods, which can be classified into two categories according to their crosslinking structure: *chemically (covalent) crosslinked nanogels* which form crosslinking points by covalent bonds and *physically crosslinked nanogels* with noncovalent bonds (such as hydrogen bonds), electrostatic and hydrophobic interactions.[4] Typically, chemically crosslinked nanogels are synthesized under dilute conditions by a crosslinking reaction of polymers modified with reactive groups, such as vinyl and thiol groups. Nano- or microemulsion polymerization methods are often used to obtain nanogels with a well-controlled size.

A variety of new synthetic techniques have been reported recently. They include (1) block copolymer crosslinking method in which micelles composed of amphiphilic block copolymers[5] or polyion complexes[6] are crosslinked, (2) nanotemplate method, which exploit the internal water phase of liposomes[3b] or the surface of silica[7] or gold nanoparticles,[8] and (3) lithography-based top-down method.[9] Various chemical crosslinking reactions have now been developed, including carbodiimide-mediated amide bond crosslinking,[5a] quaternization of amino groups,[10] "click" chemistry,[11] and photocrosslinking.[12] Meanwhile, nanocarriers bearing disulfide bonds have been shown to be useful for efficient gene delivery.[6] Since many reports and excellent review articles have described the methods used to synthesize chemically crosslinked (nonassociating polymer-based) nanogels (Figure 1),[3a] this method will not be discussed in detail here.

Noncovalent interactions, such as hydrogen bonds, van der Waals forces, electrostatic and hydrophobic interactions are also available to prepare nanogels. However, it is difficult to obtain stable physically crosslinked nanogels with controlled sizes using noncovalent interactions because the bonds are relatively weak. In this review, we introduce the preparation and characteristics of mainly polysaccharide nanogel and some applications of nanogel in biomedicine. In addition, our recent studies to realize nanogel tectonic engineering including construction of functional hierarchical gels through their bottom-up assembly are described.

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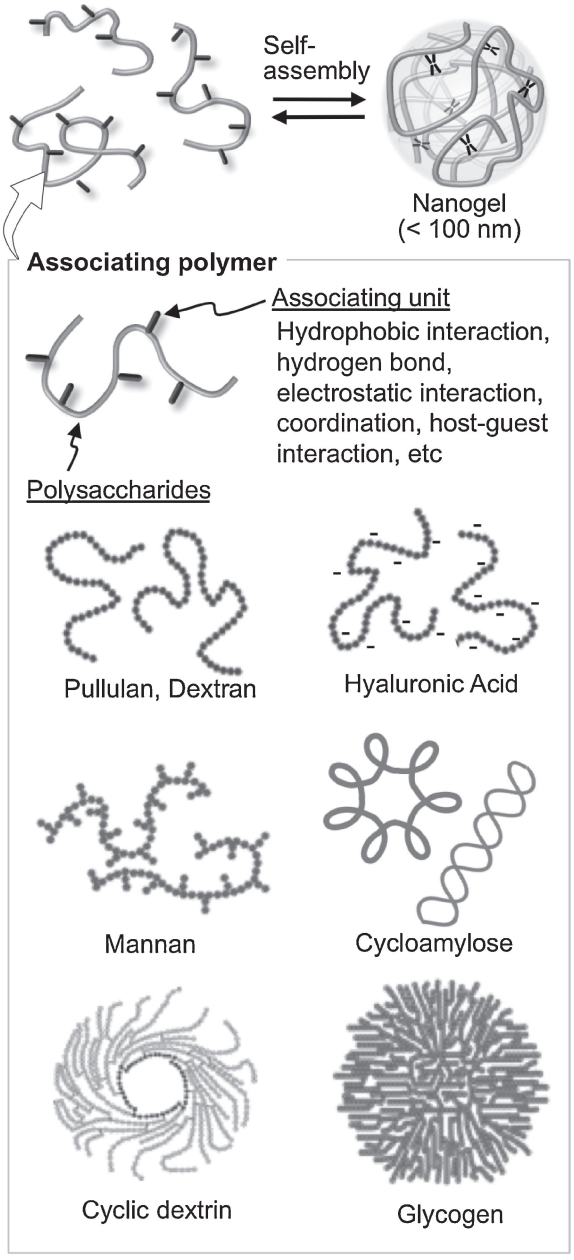

Figure 1. Physically crosslinked nanogels formed with associating polymers based on various polysaccharides.

♦ *Self-assembled Nanogels by Amphiphilic Associating Polymers*

Physically crosslinked nanogels via controlled association of hydrophobically modified polymers in a dilute aqueous solution were first reported by our groups.[13] Cholesterol-bearing pullulans (CHPs, Figure 2), which are composed of hydrophilic pullulan partially modified with cholesteryl groups, self-assemble in an aqueous solution and form stable nanogels with a diameter of 30 nm. The association of hydrophobic cholesteryl groups provides crosslinking points via hydrophobic interactions. The close association of polymers at the nanosize scale is governed by many factors that include the concentration of the

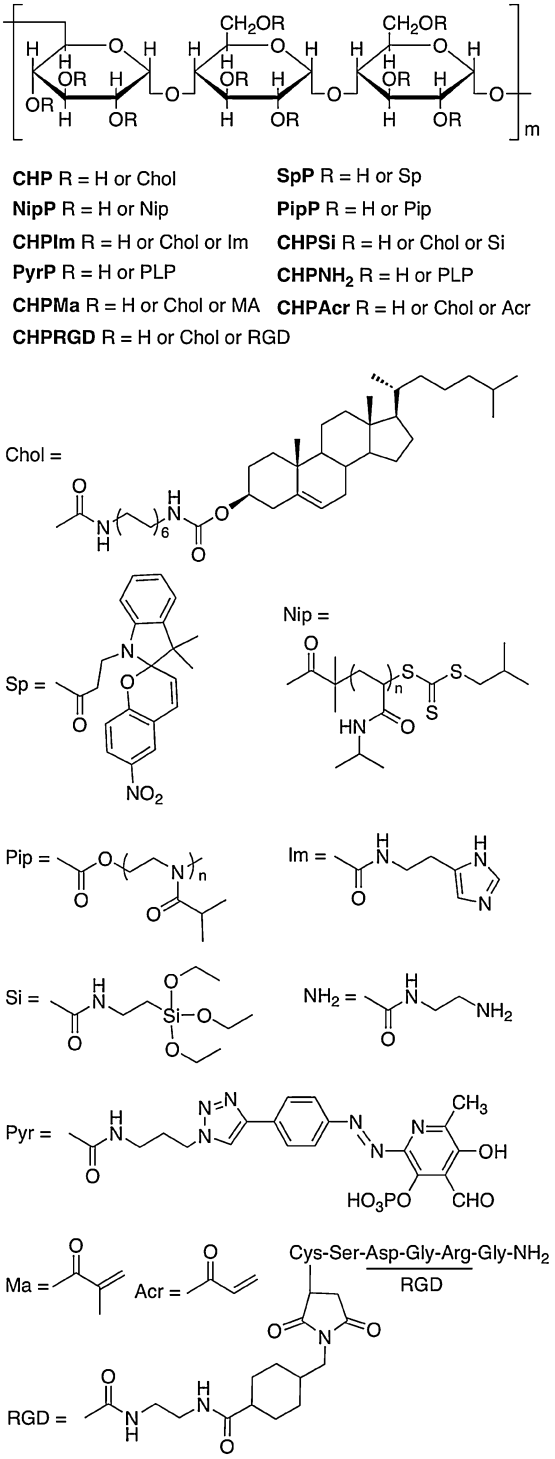

Figure 2. Library of pullulan derivatives to prepare nanogels.

associating polymers, the structure and concentration of crosslinking points, and the characteristics of the associating polymers. Self-assembled nanogel formation from hydrophobically modified polymers has also been demonstrated with other associating polymers such as poly(amino acids)[14] and hydrophilic synthetic polymers onto which associating groups are

partly grafted.[15] By grafting hydrophobic polymer chains (polylactic acids) onto polysaccharides in place of low-molecular-weight hydrophobic groups, more stable nanogels with relatively large hydrophobic domains have been obtained.[16]

Naturally occurring polysaccharides as a backbone polymer are useful for biomedical application because of their excellent biocompatibility and availability. For example, deoxycholic acid-modified glycol chitosan,[17] deoxycholic acid-modified heparin,[18] bile acid-bearing dextran,[19] cholesterol-bearing hyaluronic acid,[20] cholesterol-bearing cycloamylose,[21] and cholesterol-bearing cyclic dextrin[22] have been used as hydrophobically modified polysaccharides for nanogel formation.

The effects of the polysaccharide structures on the physicochemical properties of the resulting nanogels have been investigated in detail, leading to design rules with respect to the polysaccharide backbone. For example, cholesterol-modified highly branched mannan (CHM) formed highly hydrated nanogels in water through the association of cholesteryl moieties in a manner similar to that of CHP.[23] A comparative study of the CHM and CHP nanogels revealed that the structure and level of hydration of the polysaccharide chain significantly affect the microscopic structure of the self-aggregates and the microviscosity of the hydrophobic domain within the nanogels.

We have also reported a novel amphiphilic nanoball in which a cholesterol group was introduced to enzymatically synthesized glycogen (ESG).[24] ESG is a highly branched (1→4)(1→6)-linked α-glucan and is a monodisperse spherical hyperbranched nanoparticle. Cholesterol-modified ESG assembled into a structure containing a few molecules in water to form a cluster of nanogels has great potential as a new building block for nanogel engineering.

♦ Design of Functional Nanogels: Response to Environmental Stimuli

Stimuli-responsive materials have been developed and applied in biology and medicine as DDS and for tissue engineering recently.[25] Nanogels with stimuli-responsiveness have also attracted much attention because nanogels show an unusually rapid response to microenvironmental stimuli such as temperature and pH because of their nanoscaled dimension. Amphiphilic associating polymers can be used to prepare stimuli-responsive nanogels because of the formation of noncovalent crosslinks, which can be easily modified by external stimuli.

To date, several stimuli-responsive nanogels have been developed by introducing stimuli-responsive molecules to the polymers. Especially, photoresponsive or thermoresponsive nanogels have been extensively developed. For example, a photoresponsive nanogel was synthesized by substituting pullulan with a spiropyran molecule (SpP), a hydrophobic group that changes the polarity of the molecule in response to light and heat.[26] The assembly is controlled by changing the amphiphilicity of the spiropyran molecule due to exposure to photoirradiation or heat. In addition, refolding of the chemically denatured proteins after dilution of the protein solution is controlled by photoirradiation. Photoresponsive nanogels also act as novel photoresponsive artificial molecular chaperones. Thermoresponsive nanogels that enable heat-induced association and dissociation of polysaccharides partially grafted with short

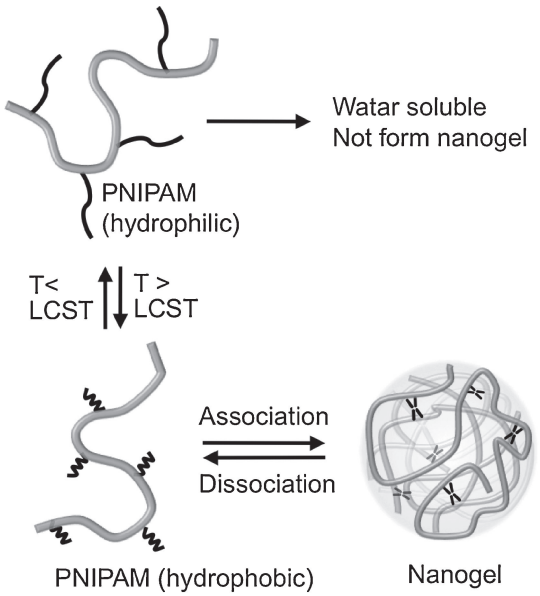

Figure 3. Nanogels enable heat-induced association of polysaccharides partially grafted with short poly(*N*-isopropylacrylamide) (PNIPAM) chains.

poly(*N*-isopropylacrylamide) (PNIPAM) chains (NipP) have been prepared.[27] Above the LCST, PNIPAM-*g*-polysaccharides form nanogels that are physically crosslinked by the hydrophobic nanodomains generated by dehydration of PNIPAM (Figure 3). Biocompatible heat-induced nanogels have also been obtained by grafting poly(2-isopropyl-2-oxazoline) (PipP), which is a biocompatible, thermoresponsive crystalline molecule, onto a polysaccharide.[28]

Stimuli-responsive nanogels that respond to changes in environmental stimuli other than light or temperature has also been reported. Redox-sensitive nanogels have been found to be useful for efficient gene delivery.[6] These nanogels can be obtained by using disulfide bonds as a crosslinking point.[27] More recently, redox-sensitive metal-coordinative crosslinked nanogels were prepared by introducing a metal–ligand to CHP (CHPIm).[29] As a metal ligand, imidazolyl groups were conjugated with pullulan. The imidazolyl group has a high affinity for divalent transition-metal ions and forms a complex. Complexation of more than one ligand group per metal ion can link two or more polymer chains at a metal-centered crosslink. In addition, the redox couple of Co(II) and Co(III) afforded this metal-coordinative nanogel system with good redox sensitivity. A dual stimuli-responsive nanogel that responds to changes in both environmental temperature and redox potential has also been reported.[27] That nanogel was developed by associating a redox-responsive polymer containing a thiol group with the terminus of the PNIPAM chain, which forms a disulfide bond after nanogel formation. More recently, we reported a cyclodextrin-responsive nanogel regarded as an example of chemical-responsive systems for use in an artificial molecular chaperone.[30]

♦ Design of Functional Nanogels: Hybrid with Inorganic Components

Hybrid organic–inorganic materials offer great advantages

because of their wide variety of properties.[31,32] One of the most valuable inorganic constituents is calcium phosphate, which is a major mineral component of bone and teeth. Calcium phosphate shows excellent biocompatibility and biodegradability[33] and thus has been used as a biomaterial for bone tissue, artificial bone and joints.[34]

Recently, nanosized calcium phosphate materials have attracted attention as carriers in drug delivery systems (DDSs).[35] The functionalization of calcium phosphate nanomaterials with organic molecules could provide new advanced DDS materials. There have been several reports on such hybrid nanomaterials prepared by the use of surfactants,[36] block copolymers,[37] and liposomes[38] as organic components. Block copolymers with calcium phosphate nanoparticles showed enhanced cellular uptake.[37] Hollow calcium phosphate nanospheres formed by using surfactants and calcium phosphate-coated liposomes provided controlled-release properties for drugs. It is important that we are able to control the structural properties of calcium phosphate hybrid nanomaterials. Size, colloidal stability, surface properties, and crystallinity greatly affect nanomaterial's functions, such as controlled release, cell-material interactions, and biodegradability. Organic-mediated mineralization is one of the more effective methods for the preparation of desired calcium phosphate hybrid nanomaterials.

On these grounds, we have reported that CHP nanogel acts as a template for calcium phosphate mineralization to form well-dispersed hybrid nanoparticles (ca. 30 nm) in solution (Figure 4A).[39,40] The formation of hybrids, consisting of nanogels with calcium phosphate, may provide more stable DDS materials with controlled release properties. However, one of the drawbacks of calcium phosphate–CHP nanogel hybrid materials is that we only obtained amorphous or low-crystalline calcium phosphate as an inorganic component and could barely control the crystallinity. The amorphous calcium phosphate (ACP) is relatively easy to dissolve in water. The crystallinity of calcium phosphate is an important factor for developing adjustable controlled release systems with nanogel hybrid materials.

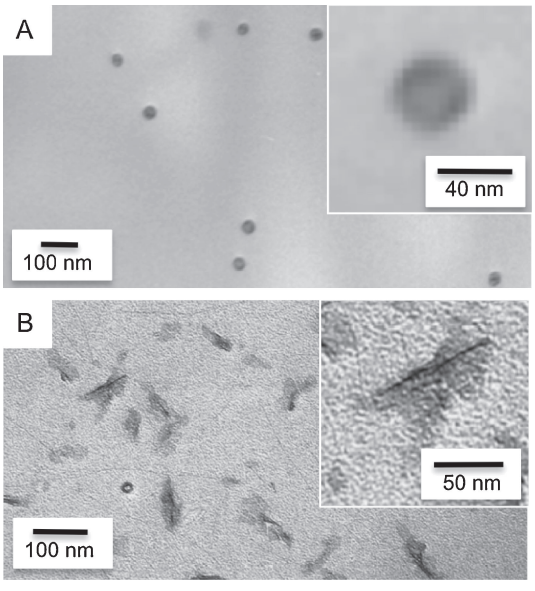

Figure 4. Transmission electron microscopic images of calcium phosphates prepared in the presence of CHP (A: ACP was observed) and CHM (B: HAp was observed). Insets show magnified images.

Low crystallinity, using CHP nanogel, may be possible due to the lack of strong interaction between neutral CHP nanogels and calcium phosphate. Anionic functional moieties, such as carboxylic acid, sulfonic acid, silanol, and phosphate, are known to facilitate the mineralization of calcium phosphates.[41] Nanogels, with these functional moieties, are expected to behave as an improved template for calcium phosphate nanomaterials. Nanogel-templated mineralization using anionic nanogels that consist of CHM was explored. Mannan, from *Saccharomyces cerevisiae*, has a highly branched structure, and phosphodiester forms bonds to these branch points. The crystallinity (amorphous vs. crystal) in the nanoparticles could be controlled by the ratio between calcium ions and CHM nanogels as templates in the mineralizing solution. Dilute calcium solution and a higher calcium ions led to the formation of spherical ACP nanoparticles ca. 20 nm in size and needle-shaped hydroxyapatite (HAp) ca. 80 nm long, respectively (Figure 4B).[42] CHM nanogels prevented the aggregation of calcium phosphate nanoparticles during the phase transition of ACP to HAp.

The hybrid nanogels are useful as intracellular protein carriers because of their excellent biocompatibility and biodegradability, in addition to their mechanical stability. In another example, hybrid nanogels were prepared by condensation of inorganic silanol groups (sol–gel reaction) grafted onto polysaccharides (CHPSi) at ambient temperature and pH without any catalysts or organic solvents.[43] The hybrid nanogels were made rigid upon covalent crosslinking with inorganic siloxanes, which provides an additional platform for mineralization with other silane-coupling agents, titania and hydroxyapatite, for example. These stable hybrid nanogels are promising candidates for controlled-release DDS. In fact, using hybrid nanogels based on PNIPAM gels and tailored nanoporous silica, the carried drug can slowly diffuse out of the porous channels via a nanodiffusion mechanism.[44]

♦ *Design of Functional Nanogels: Conjugate with Biomolecules for DDS*

The cytosolic delivery of exogenous proteins is a topic of growing interest in bioscience and for medical applications. For example, we previously described a protein nanocarrier composed of cationic nanogels formed by the self-assembly of ethylenediamine- and cholesteryl group-modified pullulan ($CHPNH_2$).[45] The nanogel was found to strongly interact with cells, and proteins were effectively internalized, compared with other carriers. Protein-coated quantum dots (QDs) have also been effectively delivered to cells and shown to act as an imaging reagent.[46] One advantage of nanogels is that they can form a colloidally stable complex with protein with an overall complex size of about 50 nm, which is suitable for effective intracellular uptake. They also act as artificial chaperones to protect against the aggregation of denatured protein and assist in refolding of the protein.[47] These unique properties of nanogels have proven valuable for cytokine delivery,[48] cancer vaccines,[49] and adjuvant-free intranasal vaccines.[50] However, the utility of these nanogels is limited by their low target site specificity. To address this issue, a cell-specific peptide (Arg–Gly–Asp; RGD)-

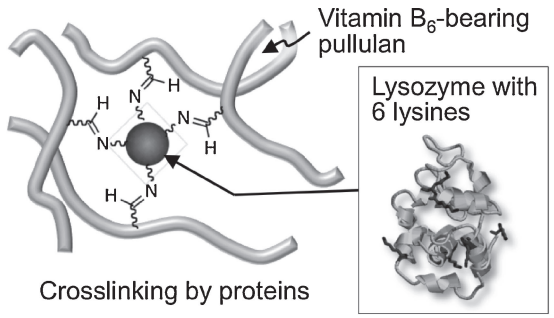

Figure 5. Formation of crosslinking point by Schiff-base formation between vitamin B_6 grafted to polysaccharide and protein (lysozyme).

modified nanogel (CHPRGD) was prepared and evaluated for its potential to act as a protein delivery carrier.[51] This nanogel effectively internalized into the cell via integrin receptor-mediated endocytosis, specifically clathrin-mediated endocytosis and macropinocytosis. Recently, RGD-modified carriers have been shown to be efficient for gene delivery, tumor imaging, and tumor targeting of chemotherapeutic drugs. This tumor targeting peptide-modified polysaccharide nanogel should prove useful for applications in various types of drug delivery.

Biomolecules as drug candidates themselves also could be utilized as a crosslinker to form nanogels. Macroscale hydrogel has been prepared by using biomolecules such as oligopeptides, proteins, antibodies, and oligonucleotides as crosslinkers.[52] One of the main advantages of biomolecule-crosslinked hydrogels is that their response to stimuli is controlled and modulated by the complexed biomolecule. Despite the promising utility of crosslinked biomolecules, there are few examples of biomolecule-crosslinked nanogels, perhaps because of the difficulty in conjugating the biomolecules with polymer chains in an aqueous environment. We recently prepared protein-crosslinked nanogels by introducing vitamin B_6 (pyridoxal) to hydrophilic polysaccharide (PyrP).[53] One of the important chemical characteristics of the pyridoxal moiety is that it acts as an active aldehyde to form a Schiff base between the amine and the pyridoxal formyl group. By using the Schiff base, the pyridoxal-modified polysaccharides were held together to form nanogels that crosslinked with lysozyme containing six lysine residues (Figure 5). The pH-dependence of Schiff base formation was exploited to construct a pH-sensitive nanogel system. These pH-sensitive hybrid nanogels, upon Schiff base formation with various biomolecules, are useful nanocarriers in DDS and could be used for cytosolic protein delivery.

♦ *Organization of Nanogels toward Nanogel Tectonic Engineering*

Conventional hydrogels have been widely used as functional materials in biotechnological and biomedical applications. However, designing hydrogels with a well-controlled nanodomain structure remains challenging. Meanwhile nanogels (nanometer-sized hydrogel nanoparticles, <100 nm) have attracted increasing interest over the past several years due to their applications in DDS. Recently, we proposed nanogel tectonic engineering for construction of functional hierarchical gels or interfaces through their bottom-up assembly (Figure 6). Hierarchical integration of individual components (nanogel tecton) for controlling nanostructure is a novel strategy for construction of gel material and functional nano-/micro-soft gel interfaces. This methodology will overcome the limitations of conventional gel materials and open up new possibilities for gel research.

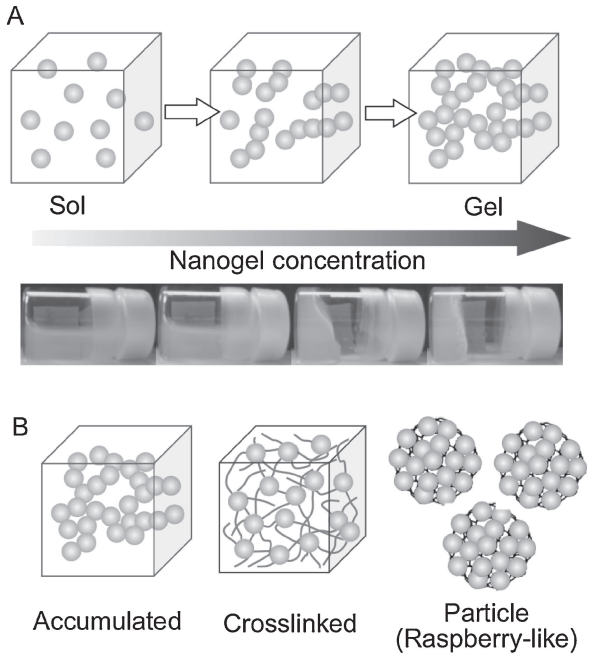

Figure 6. Macroscopic gelation of nanogels to obtain nanogel-accumulated macrogels (A) and organization of nanogel tectons for nanogel tectonic engineering (B).

We found that a nanogel-accumulated hydrogel could be formed when nanogels were dispersed in relatively high concentration ($30\,\mathrm{mg\,mL^{-1}}$, Figure 6A).[54] To further develop nanogel engineering, polymerizable nanogels can be employed as building blocks to control nanostructure in the macrogel. For this purpose, a nanogel containing methacryloyl groups as polymerizable units was designed.[55] Novel nanogel crosslinked macrogels (Figure 6B) with immobilized artificial molecular chaperone functions[47c] and quick thermoresponsivity[56] have been produced by polymerizing methacryloyl-bearing CHP (CHPMa) nanogels with various water-soluble monomers. Macrogels consisting of network structures of nanogels connected by biocompatible polymers can be formed by polymerization between nanogels and 2-methacryloyloxyethylphosphocholine (MPC) in relatively high concentration of the aqueous solution.

Macroscopic hydrogels have been used as an artificial extracellular matrix capable of controlled release of a drug in regenerative medicine. Efficient tissue regeneration is possible by precisely controlling the release of multiple cytokines and hormones. Therefore, development of a durable, long-lasting matrix is essential for applications in regenerative medicine. However, unfavorable rapid and bursts of drug release were observed in many cases because of the difficulty in controlling the crosslinking point in the gel, which leads to heterogeneity of the nanoscale structure. Another problem is the denaturation of proteins inside the hydrogels and their loss of function through irreversible aggregation.

Biodegradable hybrid hydrogels consisting of acryloyl-substituted CHP (CHPAcr) and PEG with four branched terminal thiol groups (CHP–PEG) were used as a matrix for bone regeneration. By allowing the acryloyl group-modified CHP nanogel to absorb prostaglandin E2 (PGE2), a PGE2-encapsulated CHP–PEG hydrogel was prepared.[57] In that study, new bone formation was detected 4 weeks after implantation of the gel into the calvaria of mice. Furthermore, the CHP–PEG hydrogel system exhibited no side effects, such as increases in the cancellous bone of the femur, while bone formation was facilitated specifically at the target site. A CHP–PEG hydrogel encapsulating BMP2 within the nanogel markedly facilitated bone formation.[58]

It was found that nanoparticles (ca. 150 nm) consisting of several hundred nanogels (ca. 30 nm) crosslinked with PEGSH were formed when the nanogel crosslinking reaction was performed at low nanogel concentration,[48a] a process termed raspberry-like nanogel assembly. The resulting raspberry-like nanoparticle was capable of encapsulating interleukin-12 as an immunostimulatory cytokine and was able to keep it even in the presence of BSA in vitro. More importantly, the raspberry-like nanoparticle had a protracted release profile after subcutaneous injection in mice because of hydrolytic degradation under physiological conditions to dissociate back to an original nanogel. Therefore, this system enables sustained release of proteins. The simplicity of the preparation and the high encapsulation efficiency will be very advantageous in practical applications.

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