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One-pot aqueous synthesis of sub-10 nm responsive nanogels†

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A strategy involving free radical copolymerization of a difunctional oligomer and a small-molecule crosslinker to give sub-10 nm nanogels is proposed. These nanogels can adapt to surrounding temperatures and regulate the release of a preloaded model anticancer drug 5-fluorouracil.

Responsive polymer nanogels with three-dimensional crosslinked network structure are very promising as drug carriers because of their high stability, high loading capacity, and responsiveness to environmental factors, which are unprecedented for common pharmaceutical carriers.[1] Recently, this novel family of nanomaterials has attracted increasing attention for co-delivery of pharmaceuticals, labels, and sensing agents towards simultaneous diagnosis, therapy, and feedback.[2] However in the current state of development, such nanogel-based carriers have not yet reached clinical trials. One of the reasons is that they typically need a fairly long time to degrade under physiological conditions, resulting in potential particle accumulation in the body, which may in turn cause long-term toxicity. Even in a few cases where nanogels may degrade rapidly,[1–3] questions about the degradation mechanism, biodistribution, and toxicity remain.

One way to overcome these problems is to design nanogels with sizes smaller than 10 nm, *i.e.*, below which is demonstrated to be a threshold for renal clearance.[4] Generally, two strategies are widely employed for preparing nanogels: (i) a top-down approach where emulsion polymerization techniques are further refined and optimized, leading to the development of micro-/mini-emulsion procedures; (ii) a bottom-up approach which either relies on the synthesis of discrete spherical macromolecules or the self-assembly of linear block copolymers into polymeric micelles followed by chemical crosslinking.[5] In both of these two approaches, however, gels with typical sizes ranging from 20 nm to several micrometers are obtained. As a consequence, the ability to routinely prepare nanogels of sub-10 nm is quite limited. Only recently, an approach developed by Mecerreyes's group and other groups led to nanogels with the size ranging between 3–15 nm by intra-chain crosslinking of macromolecules in an ultra-dilute solution (*ca.* 10^{-5}–10^{-6} mol L^{-1});[6] later, its preparation efficiency was improved by combining a continuous addition strategy and thermally activated benzocyclobutene coupling chemistry.[7] Another approach developed by Jiang's group involves free radical polymerization of a small-molecule crosslinker divinylbenzene (DVB) in a glassy polymer matrix of poly(4-vinylpyridine), owing to the successful prohibition of aggregation between the primary nanogels by the solid matrix.[8] Despite the exciting progress, the challenge of synthesizing sub-10 nm nanogels still remains. The sub-10 nm nanogels fabricated by those multistep approaches also lack responsiveness to the changes in environmental factors. Moreover, no attempt has yet been made to utilize sub-10 nm nanogels as carriers.

Herein, we report a novel one-pot aqueous approach (Fig. 1a; see ESI† for the detailed experimental procedures) to synthesize the sub-10 nm responsive nanogels, starting from poly(ethylene glycol) dimethacrylate (PEGDMA, $M_n \approx 550$ g mol^{-1}), which is a commercially available difunctional oligomer that has been used for preparing temperature-responsive polymers,[9] and the small-molecule crosslinker DVB. Unlike conventional approaches that focus on controlling the diffusion rate of the reaction reagents (using ultra-dilute solution[6,7] or a solid matrix as the reaction medium[8]), we try to exploit functional oligomers that are able to form polymers with appropriate compact conformational characteristics, which are favorable for the formation of the sub-10 nm nanogels.

More specifically, such a typical sub-10 nm nanogel (denoted as SPN-1) can be synthesized by free radical copolymerization of PEGDMA (4.2×10^{-3} mol L^{-1}) and DVB (2.8×10^{-3} mol L^{-1}) using ammonium persulfate (APS; 4.5×10^{-3} mol L^{-1}) as an initiator in the presence of sodium dodecyl sulfate (SDS; 7.1×10^{-3} mol L^{-1}, which is below the critical micelle concentration of 8.0×10^{-3} mol L^{-1} (ref. 10)) at 70.0 °C. We monitored the whole synthesis process using *in situ* dynamic light scattering (DLS). As shown in Fig. 1b, two stages can be observed after addition of the initiator into the reaction mixture: (i) DLS intensity remained nearly the same in the first *ca.* 10 min and then (ii) increased gradually and reached a stable value within the reaction time of 7 h. It is possible that after the free radical copolymerization was initiated at the reaction temperature of 70.0 °C, the poly(PEGDMA-*co*-DVB) fragments formed at the early

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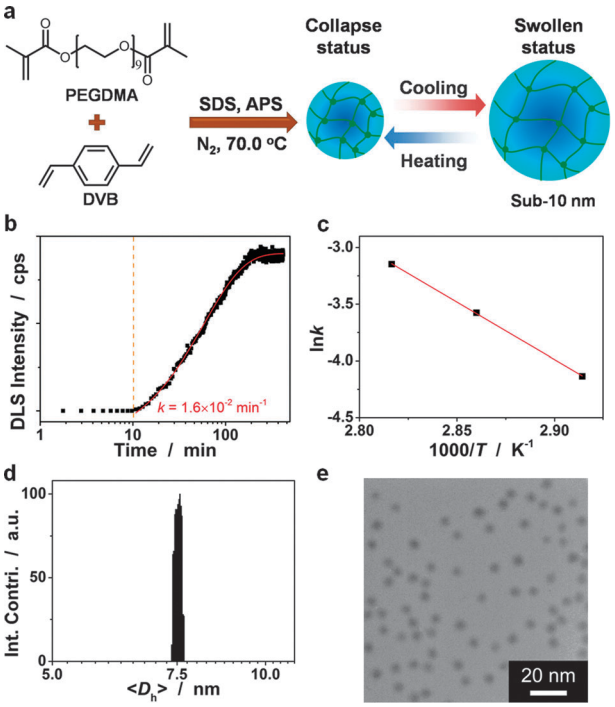

Fig. 1 (a) Schematic illustration of the one-pot aqueous approach to prepare sub-10 nm responsive nanogels. (b) Kinetic curve of SPN-1's formation at 70.0 °C. Solid line: 1st-order kinetic fit. (c) An Arrhenius plot for SPN-1. (d) DLS size distribution of SPN-1 at 25.0 °C. (e) TEM image of SPN-1.

stage of reaction would undergo coil-to-globule transition to form very tiny nuclei, which were stabilized by SDS; as the copolymerization reaction proceeded, more poly(PEGDMA-*co*-DVB) fragments were added onto the initially formed nuclei, leading to a continuous growth in the size of the gel until the reaction was completed.[5,11] Based on the evolution of DLS intensity, the apparent rate constant k of 1.6×10^{-2} min^{-1} was derived from the fitting of the second-stage of the time-dependent DLS intensity with an exponential growth (1st-order). The kinetic evolution was further substantiated by *in situ* DLS measurement of the average hydrodynamic diameter, $\langle D_h \rangle$, *vs.* reaction time (Fig. S1 in ESI†). The k value increased to 2.8×10^{-2} and 4.3×10^{-2} min^{-1}, respectively, if the reaction temperature was set to 76.5 °C and 81.9 °C (see Fig. S2 and S3 in ESI† for kinetic curves). Based on the linear fitting of ln k *versus* T^{-1} (Fig. 1c) and the Arrhenius equation

$$\ln k = \ln A - E_a/RT \quad (1)$$

the activation energy (E_a) was determined to be 84.2 kJ mol^{-1}. However, a negligible effect of the reaction temperature on the size of the produced nanogels was observed. The $\langle D_h \rangle$ for the nanogels was *ca.* 7.6 nm, as measured at 25.0 °C (Fig. 1d, also see Fig. S4 and S5 in ESI†). The $\langle D_h \rangle$ distribution shows only a single peak for the narrowly distributed nanogels, with polydispersity index $\mu_2/\langle \Gamma \rangle^2 = 0.001$. Moreover, the TEM image (Fig. 1e) indicates sphere-like morphology of the nanogels.

Since the conformational characteristic of the polymer chains is a consequence of the counterbalance of the hydrophilic and hydrophobic forces in the polymers at a particular temperature,[11] an appropriate control of the feeding mol ratio $f_{P\text{–}D}$ of PEGDMA to DVB should play a key role in the synthesis of such sub-10 nm nanogels (Fig. 2a). The homopolymers of PEGDMA have a lower critical solution temperature (LCST) as high as ~90 °C.[9] In our approach, when a very high content of PEGDMA ($f_{P\text{–}D} \geq 19.0$; the feeding amount of DVB was set to 2.8×10^{-3} mol L^{-1}, the same below) was fed, the polymer chains may remain very hydrophilic and adopt a random coil conformation, resulting in the formation of large particles ($\langle D_h \rangle \geq 269.3$ nm) or even bulky gels from the reaction. An appropriate functionalization of the temperature-responsive polymer with hydrophobic moieties should be able to reduce its LCST.[2c,11] When the $f_{P\text{–}D}$ was decreased to 9.0, a much smaller nanogel of 36.2 nm was obtained. Interestingly, when the $f_{P\text{–}D}$ fell in a quite wide range of 0.5–6.2, the size of the obtained nanogels can be readily controlled to be very small ($\langle D_h \rangle$ = 6.1–8.5 nm), demonstrating a favourable window to synthesize sub-10 nm nanogels. All nanogels were well reproducible from batch to batch, with a high yield of ≥89%. All nanogels show good stability, due to the highly hydrophilic surface created by the ionic sulfate groups (SI1 and Fig. S6 in ESI†) *via* using an extraordinary quantity of initiator APS in the synthesis.[2d] No sediment was observed after 2 months storage at *ca.* 25.0 °C. In contrast, at a very low $f_{P\text{–}D}$ (≤0.3), the polymer chains may became very hydrophobic to form large aggregates, leading to the formation of large nanogels of $\langle D_h \rangle \geq 15.3$ nm.

For a nanogel, the volume phase transition can be thought of as the summation of the phase/conformational transitions of many different sub-network chains inside the nanogel.[12] Fig. 2b shows the temperature-induced volume phase transitions of the nanogels dispersed in phosphate buffered solution (PBS; 5.0×10^{-3} mol L^{-1}, pH = 7.4). It is clear that the temperature of dispersion medium can influence the size of the nanogels (see Fig. S7 in ESI† for $\langle D_h \rangle - T$ plots). More importantly, the equilibrium deswelling ratio, DSR = $\langle D_h \rangle_T / \langle D_h \rangle_{T=20°C}$, of the nanogels can be regulated by controlling the $f_{P\text{–}D}$, leading to a significant shift in the critical volume phase transition temperature (VPTT) of the nanogels. The lower the $f_{P\text{–}D}$ is, the lower the VPTT of the nanogels. For the nanogels synthesized with a high $f_{P\text{–}D}$ of 19.0, the DSR remained nearly constant until the temperature of above *ca.* 80 °C and then dropped, which confirms that the sub-network polymer chains are indeed too hydrophilic to undergo a coil-to-globule transition at 70 °C. While an appropriate control of the $f_{P\text{–}D}$ at 9.0, 6.2, 3.0, and 1.5, respectively, resulted in a continuous change in the DSR but in tunable slopes across 70 °C, indicating different degrees of coil-to-globule transition of the sub-network polymer chains at 70 °C, the nanogels synthesized with $f_{P\text{–}D}$ = 0.1 exhibited a VPTT of *ca.* 25 °C, revealing that the sub-network polymer chains should be very hydrophobic at 70 °C. The reversibility

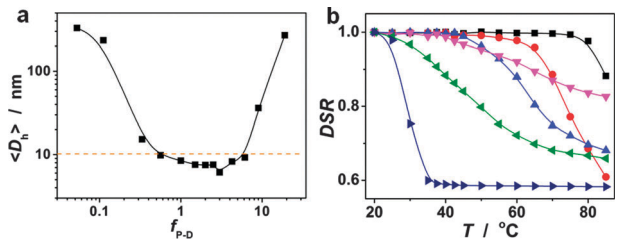

Fig. 2 (a) A plot of $\langle D_h \rangle$ (measured at 25.0 °C) against $f_{P\text{–}D}$. (b) Temperature-dependent DSR of the nanogels synthesized with $f_{P\text{–}D}$ of: ■, 19.0; ●, 9.0; ▲, 6.2; ▼, 3.0; ◀, 1.5; and ▶, 0.1.

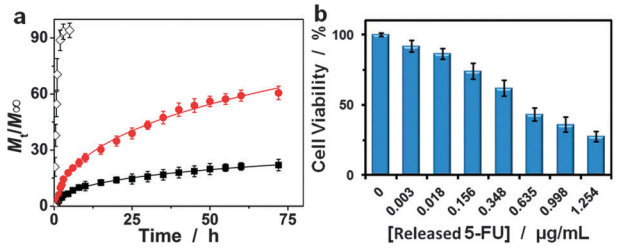

Fig. 3 (a) Release profiles of 5-FU from SPN-1 at 37.0 °C (■) and 42.0 °C (●), respectively. Solid lines: fits to Peppas's model. In the blank release (◇), free 5-FU without nanogels was released at 37.0 °C. (b) B16F10 cell viability following treatments with 5-FU-loaded SPN-1 for 24 h, where [released 5-FU] is the calculated concentration of 5-FU released from 5-FU-loaded SPN-1 according to the release profiles.

of the volume phase transition is demonstrated by the perfect match of heating–cooling curves, as well as the repeatable size distribution during heating–cooling cycles. Moreover, some types of the sub-10 nm nanogels (*e.g.*, SPN-1) can also exhibit a continuous change in the DSR across the physiologically important temperature range of 37–42 °C, which is found in many pathological zones (*e.g.*, tumor).[13] Therefore, these results can not only provide direct experimental proof of the conformational characteristic of the polymer chains at the reaction temperature of 70 °C, but also foreshadow a responsive nanocarrier with direct implications in biomedicine.

We carried out loading and release tests of 5-fluorouracil (5-FU), a widely used anticancer drug,[14] in order to evaluate the drug-delivery performance of the sub-10 nm nanogels. The ether oxygens of the PEGDMA units can form hydrogen bonds with the amide groups in 5-FU molecules to form intermolecular complexes,[15] resulting in a high drug loading efficiency. A drug loading capacity of 59.8 wt% was determined for SPN-1. The nanogels had a narrow size distribution with a negligible change in size after drug loading (Fig. S8 in ESI†). The drug release from the nanogels was determined in PBS at different temperatures (Fig. 3a). The much slower drug release from the nanogels than from the free drug solution indicates a sustained release of 5-FU from the nanogels. The temperature of the releasing medium can trigger the drug release. The increase in temperature induces a gradual transition from hydrophilic to hydrophobic polymer chains (Fig. 2b), which will not only break the PEGDMA–5-FU hydrogen bond complexes and enhance the mobility of guest 5-FU molecules, but also shrink the mesh size of the nanogels so that the hydrophilic 5-FU molecules are expelled and diffuse out of the gel. The release mechanism was confirmed by analysis of the release profiles using Peppas's model:[16]

$$M_t/M_\infty = A't^n \qquad (2)$$

where M_t and M_∞ are the absolute cumulative amount of the drug released at time t and infinite time, respectively. While $n = 0.55$ for free 5-FU indicates a diffusion controlled transportation, the low n values (for release at 37.0 °C and 42.0 °C, $n = 0.24$ and 0.33, respectively, both of which are smaller than the critical value of 0.43 for spherical devices[16]) for the spherical nanogels demonstrate that the release of 5-FU from the nanogels obeys two correlated processes: one is a chemically controlled event related to the breakage of hydrogen bonds; another is a diffusion-controlled step.[16] The investigated temperature variables did not change the drug release mechanism. This release profile may be exploited for biologically controlled drug delivery utilizing the abnormal temperatures in pathological zones.[13] Drug release by a stepwise treatment can be achieved with surrounding temperature alternately changed between 37.0 and 42.0 °C (Fig. S9 in ESI†).

Interestingly, 5-FU released from the nanogels ($M_t/M_\infty \approx 15\%$ after 24 hours of release at 37.0 °C) has an unexpectedly high anticancer activity with $IC_{50} = 0.6$ μg mL^{-1} (Fig. 3b and Fig. S10 in ESI†; Fig. S11 indicates low-toxicity of empty nanogels, ESI†), which is even lower than that of free 5-FU ($IC_{50} = 1.9$ μg mL^{-1}; Fig. S12 in ESI†). The improvement in anticancer activity of an anticancer drug has been observed using responsive nanogels with larger sizes (>20 nm).[3–6,15] In our case, the temperature-dependent cell viability assays indicated the potential use of sub-10 nm nanogels as temperature-responsive carriers (Fig. S13 in ESI†). Further, in comparison with the large nanogels (>20 nm), sub-10 nm nanogels as carriers exhibited a significantly enhanced anticancer activity (Fig. S14 in ESI†), implying another advantage (besides being renal clearable, which is documented in the literature[4]) of sub-10 nm nanogels over their large counterparts.

In conclusion, a simple free radical copolymerization of a difunctional oligomer and a small-molecule crosslinker can provide a versatile approach to prepare sub-10 nm responsive nanogels. It is readily conducted at relatively high concentrations, making it a practical approach for large-scale synthesis. This approach may serve as a starting point for the synthesis of controllable and renal clearable nanogel carriers with huge potential in biomedicine.

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