High-Throughput Screening

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Combinatorial Low-Volume Synthesis of Well-Defined Polymers by Enzyme Degassing

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Abstract: *The synthesis of well-defined polymers in a low-volume, combinatorial fashion has long been a goal in polymer chemistry. Here, we report the preparation of a wide range of highly controlled homo and block co-polymers by Enz-RAFT (enzyme-assisted reversible addition–fragmentation chain transfer) polymerization in microtiter plates in the open atmosphere. The addition of 1 μM glucose oxidase (GOx) to water/solvent mixtures enables polymerization reactions to proceed in extremely low volumes (40 μL) and low radical concentrations. This procedure provides excellent control and high conversions across a range of monomer families and molecular weights, thus avoiding the need to purify for screening applications. This simple technique enables combinatorial polymer synthesis in microtiter plates on the benchtop without the need of highly specialized synthesizers and at much lower volumes than is currently possible by any other technique.*

High-throughput combinatorial synthesis of polymers is emerging as an important method for the discovery and development of novel biomaterials.[1–3] By rapidly screening a large library of structures and compositions, promising candidates can often be much more reliably found than by a rational design approach. High-throughput synthesis and screening of polyacrylates[4] and polyesters[5] have been used to investigate the interaction of polymeric biomaterials with stem cells,[6,7] to develop polymers for the delivery of RNA and other therapeutics,[8] and for the investigation of various structure–property relationships.[9] However, these polymerization reactions are not well-controlled and therefore cannot be used to screen precise structures. While controlled/living polymers and polymer conjugates with well-defined architectures have been widely exploited in the biomedical field to great effect, the combinatorial synthesis of such polymers is relatively unexplored. A few automated synthesizers for anionic and cationic polymerization processes do exist,[10,11] but the sensitivity of these reactions, and the oxygen sensitivity of controlled radical polymerizations in particular, makes combinatorial synthesis extremely challenging and has traditionally limited the number of structures that can be investigated for any given application. Post-functionalization is one way of rapidly generating a large library of compounds from a smaller library of materials,[12] but each polymer still needs to be prepared individually making this approach hard to automate. Seeking to prepare libraries of controlled radical polymers, Guerrero-Sanchez, Moad and co-workers have recently developed an elegant parallel synthesis method for the rapid synthesis of homo[13,14] and quasi block copolymer libraries[15–17] using reversible addition–fragmentation chain transfer (RAFT) chemistry. However, their technique still requires an inert atmosphere, and uses robotics to remove oxygen via a conventional freeze–pump–thaw mechanism.[13] Reactions are typically performed in volumes of 1–10 mL.

We propose that the ideal high-throughput polymer synthesis setup would enable polymerization in microtiter well plates, at low volume, and on the benchtop. This would allow reagents to be transferred with multichannel and simple automated pipettes, and the construction of large libraries of polymers from relatively little material. Ideally block extensions would be performed by simply adding the next desired monomer to the well after completion of each reaction. Provided polymerizations could be taken to near full conversion, no purification would be required, and the polymers would already be in the correct format to be diluted and used for whatever high throughput screening assay was desired. For such a system to work, a mechanism is needed for removal of oxygen that does not involve a glovebox, complicated robotics, or the use of freeze–pump–thaw or inert gas sparging to remove the oxygen. We recently reported the use of the enzyme glucose oxidase (GOx) to scrub oxygen from controlled radical polymerization reactions such as RAFT (Enz-RAFT).[18,19] The activity of the enzyme is very high and can remove dissolved oxygen faster than diffusion from the surface can occur, even at very low enzyme concentrations (<1 μM). This enables polymerizations to proceed at low radical concentrations in open vessels without inhibition from oxygen. We hypothesized that the technique may enable low volume combinatorial polymer synthesis in microtiter plates (Figure 1). In a well-mixed solution, the GOx degassing mechanism is theoretically independent of the volume, and should facilitate polymerization at the low radical concentrations necessary for good control even when the reaction volume is very low.

We first tested this hypothesis by investigating the kinetics of *N*,*N*-dimethylacrylamide (DMA) polymerization in mixtures of PBS buffer and either methanol or *t*-butanol.

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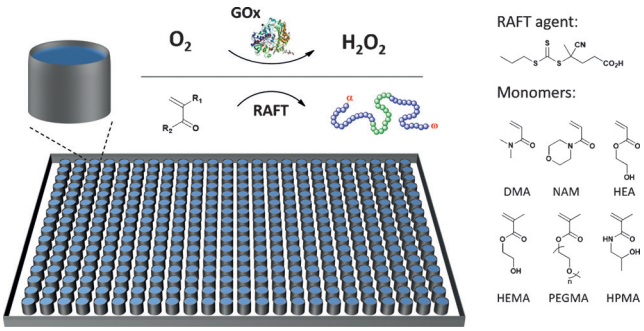

Figure 1. Representation of enzyme-degassed RAFT polymerization (Enz-RAFT) performed in 40 µL water/solvent mixtures in 384 well plates, showing the structure of the RAFT agent and monomers investigated. With this technique, well-defined polymers can easily be prepared in high throughput.

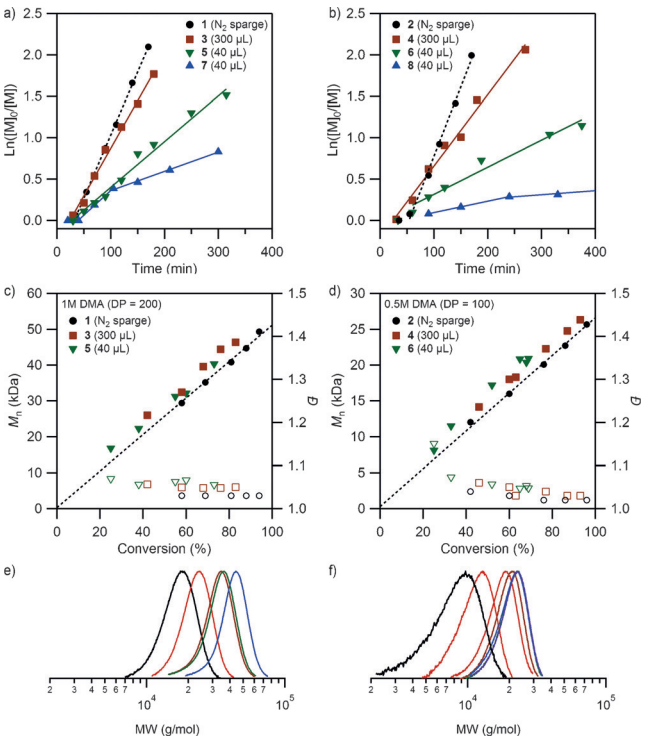

Figure 2. Kinetics of *N*,*N*-dimethylacrylamide (DMA) polymerizations performed under standard RAFT conditions and by Enz-RAFT in 300 µL and 40 µL wells. Polymerizations were conducted at 45 °C in either 15 % *t*-butanol (**1**–**6**) or 25 % (v/v) methanol (**7**, **8**)in PBS, and the concentrations of VA-044 and RAFT agent were kept constant at 0.5 and 5 mM, respectively. The graphs show the pseudo first-order kinetic plots at 1 M (a) and 0.5 M DMA (b); the evolution of molecular weight (closed symbols) and dispersity (open symbols) vs. conversion for polymerizations at 1 M (c) and 0.5 M (d) DMA; and example SEC traces for the 40 µL Enz-RAFT case at 1 M (e) and 0.5 M (f) DMA. Molecular weights are reported relative to pMMA standards without correction.

Reactions were performed in 300 µL solutions on 96 well plates or 40 µL solutions on low-volume 384 well plates, and the kinetics were compared to polymerizations degassed by conventional nitrogen sparging. While it is possible to use metals or enzymes to generate radicals from the peroxide produced by the GOx,[19,20] it is easier to control the radical flux if the GOx degassing mechanism is kept independent of the polymerization mechanism. We have found GOx to be fairly stable at up to about 55 °C in aqueous buffers (Supporting Information, Figure S1), so we chose to work at 45 °C with the low-temperature thermal initiator VA-044. The ratio of RAFT agent / VA-044 was kept constant at 10:1 to ensure good control and end group fidelity, even at high conversion. The GOx concentration was maintained at 1 µM, as in our previous work. At monomer concentrations of both 1 and 0.5 M in 15 % (v/v) *t*-butanol/PBS, the enzyme degassed polymerizations in 300 µL wells proceeded at a similar rate to large scale polymerizations degassed by traditional means (Figure 2 a,b). The well-plate reaction demonstrated a slightly shorter inhibition time in both cases, indicating the RAFT equilibrium was reached very rapidly. This suggests that the oxygen in solution was consumed by the enzyme almost immediately, and faster than oxygen could diffuse in from the surface. When the well volume was reduced to 40 µL, the polymerization rate was slightly retarded at both monomer concentrations (Figure 2 a,b), but the molecular weight evolution was unchanged (Figure 2 c,d). Regardless of the solution volume, a linear evolution of molecular weight with conversion and very low dispersities ($Đ < 1.1$) were observed, as is characteristic of a well-controlled system. The plotted M_n (GPC) was calculated from a pMMA calibration without correction, and so differs from the theoretical M_n (Supporting Information, Tables S1–S3), but the linear trend in Figures 2 c,d nonetheless confirms that the polymerization proceeds according to the RAFT mechanism. The slight differences in rate between the 300 µL and 40 µL wells may be due to imperfect degassing or mixing in the 40 µL case, but whatever the cause the resulting polymers are unaffected. It is worth noting that in both cases investigated a high degree of polymerization (DP) was targeted, and thus a low concentration of radicals was used. For lower target DPs utilizing higher radical concentrations, the effect of any small amount of oxygen contamination should be reduced.

Such a simple route to controlled polymers at such small volumes has to our knowledge not been shown, and is facilitated by the extraordinary efficiency of the enzyme degassing. Slower polymerization rates were observed when methanol was used as a co-solvent (Figure 2 a,b). In both the 1 M and 0.5 M DMA reactions, a sharp change in rate was observed after about 4 h, which is presumably due to loss in enzyme activity, thus allowing oxygen to quench some of the propagating radicals. An inhibition period was also observed, suggesting the enzyme takes longer to fully remove the oxygen at the start of the reaction. This is explained by the reduced stability of the enzyme in methanol compared to *t*-butanol, which we measured using a benzoquinone GOx activity assay. The GOx activity was found to drop sharply over the first few hours in even 25 % (v/v) methanol (Supporting Information, Figure S2), but was retained in up to 75 % (v/v) *t*-butanol (Supporting Information, Figure S3) over the same time frame. This technique for polymerizing in small volumes was found to be extremely versatile, and the enzyme maintained its activity at high monomer concentrations of up to 1.6 M (see kinetics in the Supporting Information, Figure S4) and across

a range acrylamide, acrylate, methacrylamide, and methacrylate monomers. To access acceptable reaction rates, monomers with a low propagation rate coefficient, such as *N*-2-hydroxypropyl methacrylamide (HPMA), needed to be polymerized at concentrations of 1 M or more, and at a higher temperature of 50 °C (Supporting Information, Figure S5a), but even at this higher temperature the enzyme activity was retained for at least 8 h. As with the DMA, HPMA polymerizations showed excellent control, as evidenced by the pseudo log plot (Figure S5b) and low dispersity SEC traces (Figure S5c). To investigate the utility of this technique for generating large polymer libraries, we prepared homopolymers of varying chain lengths from DMA, HPMA, as well as *N*-acryloylmorpholine (NAM), 2-hydroxyethyl acrylate (HEA), 2-hydroxyethyl methacrylate (HEMA), and poly(ethylene glycol) methyl ether methacrylate (PEGMA). To maximize the number of different polymers that can be produced from a small amount of starting material, for many screening applications it is important to work at low concentrations of monomer and RAFT agent, and for this reason all monomers were polymerized for 20 h at 45 °C and 0.5 M using a RAFT agent / VA-044 ratio of 10:1. To target different molecular weights, the monomer concentration was held constant and the concentration of RAFT agent and initiator was reduced. For the acrylates and acrylamides, high conversion (> 80 %) were achieved at 0.5 M for degrees of polymerization (DPs) between 20 and 400 (Figure 3 a; Supporting Information, Table S2), requiring concentrations of initiator down to 0.1 mM. However, for the slower methacrylates and methacrylamides, when working at 0.5 M monomer, high conversions could only be achieved with target DPs of 200 and below, and access to higher DPs required increasing the monomer concentration. In the case of HPMA, conversions of 80 % of more over the entire 20–200 DP range were only obtained when the monomer concentration was increased to 1 M (Figure 3 b). In all cases excellent control was observed, as seen by low dispersity (Figure 3 a,b) and monomodal SEC traces (Figure 3 c), demonstrating the versatility of the technique for access a broad library of different polymers in low volume.

This technique can also be expanded to prepare block copolymers. One pot synthesis of RAFT block copolymers, without intermediate purification, is becoming increasingly popular and a necessary feature of any high-throughput screening synthetic technique.[15,16,21,22] In our system, because the GOx is active throughout the polymerization, it should be able to degas a solution of fresh monomer added to the reaction after consumption of all of the first monomer. To test this we attempted to synthesize two triblock copolymers by block extension of pDMA (DP20 and 30) with NAM and then again with DMA. Because each addition of the monomer will dilute the solution we began these polymerizations at 1 M DMA and 2 μM GOx in 30 μL of 15 % (v/v) *t*-butanol/ PBS. After 2 h at 50 °C more than 95 % of the DMA was consumed (Supporting Information, Table S3), and 10 μL of NAM (3 M) was added, without any additional initiator or enzyme. A further 2 h resulted in almost complete conversion of the NAM, after which another 10 μL of DMA (3 M) was added for a final 2.5 h. The rapid polymerization to full conversion at each block extension demonstrates the successful removal of

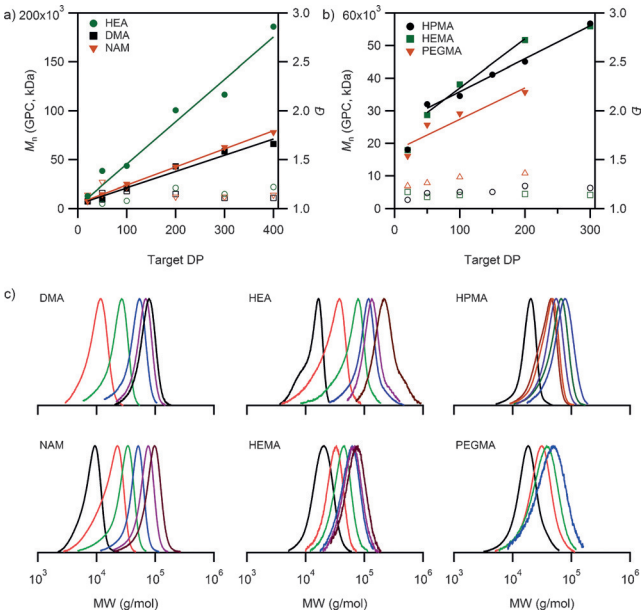

Figure 3. a), b) Molecular weight (closed symbols) and dispersity (open symbols) of homopolymers prepared in 40 μL of 15 % (v/v) *t*-butanol/ PBS as measured by GPC for DMA, NAM, and HEA (a), and for HEMA, PEGMA, and HPMA (b). c) GPC traces for each polymerization. DMA, HEA, NAM, HEMA, and PEGMA polymerizations were conducted at 0.5 M monomer at 45 °C for 20 h with a [RAFT]/[VA044] ratio of 10, and HPMA polymerizations were conducted at 1 M monomer at 50 °C for 20 h with a [RAFT]/[VA044] ratio of 5. Degrees of polymerization (DP) of 20–400 were targeted by varying the RAFT agent concentration. Molecular weights are reported relative to pMMA standards without correction.

the injected oxygen by the GOx at each step. Clean shifts in the molecular weight distribution, with low dispersity ($Đ <$ 1.2) and almost no tailing was observed by SEC for each block copolymer (Figure 4), indicating excellent control and good chain end fidelity. These experiments also demonstrate that the concentration of peroxide is too low to cause degradation of the RAFT agent at these temperatures and timescales, as we have shown by UV/Vis experiments in our previous work.[18] This approach could be used to rapidly synthesize any combination of blocks desired, as each addition is performed on a well plate by simple addition of the next monomer solution.

In summary, we have shown for the first time an accessible method to generate large libraries of polymers without complicated robotics or degassing steps. Polymers can be made in extremely low volume (40 μL) and at low monomer concentrations (0.5 M) without compromising control, as cannot be achieved by any existing method. The activity of the GOx is so high that very low initiator concentrations can be used, facilitating high conversion under these conditions at DPs of up to 400 in the case of acrylates and acrylamides, and up to 200 for methacrylates and methacrylamides. Block copolymers can be prepared by simple addition of the subsequent monomer to the same well after completion of the previous reaction. We expect this technique will revolutionize the ability of the non-specialist to synthesize large libraries of polymers, enabling the combinatorial screening of

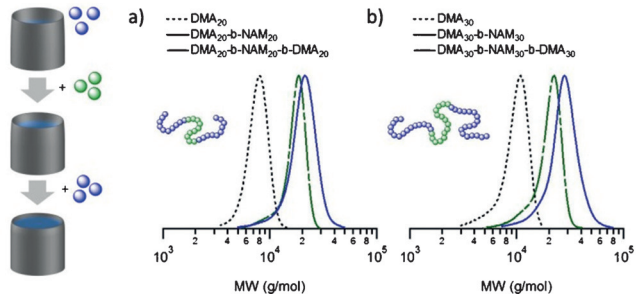

Figure 4. GPC traces of DMA polymers block extended with NAM, and then with DMA. Polymerizations were conducted at 50 °C with an initial DMA concentration of 1 M, and a RAFT concentration of 50 mM (a) and 33 mM (b), to target block lengths of 20 and 30 units, respectively. [RAFT]/[VA044] = 10. Polymerizations were begun in a volume of 30 μL in 15% (v/v) *t*-butanol/ PBS, and 10 μL of monomer (3 M in PBS) was added for each block extension.

polymers for desired physical and biological properties and the investigation of structure–activity relationships.

Experimental Section

Materials: Glucose oxidase (GOx) from *Aspergillus niger* was purchased from Sigma Aldrich as a lyophilized powder, dissolved in phosphate-buffered saline (PBS), and stored in aliquots at −20 °C to avoid successive freeze–thaw cycles. *N*,*N*-dimethyl acrylamide (DMA), *N*-acryloylmorpholine (NAM), 2-hydroxyethyl methacrylate (HEMA), and poly(ethylene glycol) methyl ether methacrylate (PEGMA, $M_n = 300$), were purchased from Sigma Aldrich and de-inhibited prior to use by passing over a short column of basic alumina. 2-hydroxyethyl acrylate (HEA) was purchased from Sigma Aldrich and distilled prior to use. *N*-2-hydroxypropyl methacrylamide (HPMA) and the RAFT agent were both synthesized following previously reported procedures (see the Supporting Information). VA-044 was purchased from Wako chemicals and recrystallized from methanol prior to use.

Instrumentation: NMR spectroscopy was performed using a Bruker Avance 400 (^{1}H 400.13 MHz, ^{13}C 75.5 MHz), with a BBFO probe, and spectra were processed using the Bruker TOPSPIN 3.0 software. Gel permeation chromatography (GPC) was performed using dimethylacetamide (DMAc) + 0.01% (w/v) LiBr as the eluent on a Shimadzu modular system comprising an auto injector, a Phenomenex 5.0 μm beadsize guard column (50 × 7.5 mm), followed by three Phenomenex 5.0 μm bead-size columns (10^5, 10^4 and 10^3 Å), and a differential refractive index detector and a UV detector. Molecular weights were estimated relative to narrow molecular weight distribution polymethyl methacrylate (200 to 1×10^6 g mol^{-1}) calibration standards without correction.

General polymerization method: Stock solutions were prepared of the RAFT agent (50 mM) and VA-044 (5 mM) in 30% (v/v) *t*-butanol/PBS or 50% methanol/PBS, glucose (0.8 M) in PBS, monomer (2 M) in PBS, and GOx (8 μM) in PBS. In all cases, the pH of the solutions was buffered at pH 6.0. For a typical reaction, monomer stock (10 μL) was added to the RAFT agent stock (20 μL) diluted to the appropriate concentration in 30% (v/v) *t*-butanol/PBS or 50% methanol/PBS in a 384 Corning low-volume round-bottom polystyrene NBS well plate. To this was added glucose stock (5 μL), and GOx stock (5 μL). The wells were sealed with a plastic well plate sealing film and heated in a water bath, with agitation at 100 rpm, at 45 or 50 °C. At the completion of the reaction, 15 μL of the solution was diluted in D_2O for measurement of conversion by ^{1}H NMR spectroscopy, and 15 μL was diluted in DMAc for measurement of the molecular weight distribution.

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