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# Arrays of Polyacrylamide Hydrogels Using a Carbodiimide-Mediated Crosslinking Reaction

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**ABSTRACT:** This study introduces a radical-free approach for generating polyacrylamide (PAM) hydrogels with no toxic residues remaining in the networks. Acrylamide and bisacrylamide, which are neurotoxins, are not used during the hydrogel synthesis and only nontoxic side products are generated. This is achieved using a gentle carbodiimide-mediated crosslinking (CMCL) reaction that does not require complex initiation systems and is effective in the presence of oxygen. This overcomes some of the key limitations related to PAM hydrogel synthesis using free-radical routes and maintains the advantages of synthetic hydrogels over biopolymers. In addition, the CMCL reaction allows for accurate placement of functional groups, which controls hydrogel structure and performance including mechanical strength, swelling capacity, and hydrophobic balance. This flexibility is demonstrated through the synthesis and rheological characterization of a library of structurally diverse hydrogels as well as spherical hydrogels. PAM-based hydrogels are used extensively in a broad number of applications, and this study demonstrates the applicability of this method as a nontoxic and radical-free complementary alternative route that can generate structures analogous to those prepared using free-radical routes. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40416.

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## INTRODUCTION

Hydrogels are formed when hydrophilic polymer chains are loosely crosslinked with chemical or physical bonds and are typically applied in areas that require materials that are compatible with aqueous environments but do not dissolve. They consist of a high percentage of water so they resemble living tissues and are permeable to small molecules such as oxygen, nutrients, and metabolites.[1] Polyacrylamide (PAM) is a water soluble polymer that forms the basis for an extensive range of synthetic hydrogels due to the unique properties it contributes. For example, PAM hydrogels find use as a superabsorbent polymer,[2,3] in gel electrophoresis,[4–6] chromatography,[5] cosmetics,[7] biomaterials,[8–16] as well as soil conditioners.[17] The most common method to prepare PAM hydrogels suffers from a number of disadvantages including the use of potent neurotoxins during the hydrogel synthesis. Alternatively, biopolymers have been extensively studied to generate hydrogels without using toxic monomers; however, the current state-of-the-art cannot replace synthetic hydrogels in all applications.[18] As a result, synthetic hydrogels continue to find widespread application but complementary methods to access PAM hydrogels without using toxic monomers during the hydrogel synthesis are desirable.

The most common synthesis method of PAM-based hydrogels involves the aqueous free-radical polymerization of acrylamide monomer with a crosslinker such as *N*,*N*-methylenebisacrylamide (MBA). The reaction includes a free-radical initiator such as ammonium persulfate and requires heating or UV irradiation. Alternatively, tetramethylethylenediamine can be used as a redox catalyst in combination with a free-radical initiator; in this case no heating or UV irradiation is required.

A large number of hydrogels with unique properties can be generated using this approach; however, there are a number of limitations including unequal incorporation of target groups, as mentioned the use of acrylamide and bisacrylamide monomers which are neurotoxins,[19] multiple side products from residual initiators and crosslinkers, remaining initiators can contribute

Additional Supporting Information may be found in the online version of this article.

to toxicity by promoting network degradation,[20] oxygen inhibits the reaction, and the use of free radicals (or UV irradiation) can be detrimental in many applications.[21] As such there is a need for alternative radical-free approaches to generate synthetic hydrogels based on PAM that can minimize, or eliminate, toxic residues while providing accurate control over polymer structure. In addition, due to the widespread use of PAM hydrogels alternative methods must also be scalable and effective under a number of different conditions (i.e., in the presence of oxygen). Biopolymers can be used to overcome some of these limitations because nontoxic hydrogels can be formed. However, they suffer from a number of limitations as they differ in composition from batch to batch, large scale production is limited, and they cannot easily be tailored to the specific application as their properties are determined by the living species which produce them.[18] By contrast, synthetic polymer matrixes such as PAM hydrogels can be prepared in large volumes and their composition can be controlled. Therefore, the aim of this study was to bridge the gap between synthetic and bio-based hydrogels by avoiding radical-based approaches. This would avoid the use of neurotoxins or components during the hydrogel synthesis that could impart toxicity. Structural flexibility was another target along with scalability and insensitivity to oxygen.

Carbodiimide coupling was chosen as a possible alternative because it is a nontoxic transformation[22] that facilitates the formation of an amide linkage between a carboxylic acid and an amine group and can occur in aqueous media using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). It has been used to modify polymers[23,24] and form hydrogels from a range of polymers including alginate,[25,26] ultrathin synthetic hydrogels,[27,28] gelatin,[29,30] hyaluronic acid,[31,32] collagen,[33,34] chitosan,[35] and polyglutamic acid.[36,37] In most cases, the carbodiimide is capable of crosslinking the polymers without the addition of other crosslinkers but in some cases diamines have been used with alginate,[38] poly(gamma-glutamic acid),[36] hyaluronic acid,[32] and poly(methacrylic acid) thin films.[28] However, carbodiimide coupling has not been used for PAM hydrogel synthesis and has the potential to overcome the limitations of free-radical routes.

Here, we introduce an alternative approach to generate stable PAM-based hydrogels using a carbodiimide mediated crosslinking (CMCL) reaction of a PAM-based polymer that is activated to crosslink with a broad range multifunctional amines. This radical-free hydrogel synthesis route does not generate toxic side products and control over polymer structure is demonstrated through rheology studies. It is applicable on a large scale because it uses commercially available reagents and does not require heating or UV irradiation and proceeds under a number of different conditions, for example, in the presence of oxygen. As a result the CMCL reaction has been used to generate an array of novel hydrogels in a single study. Moreover, the method is applicable to bulk hydrogel synthesis and the preparation of microspheres using microfluidics and an inverse suspension.

## EXPERIMENTAL

### Materials

All of the chemicals for the hydrogel synthesis were purchased from Sigma Aldrich and were used as received including: polyacrylamide-*co*-acrylic acid partial sodium salt (PAM-*co*-AA), $M_w$ 520,000, $M_n$ 150,000, typical acrylamide level 80%; *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, commercial grade); *N*-hydroxysuccinimide (NHS, 98%); 1,2-diamino ethane (EDA >99%); 1,4-diaminobutane (DAB); poly(propylene glycol)bis(2-aminopropyl ether) average $M_n$ 400 (PPG); 1,10-diaminodecane ($C_{10}$-diamine), 97%; 2,2′-(ethylenedioxy)bis(ethylamine) (EDOBA); tris(2-aminoethyl)amine (TREN); 1,2,4,5-benzenetetramine tetrahydrochloride (Ph-tetramine), technical grade; crystal violet (>90% dye content); heptane (high pressure liquid chromatography (HPLC) grade, >99.5%); and sorbitan monostearate (span 60).

For the fabrication of polydimethylsiloxane (PDMS) microfluidic devices, the following chemicals were used: Su-8 2050 photoresist and developers (MicroChemicals, Germany), octadecyltrichlorosilane (OTS, ABCR Germany), PDMS and curing agent (Dow Corning, Sylgard 184). Mineral oil (Sigma-Aldrich) was used as the oil phase with the addition of 3 w/w % Abil EM90 (Goldschmidt GmbH, Germany) and 0.5 w/w % Span 80 (Fluka) as surfactants. The microfluidic device was located on an inverted microscope (TE2000U, Nikon) equipped with a thermoplate (Tokai Hit, Japan) microscope stage. Three syringe pumps (neMESYS, Cetoni, Germany) were used to deliver the reagents into the microfluidic chip. Soft silicon tubing (Gecko Optical) and hard polytetrafluoroethylene (PTFE) microbore (Cole-Parmer) were also used.

### Preparation of Bulk Hydrogels

EDC was dissolved in 1 mL of distilled water and added to 12 mL of an aqueous solution of PAM-*co*-AA (5, 7.5, 10, or 15 w/v %), and the resulting solution was shaken. After 3 min, NHS dissolved in 1 mL of distilled water was added, and the solution was mixed by shaking in a closed vessel. The reaction was left to proceed for a further 3 min, this process activated the polymer to crosslinking. After this time the requisite crosslinker was added in 1 mL of distilled water and the solution was mixed, the total volume was 15 mL. The CMCL reaction was carried out in open vials in the presence of oxygen. Representative synthesis conditions are shown in Table I for an array of structurally unique gels prepared at one polymer concentration (5 w/v %), with seven different crosslinkers, at four crosslink concentrations.

For difunctional amines, the molar ratio was 1AA : 1EDC : 1NHS : 0.5 crosslinker, and with trifunctional amine 1AA : 1EDC : 1NHS : 0.33 crosslinker. The manufacturer stated that percentage of acrylic acid groups in the polymer was 20 wt % and this was used for calculating the concentration of EDC and NHS. $^{13}$C NMR was used to confirm the incorporation of acrylic acid groups (Supporting Information Figure S1) in the polymer, however, it is challenging to accurately determine the amount. 20 wt % was used because rheological evidence showed that the gel strength continually increased at higher crosslinker concentrations up to 20 wt % (Supporting Information Table S1) but not beyond. For example, hydrogels prepared with 20% or 25% of the polymer repeat units (or mer- units) activated to crosslinking resulted in materials with the same storage modulus. When 1,10-diaminodecane ($C_{10}$ diamine) was used an additional 1.5 mL of tetrahydrofuran was added to the 1 mL crosslinker solution to ensure that the diamine was dissolved

**Table I.** Representative Synthesis Conditions Based on 12 mL of a 5 w/v % PAM-*co*-AA Aqueous Solution

| | Activators | | Crosslinkers | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| % Crosslinker[a] | Mass EDC (g) | Mass NHS (g) | Mass EDA (g) | Mass DAB (g) | Mass EDOBA (g) | Mass $C_{10}$ (g)[b] | Mass Ph Tetra (g) | Mass PPG (g) | Mass TREN (g) |
| 20 | 0.3194 | 0.192 | 0.048 | 0.0701 | 0.1234[c] | 0.1375 | 0.1135 | 0.3187 | - |
| 15 | 0.2396 | 0.144 | 0.036 | 0.0526 | 0.0926 | 0.1031 | 0.0851 | 0.239 | 0.0603 |
| 10 | 0.1597 | 0.096 | 0.024 | 0.0351 | 0.0617 | 0.0688 | 0.0568 | 0.1594 | - |
| 5 | 0.0799 | 0.048 | 0.012 | 0.0175 | 0.0301 | 0.0344 | 0.0284 | 0.0797 | - |

Activators (EDC and NHS) and crosslinkers are dissolved in 1 mL of water.
Crosslinkers: 1,2 diamino ethane (EDA >99%); 1,4 diaminobutane (DAB); 2,2′-(ethylenedioxy)bis(ethylamine) (EDOBA); 1,10-diaminodecane ($C_{10}$); 1,2,4,5-benzenetetramine tetrahydrochloride (Ph-tetramine); poly(propylene glycol) bis (2-aminopropyl ether) average $M_n$ 400 (PPG); and tris (2-aminoethyl)amine (TREN).
[a] Mol % of polymer repeat units (or mer- units) activated with EDC and NHS that undergo subsequent crosslinking, 20 mol % is the maximum because the polymer contains 20% acrylic acid (1AA : 1EDC : 1NHS).
[b] $C_{10}$ diamine was not soluble in 1 mL of water so an additional 1.5 mL of THF was added.
[c] System was used for microfluidic synthesis of microspheres.

(the overall volume of the solution was 16.5 mL). The resulting crosslinker solution was miscible with the activated aqueous polymer.

### Fabrication of Microfluidic Chips

The microfluidic chip was fabricated using a standard photolithographic technique as described below. The silicon substrate was initially cleaned with acetone/isopropylalcohol solutions and dried under nitrogen. It was further cleaned in a piranha solution ($H_2SO_4/H_2O_2$ 3 : 1 v/v) for 20 min, then in hydrofluoric acid (5%) for 1 min. This last step removed the native oxide on the Si wafer to enhance hydrophobicity and subsequent adhesion of the Su-8 photoresist. The Su-8 photoresist was spin-coated onto the wafer at 2000 rpm/300 rpm $s^{-1}$ for 30 s to obtain a film thickness of 50 μm. This was followed by a soft bake step at 65°C, then 95°C for 3 min and 9 min, respectively. Finally, the substrate was exposed with UV light at 200 $mJ/cm^2$ using a transparent sheet mask and postbaked at 65°C for 2 min and then for 7 min at 95°C. The photoresist was developed by immersion in a solution of Su-8 developer for 6 min. Finally, a hydrophobic monolayer of OTS was deposited onto the mould. This surface chemistry was necessary to facilitate removal of the PDMS without damaging the Su-8 mould. The deposition was performed by taking a 10 μL droplet of OTS on a separate glass slide and both the slide and mould were placed in a desiccator for 1 h. Subsequently the static water contact angle was approximately 110° indicating that a hydrophobic monolayer was present.

To fabricate the PDMS chip, the PDMS solution was mixed with the curing agent (10 : 1 w/w %) and was degassed under vacuum for 20 min. The solution was then poured on the Su-8 mould and heated in an oven for 30 min at 90°C. Holes were created into the chip with a 1.5 mm punch to accommodate the tubing. The resulting PDMS chip was bound onto a glass slide (100 μm thickness) using an oxygen plasma for 30 s, followed by a baking step at 90°C for 10 min. The microchannel from the nozzle where the beads were generated to the end of the tubing was 120 mm in length. The width of the channel was 400 μm and the height was 50 μm. It is important to note that at the nozzle, the dimension of the channel was reduced to 50 μm to provoke bead generation.

### Microfluidic Synthesis of Microspheres

Hydrogels with micron scale dimensions (100 μm) were generated on a microfluidic platform heated at 50°C. Three syringe pumps delivered the different reagents (two for the oil phase and one for the aqueous phase) into the PDMS chip. The flow rate was adjusted to 300 μL/h for the oil and 150 μL/h for the aqueous phase. The channel length provided sufficient time to crosslink the system, which was a 5% PAM-*co*-AA solution with 20% of the groups on the polymer backbone (mer- units) activated with EDC and NHS to crosslink with EDOBA (Table I). The precursor solution was injected at 25°C into a microfluidic channel at 50°C and the residence time in the chip was approximately 60 s. The resulting microspheres were collected in a 2 mL eppendorf tube containing an excess of water and *n*-heptane so the microspheres were transferred from the oil phase to the water phase.

### Inverse Suspension Synthesis of Hydrogel Microspheres

Hydrogel microspheres with larger dimensions (approximately 600 μm) were generated in an inverse suspension. EDC (0.3993 g) was dissolved in 0.5 mL of distilled water and added to 5 mL of an aqueous solution of PAM-*co*-AA (15 w/v %) and the resulting highly viscous solution was mixed. After 3 min, 0.24 g of NHS dissolved in 0.5 mL of distilled water was added, at this stage the viscosity of the solution decreased. This activated polymer solution was then added drop-wise over a 5-min period to 95 mL of heptane containing 5 w/v % Span 60 in a 250 mL round bottom flask heated to 50°C. The solution was continuously stirred at 1000 rpm using a magnetic stir bar (32 mm × 16 mm egg shaped) to provoke droplet generation. This mixture was termed as inverse suspension of activated polymer and consisted of an aqueous polymer phase suspended as droplets in heptane. After 5 min, the crosslinker (0.063 g EDA or 0.148 g EDOBA), dissolved in 0.5 mL of water, was added drop-wise to the inverse suspension which initiates the CMCL reaction. The reaction was complete after only 40 min at 50°C and the resulting hydrogel microspheres were denoted IS-EDA and IS-

EDOBA. They were isolated by filtering through a filter funnel that was heated to 60°C. Alternatively, the microspheres were added to excess ethanol (500 mL) and were then filtered.

### Gel Characterization

**Rheology.** Rheology was performed using a HR-3 Discovery Hybrid Rheometer (TA Instruments) and a smart swap recessed concentric cylinder geometry with a cup (radius 15 mm) and rotor (radius 14 mm, and height 42 mm). The gap between the bottom of the cup and rotor was set at 4 mm, and heating was achieved using Peltier heaters. The PAM-*co*-AA was first activated with EDC and NHS, then the crosslinker was immediately added. 12 mL of the resulting solution was quickly loaded into the measuring geometry so crosslinking could be monitored from the same point for each system. The temperature was set at 50°C which is not a prerequisite for gel formation (as shown in Supporting Information Tables S1 and S2). A lid was used to cover the cup to minimize evaporation of the water, and to further prevent this mineral oil was poured on the top of the solution and as a result no shrinkage of the hydrogels was observed. Crosslinking was monitored as a function of time and the oscillation frequency was 1 Hz and strain was kept at 0.01%. The experiments were performed for 19.5 h to ensure the crosslinking reaction was complete which was determined as the plateau in the modulus which occurred before 19.5 h. After these experiments, frequency sweeps were conducted on the samples to record the frequency dependence of the moduli and finally strain sweeps were performed to determine if the gels failed under strain. The gels were not removed between the three separate measurements.

**Nuclear Magnetic Resonance.** $^{13}C$ NMR spectroscopy was performed on a Bruker Av500 NMR spectrometer with a 10 mm autotuning and matching broad band observe probe. Samples were held at 40°C inside the probe. An 83° pulse was used with inverse-gated $^{1}H$ decoupling using a bilevel waltz-16 decoupling sequence. The acquisition time was 1.08 s and the relaxation delay was 10.0 s, the sum of 11.1 s being greater than 5× the longest $T_1$ measured using an inversion recovery sequence. The data were zero-filled once and exponential multiplication using 5 Hz line broadening was applied prior to Fourier transformation.

**Optical Microscopy of Microspheres IS-EDA and IS-EDOBA.** A KYOWA microscope was used to collect images of the samples. The spherical microspheres from the reaction flask were isolated in ethanol to deswell the microspheres which were collected by filtration. They were then dried and mounted on a self-adhesive vinyl sheet attached to a glass slide. Micrographs were taken for each sample using an Infinity $\chi$ camera, and particle size distributions were manually measured. The microspheres from the reaction were filtered through a funnel at 60°C and were washed three times with heptane (100 mL). The swollen microspheres were placed in a Petri dish against a pixelated background (line size 6 mm), and the particle size was manually calculated. The dye swollen samples were placed in a Petri dish and the particle sizes were also manually calculated.

**Swelling Experiments.** To estimate the uptake capacity of the microspheres (IS-EDA and IS-EDOBA) an accurate mass of dried microspheres was added to preweighed poly-prep chromatography columns (Bio-Rad). For bulk hydrogels, the samples were filtered through a 100-mesh wire gauze. 25 mL of distilled water was added to each cartridge and the samples were swelled for a specified period of time at 25°C (1 h, 18 h, and 96 h). The excess water was then removed by filtrating under pressure. The cartridge and swelled samples were reweighed to determine the water uptake. The samples were deswelled by washing with 25 mL of acetone and three separate washes with ethanol (25 mL), each time the samples were shaken and left for 20 min before filtering. The samples were then dried (in the column), reweighed, and the process was repeated by adding 25 mL of water and swelling to generate repeat measurements. The value for the standard deviation for this method is ±2.3 g which is in line with other swelling studies.[39] For dye swollen microspheres, 40 mg of a dried sample was placed in 250 mL solution of crystal violet (5 mg/L) and allowed to swell for 2 days. The particles were isolated by filtration, and the process was repeated three times by replacing the solution.

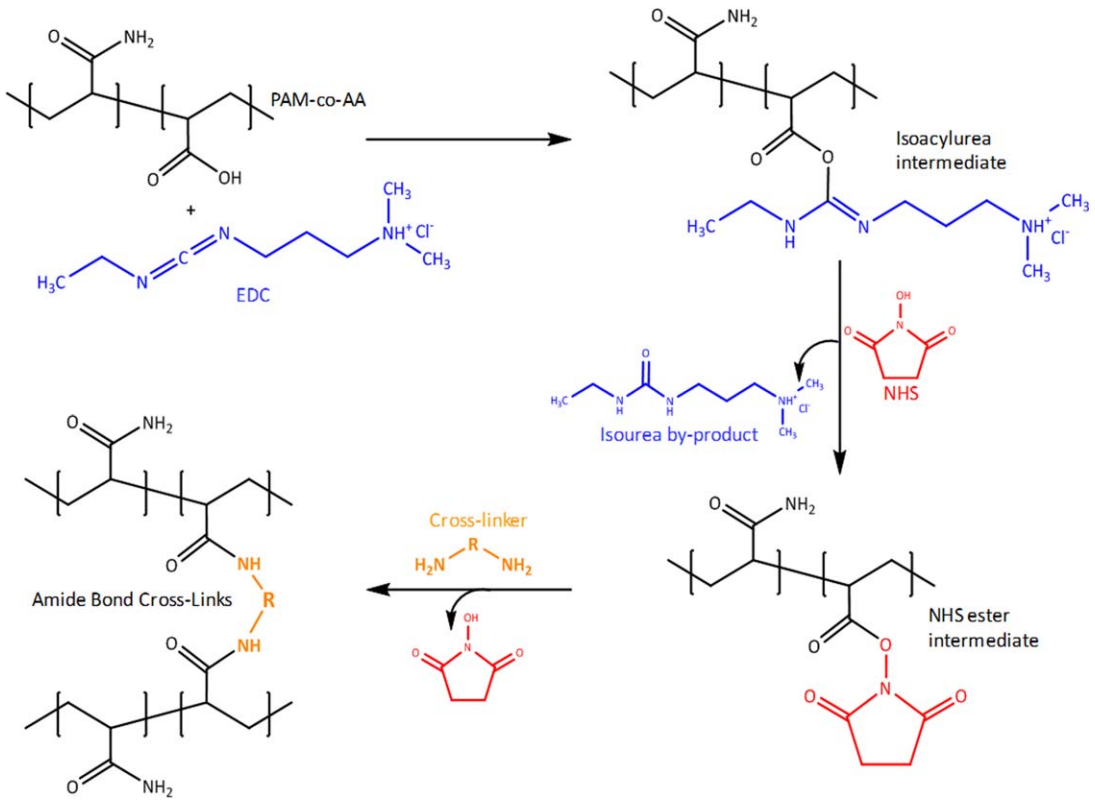
## RESULTS AND DISCUSSION

A diverse range of hydrogels were synthesized using the CMCL reaction by varying polymer concentration, crosslinker structure, and crosslinker concentration. In the following sections, a reaction mechanism is presented and the properties of the hydrogels as investigated using rheology are included. The flexibility of this approach is further demonstrated through the synthesis of hydrogel microspheres using two separate techniques.

### Crosslinking Studies

EDC and NHS are routinely used to activate carboxylic acid groups[40] to react with amines resulting in the formation of amide bonds. This occurs via the formation of an acylurea intermediate which undergoes further reaction with NHS. The resulting NHS-intermediate is more stable and can readily react with amines thus forming an amide bond.[41] This series of reactions was used during this study to activate the carboxylic acid groups in a PAM-*co*-AA copolymer (Scheme 1) to crosslink with multifunctional amines. Therefore, multiple amide bonds between the polymer chains were generated resulting in hydrogel formation and the structure is analogous to networks synthesized using free-radical chemistry.

The mechanism was confirmed using $^{13}C$ NMR of the resulting hydrogels which showed the presence of the expected nontoxic side products (isourea and NHS) and a reduction in the acrylic acid carbonyl peak as those groups were converted to amide bonds in the crosslinking reaction (Supporting Information Figure S2). In addition, unreacted crosslinker was not observed in the $^{13}C$ NMR spectra because it was consumed in the reaction (Supporting Information Figure S3). Further evidence of the mechanism of crosslinking can be seen from the gel strength which continually increased up to but not beyond, the maximum acrylic acid content (i.e., 20 wt %, Supporting Information Table S1). As a result, the hydrogels generated do not contain any toxic residues, which is a significant step forward for synthetic hydrogels. The base polymer is synthesized using free-radical routes but this can be generated in a controlled




**Scheme 1.** Reaction schematic of the CMCL mediated crosslinking or PAM-*co*-AA (shown in black) with EDC (shown in blue) and NHS (shown in red) which forms an activated polymer (NHS ester intermediate) that is crosslinked with multifunctional amines (shown in brown). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

environment and the route presented avoids the use of toxic monomers during the synthesis of the PAM hydrogels.

A series of control experiment were also performed to confirm the mechanism presented in Scheme 1. This included EDC-activated polymer only, NHS-activated polymer without crosslinker, polymer and crosslinker (no activation), and activated polymer with excess crosslinker (to promote polymer modification and not crosslinking). As expected none of the control systems formed hydrogels and remained as flowing liquids with no increase in moduli when measured using rheology.

Based on this reaction mechanism, any multifunctional amine can theoretically crosslink the activated base polymer and all of the amines that we applied were effective. Therefore, the number of hydrogels that can be readily generated using the CMCL reaction is vast because there are a broad number of multifunctional amines that are commercially available. As such, we believe that this method is extremely flexible, which is critical for developing a complementary alternative to free-radical routes. The large number of crosslinkers used in this study have generated stable hydrogels with broad structural flexibility as the R group (Scheme 1) changed. For example, EDA is a short chain diamine whereas PPG is a low molecular weight polymer ($M_n$ 400) and the hydrogels generated have significantly different networks. Alternatively, trifunctional amines such as TREN and tetrafunctional amines (Ph-tetramine) were also effective and again the structure of the hydrogels varies substantially. All of the above crosslinkers are water soluble amines but longer chain amines ($C_{10}$ diamine) were also effective. The ability to control the network structure is important in all applications. For example, in polyacrylamide gel electrophoresis which is used for protein purification controlling mesh size is essential and the CMCL reaction will have application in this area. The fact that oxygen does not need to be removed is an additional advantage. Moreover, the CMCL reaction generates urea which is a chaotropic agent that allow proteins to unfold and is often used in electrophoresis.[42]

**Residues in the Networks.** By using a purified polymer to form the networks toxic monomers were eliminated from the hydrogel synthesis. This is advantageous because removing residues from the resulting hydrogels is challenging and adds several other steps before using the material in an end application. The base polymer is still synthesized using free-radical routes and acrylamide monomer which cannot be avoided; however, this is commercially available in a pure form without toxic monomers. Overall the CMCL reaction offers an alternative to generate PAM hydrogels without handling toxic monomers.

By examining the reaction mechanism presented in Scheme 1, the only side products that were generated during the hydrogel synthesis are nontoxic isourea and NHS, which is why carbodiimide coupling is regarded as a nontoxic transformation.[22,43] The crosslinkers are amine-based so the primary hazard is their corrosiveness,[43] but unreacted crosslinker was not detected in

the hydrogels using $^{13}C$ NMR (Supporting Information Figure S3). Rheology data also shows that the elastic modulus increases rapidly and is significantly higher than the loss modulus which is indicative of a well developed network. Overall, the CMCL reaction involves polymer, isourea, NHS, and crosslinker. Three of these molecules are nontoxic and the fourth reagent is consumed in the reaction which represents a significant step forward in terms of toxicity.

In contrast, free-radical synthesis of hydrogels requires reagents which can all directly contribute to toxicity. These include acrylamide, initiators, and crosslinkers. It is impossible to avoid the use of acrylamide which is a neurotoxin, and MBA is also a neurotoxin and residues can remain in the gel. The residual initiators can also generate toxic residues via secondary mechanisms caused by degradation of the network,[20] especially in the presence of oxygen. In addition, free radicals are harmful in a number of applications,[21] and heating or UV irradiation also presents an issue if sensitive cargoes are loaded into the gel. Therefore, the CMCL reaction represents a significant step forward in eliminating toxic residues, free radicals, and extreme curing conditions such as elevated temperatures or UV irradiation while offering broad synthetic flexibility. It is worth noting that the hydrogels are structurally analogous to those produced using free-radical routes so the benefits are maintained.

### Rheological Characterization of Hydrogels

The polymer networks were well developed and resulted in the formation of stable hydrogels that endured a range of conditions including the addition of excess water, salt, acid, and base. This demonstrated that the crosslinking reaction was efficient so rheology was used to study the formation of the hydrogel networks. The following discussion focuses on gels prepared with different crosslinkers: EDA, PPG, $C_{10}$ diamine, and TREN. A number of other experiments were also performed (Supporting Information Tables S1 and S2) which are not included in this discussion. The experiments were performed at 50°C to ensure that the reactions all reached completion in a reasonable timeframe; however, heating is not a prerequisite for gel formation (Supporting Information Tables S1 and S2). The polymer concentration in each case was 5 w/v % PAM-*co*-AA and 15% of the repeat units (or mer- units) on the polymer backbone were activated to crosslinking. The rate of gel formation and resulting mechanical properties of the gels was substantially different as shown in Figure 1. Figure 1(A,B) show the same data on different time scales.

The crosslinking reaction was efficient as evidenced by the rapid increase in modulus for all of the systems. The gel time was defined as the time required for the storage ($G'$) and loss ($G''$) modulus to intersect: for PPG this time is 14.9 min, EDA 7 min, $C_{10}$ diamine 2.7 min, and the TREN-based system gelled before the sample was loaded into the rheometer [Figure 1(A), blue curves have already intersected]. These data demonstrate that the rate of gelation can be controlled by selecting different crosslinkers due to the number of amine groups present and the reactivity (or basicity) of those groups.

The plateau moduli of the resulting gels also varied substantially with different crosslinkers (Figure 1). The hydrogel synthesized with PPG reached approximately 18 Pa, EDA reached 74 Pa, $C_{10}$ diamine 150 Pa, and 230 Pa for TREN so clearly the structure of the crosslinker also influenced the modulus. The PPG-based gel had the lowest overall modulus which was due to the longer and more flexible crosslinks that were formed. The EDA crosslinked system had a higher overall modulus which was attributed to the formation of shorter crosslinks. Interestingly, the network structure for this hydrogel is structurally analogous to hydrogels prepared free radically with MBA as the crosslinker; the only difference is the crosslinks derived from EDA contain one more methylene group. The maximum storage modulus of the hydrogel prepared with $C_{10}$ diamine was higher than the PPG- and EDA-based systems which is in line with other studies where the incorporation of hydrophobic groups improves mechanical properties.[44] The TREN-based system had the highest modulus (230 Pa) which was due to the short crosslinks that were formed combined with the high reactivity of TREN which was reflected in the rapid gel formation. Therefore, by changing the structure of the crosslinker the properties of the resulting gels was modulated over a broad range. This is reinforced in Supporting Information Table S2 which shows data that were collected at 25°C for four

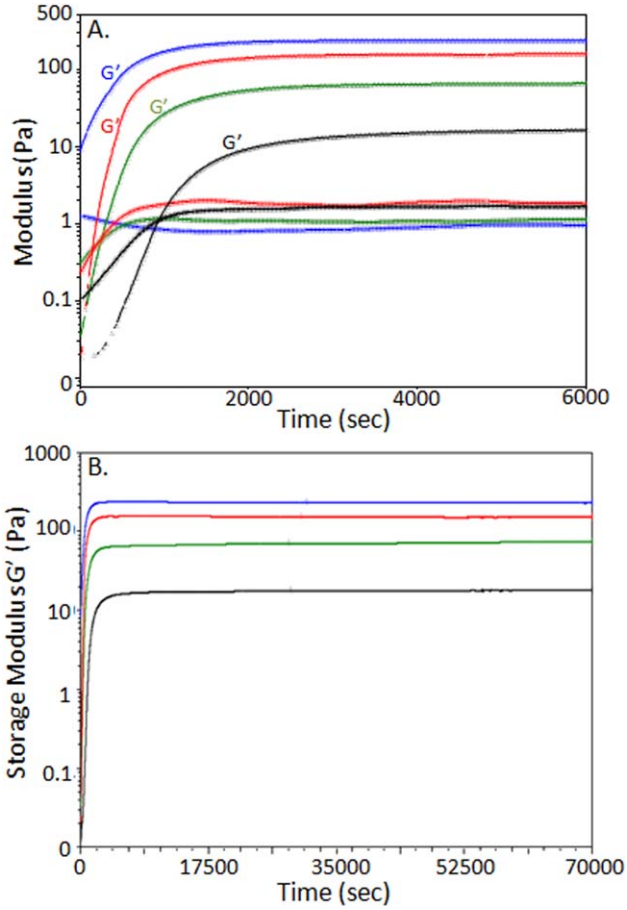



**Figure 1.** Storage modulus ($G'$) for gels crosslinked with PPG (black), EDA (green), $C_{10}$ diamine (red), and TREN (blue). (a) Early stage increase in moduli, storage modulus (indicated using $G'$) and loss modulus and (b) maximum storage moduli. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

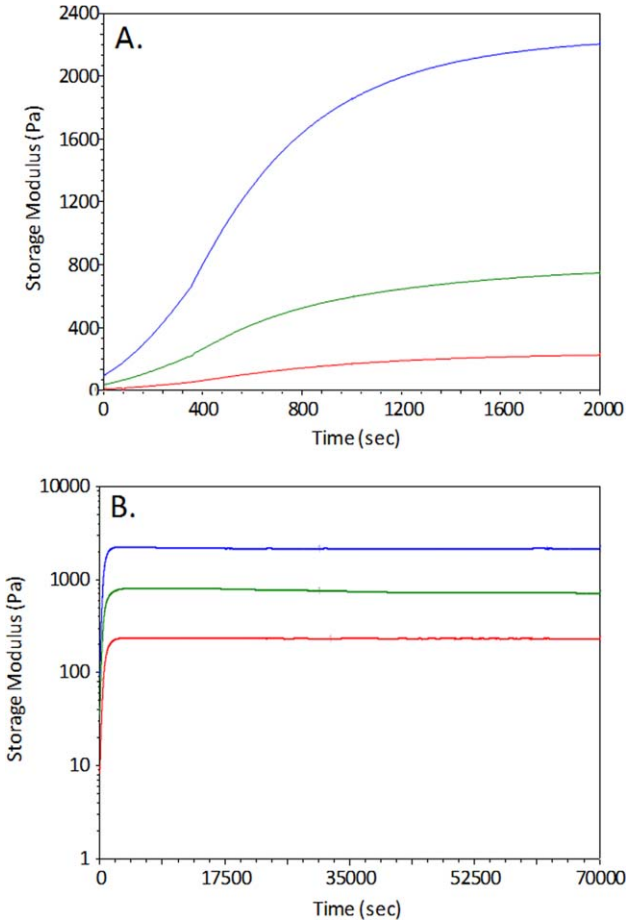



**Figure 2.** Storage modulus ($G'$) for gels crosslinked with TREN at different polymer concentrations (10% blue, 7.5% green, and 5% red). (a) Early stage increase in storage moduli and (b) maximum storage moduli. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

crosslinkers (EDA, EDOBA, $C_{10}$ diamine, and Ph tetramine), again the gel time and modulus of each sample varied substantially.

The hydrogel networks were investigated further by performing frequency and strain sweeps for the different gels (Supporting Information Figure S4, S5). The storage modulus ($G'$) for all of the gels remained constant with frequency and strain which is characteristic of a well-developed crosslinked polymer network which reinforces the efficiency of the CMCL reaction.

The effect of polymer concentration was also investigated for the system crosslinked with TREN (Figure 2) which is analogous to increasing the initial monomer concentration in conventional free-radical polymerization. The early stage increase in storage moduli for each system is shown in Figure 2(A), and the longer term maximum moduli for the same hydrogels are shown in Figure 2(B). The modulus for each system increased rapidly [Figure 2(A)] within the first 30 min and the maximum modulus [Figure 2(B)] reached 810 Pa when 7.5 w/v % polymer was used, and 2245 Pa at 10 w/v % concentration which is significantly higher than the 5 w/v % system (230 Pa). Clearly, the network and crosslink densities increased at higher polymer concentrations indicating that this route can be used to modulate mechanical properties.

### Synthesis of Hydrogel Microspheres

All of the data reported thus far have been related to bulk hydrogels and another aim of this study was to generate hydrogel microspheres because many applications require hydrogels with this morphology.[11] There are several different routes to generate discrete hydrogel microspheres including: inverse emulsion, microfluidics, precipitation, dispersion, and inverse suspension polymerization. In this study, we have applied the CMCL reaction to microfluidics and an inverse suspension route, as discussed in the following sections.

**Microfluidic Synthesis of Hydrogel Microspheres.** Microfluidics is a powerful tool for particle generation and represents a more complex scenario to apply the CMCL reaction. To generate hydrogel microspheres using microfluidics water-in-oil emulsions are generated by injecting an aqueous solution into a micron-sized channel containing an oil phase (Figure 3). If the aqueous solution contains hydrogel precursors that react when a suitable method of initiation is applied (e.g., UV irradiation) then the droplets can be converted to hydrogel microspheres. There are a number of technical challenges that must be addressed in this case, for example, the aqueous precursor solution must have a sufficiently low viscosity so that is can be injected into the microchannel. In addition, the crosslinking rate must also be controlled to balance the generation of monodisperse droplets inside the channel while the liquid precursor is converted to a hydrogel. All of the approaches reported to date generate PAM-based materials using acrylamide monomer that is polymerized free-radically using thermal-,[45,46] UV-,[47–51] or redox initiation.[52]

Our aim was to apply the CMCL reaction to generate hydrogel microspheres without using any monomers, UV irradiation, or free-radicals, while eliminating toxic residues in the microspheres. The initial target was to have a single fluid with sufficiently low viscosity that could be injected into the microchannel. Once injected the crosslinking reaction had to proceed quickly to retain the spherical morphology and form hydrogel microspheres. The system investigated for this was a 5% PAM-*co*-AA with all of the acrylic acid groups in the polymer (20 mol %) activated to crosslinking with EDOBA (Table I). This solution was injected from a syringe at 25°C into a microfluidic channel maintained at 50°C (Figure 3).

Within the channel a stable emulsion of the polymer precursor [Figure 4(A)] was formed, however, as time progressed the viscosity of the fluid being injected increased which affected the size distribution of the particles. Despite this the process was still effective and Figure 4(B) shows particles that were isolated from the chip. These particles were added to a solution of ethanol which causes shrinkage of the microspheres (from >100 μm to <40 μm) due to extraction of the water from inside the microspheres. Alternatively, particles were also isolated directly from the microchannel by dropping into excess *n*-heptane and water [Figure 4(C)]. The particles remained discrete in excess water which proved that the CMCL reaction was effective in the microfluidic channel because stable hydrogel microspheres were

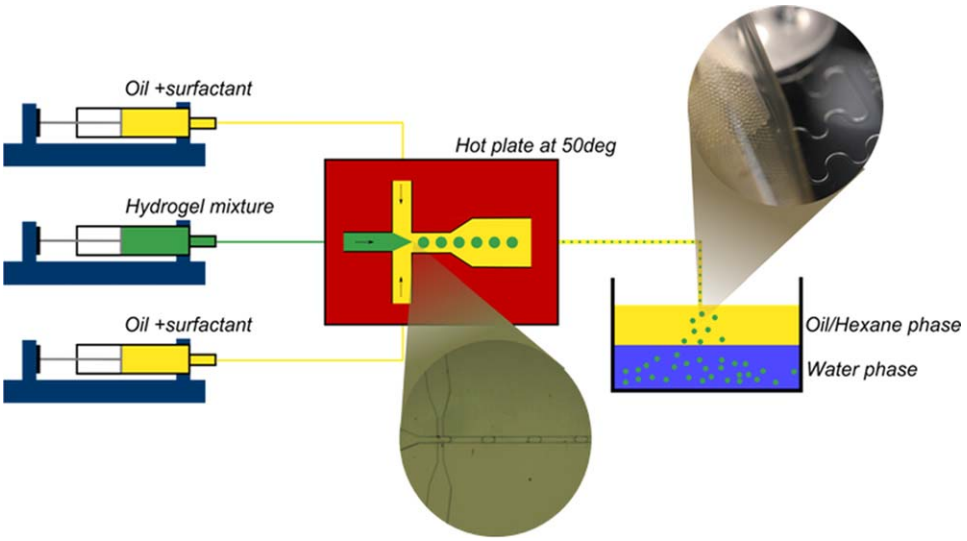



**Figure 3.** Schematic representation of the microfluidic system used for generating microspheres using a flow focusing device. At the nozzle, the height and width of the microchannel are 50 μm. The microchip is heated on a 50°C microscope stage and the resulting microspheres are collected in a 2 mL eppendorf tube containing an excess of water and *n*-heptane solution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

generated. The microfluidic synthesis using the CMCL reaction can be further optimized by changing the oil phase or injecting the activated polymer and crosslinker separately. We believe that the latter case will make a significant difference as the viscosity of the single fluid was continuously increasing during the injection which affected the droplet generation. However, these data provide a proof-of-concept study that could impact the water-in-oil microfluidic generation of PAM hydrogel microspheres. Overall, the process required minimal heating with no UV irradiation, free-radical reactions, or toxic monomers. This approach offers a flexible and gentle tool for the rational design of PAM-based microspheres using microfluidics.

**Inverse Suspension Preparation of Hydrogel Microspheres.** Microfluidics is a powerful technique although it cannot be used to rapidly generate large quantities of discrete hydrogel microspheres. Therefore, the CMCL reaction was applied to an inverse suspension to generate significant quantities of larger microspheres (600 μm). This was achieved by suspending an aqueous solution of activated PAM-*co*-AA in heptane with stirring. The suspension was maintained at 50°C to dissolve the surfactant and the droplets were then crosslinked using the CMCL reaction which converted the suspended droplets into discrete hydrogel microspheres. Significant quantities of larger microspheres were readily generated using this route. The polymer concentration in each case was 15% which was necessary for particle generation otherwise the particles agglomerated. Table II shows the properties of samples that were prepared with two different crosslinkers.

By changing the crosslinker, the resulting properties of the microspheres vary substantially. One of the key parameters that determines the performance of hydrogel microspheres in any application is particle size. To determine this for the microspheres they were isolated, however, it was challenging to filter the reaction solution directly because the surfactant is sparingly soluble in heptane at low temperatures which caused the filter to block. This was avoided by heating the filter to 60°C although the preferred method of isolation was to add the microspheres to ethanol. The hydrogels deswell in ethanol and

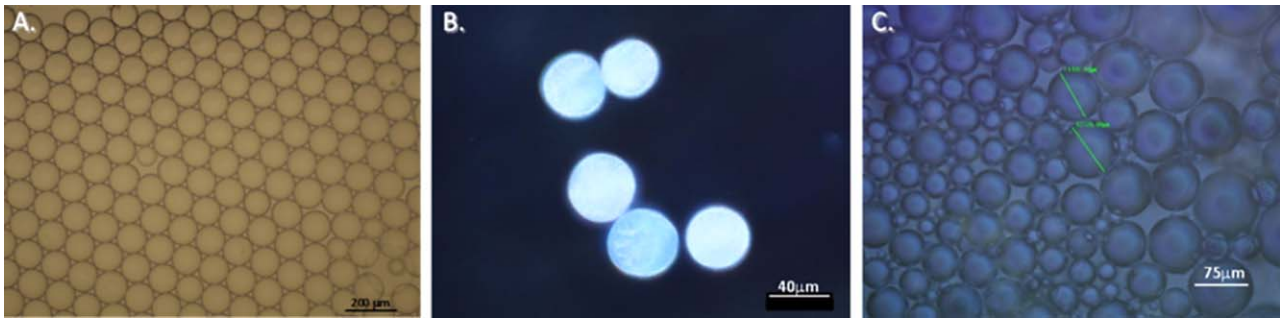



**Figure 4.** Microfluidic generation of (a) aqueous polymer phase in oil phase (scale bar 200 μm) prior to gelation, (b) microspheres isolated in ethanol to remove water (scale bar 40 μm), (c) hydrogel microspheres isolated in water/*n*-heptane (scale bar 75 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**Table II.** Resulting Properties of Hydrogel Microspheres Prepared Using the CMCL Reaction in an Inverse Suspension (IS) to Produce Hydrogel Microspheres Crosslinked with EDA or EDOBA

| Sample | Particle size (μm)[a] | Swelling[b] (g/g) | | | Gel time (s)[c] | Modulus (Pa)[d] |
|---|---|---|---|---|---|---|
| | | 60 min | 18 h | 96 h | | |
| IS-EDA | 231 (108) | 23.3 | 25.3 | 32.3 | 268.3 | 2169 |
| IS-EDOBA | 240 (59) | 39.9 | 64.4 | 163.0 | NA[e] | 567 |

[a] Determined from optical microscopy images of samples isolated in ethanol and dried, standard deviation in parentheses.
[b] Average water uptake of 1 g of dried microspheres measured over 60 min, 18 h, and 96 h—three cycles were used to determine the uptake, the standard deviation is ± 2.3 g of water per g of dry superabsorbent.
[c] Gel time was determined as the time taken for modulus to reach the maximum (Supporting Information Figure S6, measured at 25°C).
[d] Maximum modulus of bulk gels obtained (measured at 25°C).
[e] Short gel time, $G'$ and $G''$ intersected before loading into measuring geometry.

form rigid particles as the polymer collapses due to extraction of the water (Figure 5) so the overall particle size distribution remains constant with the average shifted to smaller sizes.

For suspension techniques, the size of the particles generated depends largely on the stirring speed and reactor geometry which determines the droplet size in the original suspension. Both of these parameters were constant throughout these experiments which explain why the average particle size for both sets of particles is similar (231 and 240 μm). However, the distribution of particle sizes is different which was attributed to the different rates of hydrogel formation within the suspended droplets. To estimate this, rheology experiments were carried out on the bulk gels because the kinetics within an individual droplet in a suspension matches the bulk gels. Supporting Information Figure S6 shows that the storage modulus for the EDOBA crosslinked system increased more rapidly and the maximum modulus was lower than the EDA system (567 Pa and 2169 Pa, respectively). These differences clearly affect how the droplets were generated and maintained which is reflected in the particle size distributions.

Microspheres were also isolated directly from the reaction vessel by filtering through a heated funnel which prevented the surfactant from precipitating. These particles were washed with heptane and photographs of the particles [Figure 6(B)] against a pixelated background show the spherical morphology. The particles are larger than the dried microspheres with an average particle size of 602 μm, which is representative of the particle size during the inverse suspension reaction.

The microspheres were placed in a dye solution to demonstrate that the particles could also be loaded with target cargoes. Figure 6(C) shows particles that were swollen three times in a solution of crystal violet for 2 days. The particles absorbed the dye and continued to swell to an average particle diameter of 993 μm. This demonstrates that the hydrogels can be loaded with target cargoes which is important when used for delivery applications (e.g., drug delivery). In this case, the dye was loaded into preformed microspheres and it could also be loaded simply by mixing with the polymer solution prior to crosslinking which did not affect the CMCL reaction (Supporting Information Figure S7).

Figure 6 shows that the particles could also swell to a large extent so the swelling ability of the microspheres was also investigated as a function of crosslinker type (Table II). It was found that shorter crosslinker molecules gave rise to microspheres that did not swell to the same degree (EDA swelling ratio 25.3 g/g) as those prepared with longer crosslinkers (i.e., EDOBA swelling ratio 39.9 g/g) over a 60-min period. In addition, the long term swelling capacity was also different with the system crosslinked with EDA reaching 32.3 g/g and the EDOBA system 163.0 g/g which is in line which with previous studies.[39] The longer and

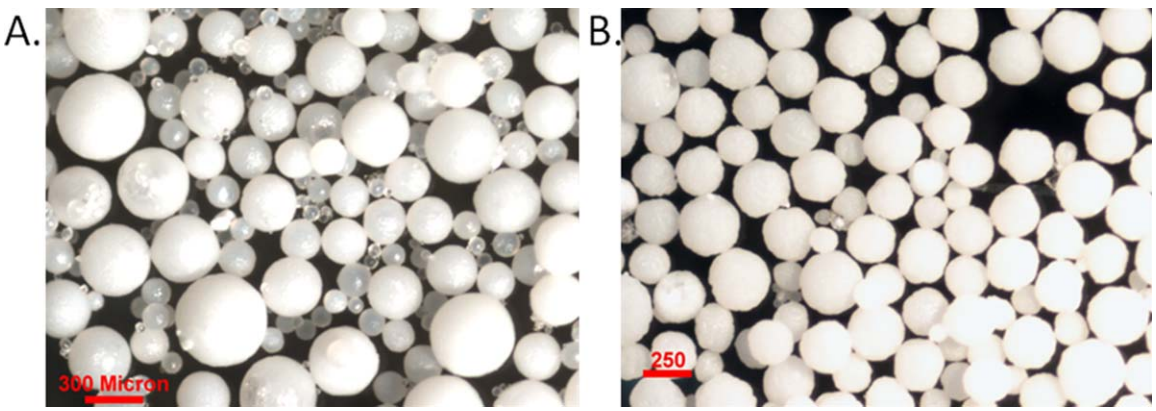



**Figure 5.** Optical microscopy images of (a) dry microspheres IS-EDA isolated from ethanol (scale bar 300 μm) [also shown in Figure 6(A) for comparison]. (b) Dry microspheres IS-EDOBA isolated from ethanol (scale bar 250 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

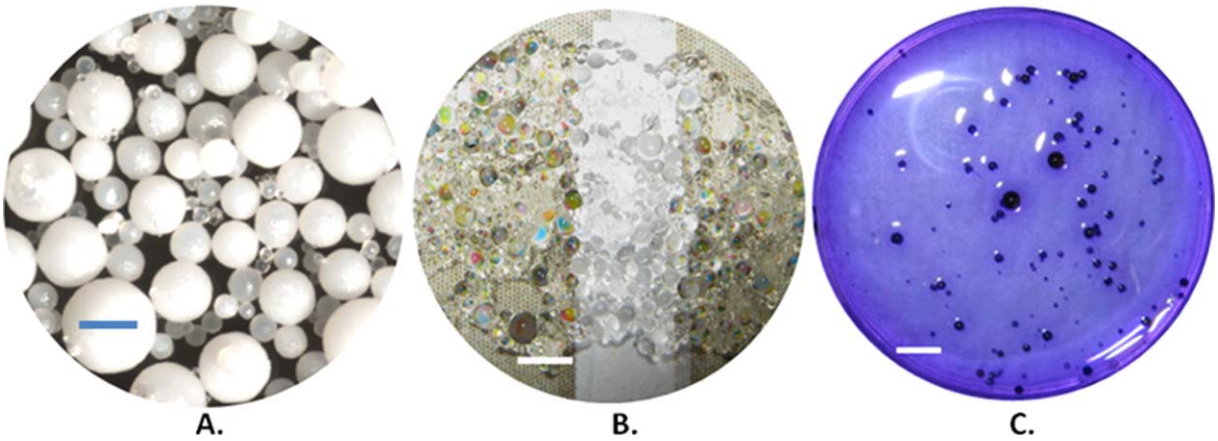



**Figure 6.** Optical images of (A) dry microspheres crosslinked with EDA isolated from ethanol (scale bar 300 μm) image matches Figure 5(A), (B) hydrogel microspheres isolated from the inverse suspension crosslinking reaction in a slightly swollen state (scale bar 2 mm), and (C) microspheres swollen in a 5 mg/L solution of crystal violet (scale bar 6 mm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

more hydrophilic crosslinker (EDOBA) generated materials that could swell to a higher degree. Further swelling studies were performed on four structurally different bulk gels (Supporting Information Table S3) to reinforce how the crosslinker structure could influence the properties of the materials. The shorter crosslinkers (EDA and TREN) produced materials that could swell to a lesser extent than those produced with longer crosslinkers ($C_{10}$ diamine and EDOBA). Again EDOBA generated materials that could swell to a higher degree. The swelling ability of the hydrogels is critical in all applications and in this case is influenced by the structure of the crosslinker. Structurally unique PAM-based hydrogel microspheres were also generated in an inverse suspension in significant quantities. The swelling ability and loading of the particles can also be tuned by changing the crosslinker or adding target cargoes.

## CONCLUSIONS

This study introduces a flexible radical free approach to generate arrays of structurally unique PAM-based hydrogels. The method avoids the use of acrylamide monomer and the side products are nontoxic, which represents a significant step forward in generating PAM-based hydrogels. In addition, no heating, UV irradiation, or free-radicals are required which is important for applications where sensitive cargos are loaded into the hydrogel. The structure, properties, and loading of the resulting gels can be modulated over a wide range by choosing from a vast array of commercially available starting materials. Hydrogels with high strength can be generated as well as materials with hydrophobic groups incorporated into the networks, both of which are a major challenge in synthesizing hydrogels. This study has broad reaching applications where PAM-based hydrogels are utilized as demonstrated through microfluidics and an inverse suspension technique.

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## REFERENCES

1. Mathur, A. M.; Moorjani, S. K.; Scranton, A. B., *J. Macromol. Sci. Rev. Macromol. Chem. Phys. C* **1996**, *36*, 405.
2. Dubrovskii, S. A.; Rakova, G. V., *Macromolecules* **1997**, *30*, 7478.
3. Zhang, J. P.; Li, A.; Wang, A. Q., *React. Funct. Polym.* **2006**, *66*, 747.
4. Raymond, S.; Wang, Y. J., *Anal. Biochem.* **1960**, *1*, 391.
5. Patel, S. K.; Rodriguez, F.; Cohen, C., *Polymer* **1989**, *30*, 2198.
6. Raymond, S.; Aurell, B.; Nakamichi, M., *Nature* **1962**, *195*, 697.
7. Andersen, F. A., *Int. J. Toxicol.* **2005**, *24*, 21.
8. Christensen, L. H., *Dermatol. Surg.* **2009**, *35*, 1612.
9. Hussain, M. D.; Rogers, J. A.; Mehvar, R.; Vudathala, G. K., *Drug Dev. Ind. Pharm.* **1999**, *25*, 265.
10. Sairam, M.; Babu, V. R.; Naidu, B. V. K.; Aminabhavi, T. M., *Int. J. Pharm.* **2006**, *320*, 131.
11. Gao, D.; Xu, H.; Philbert, M. A.; Kopelman, R., *Angew. Chem. Int. Ed. Engl.* **2007**, *46*, 2224.
12. Patton, J. N.; Palmer, A. F., *Biomacromolecules* **2005**, *6*, 2204.
13. Patton, J. N.; Palmer, A. F., *Langmuir* **2006**, *22*, 2212.
14. Patton, J. N.; Palmer, A. F., *Biomacromolecules* **2005**, *6*, 414.
15. Risbud, M.; Bhonde, R., *Drug. Deliv.* **2000**, *7*, 69.
16. Yang, T., Recent Patents on Materials Science; Bentham Science Publishers: **2008**; Vol. *1*, p 29.
17. Ferruzzi, G. G.; Pan, N.; Casey, W. H., *Soil Sci.* **2000**, *165*, 778.
18. Perez-Castillejos, R., *Mater. Today* **2010**, *13*, 32.
19. Li, W. W.; Li, H.; Liu, Z. F.; Qiao, Q., *Biomed. Environ. Sci.* **2009**, *22*, 28.
20. Caulfield, M. J.; Qiao, G. G.; Solomon, D. H., *Chem. Rev.* **2002**, *102*, 3067.

21. Rossow, T.; Heyman, J. A.; Ehrlicher, A. J.; Langhoff, A.; Weitz, D. A.; Haag, R.; Seiffert, S., *J. Am. Chem. Soc.* **2012**, *134*, 4983.
22. Park, S. N.; Park, J. C.; Kim, H. O.; Song, M. J.; Suh, H., *Biomaterials* **2002**, *23*, 1205.
23. Akagi, T.; Piyapakorn, P.; Akashi, M., *Langmuir* **2012**, *28*, 5249.
24. Suzuki, D.; Tsuji, S.; Kawaguchi, H., *J. Am. Chem. Soc.* **2007**, *129*, 8088.
25. Kong, H. J.; Kaigler, D.; Kim, K.; Mooney, D. J., *Biomacromolecules* **2004**, *5*, 1720.
26. Chhatbar, M. U.; Prasad, K.; Chejara, D. R.; Siddhanta, A. K., *Soft Matter* **2012**, *8*, 1837.
27. Serizawa, T.; Matsukuma, D.; Nanameki, K.; Uemura, M.; Kurusu, F.; Akashi, M., *Macromolecules* **2004**, *37*, 6531.
28. Kozlovskaya, V.; Kharlampieva, E.; Mansfield, M. L.; Sukhishvili, S. A., *Chem. Mater.* **2006**, *18*, 328.
29. Liang, H. C.; Chang, W. H.; Liang, H. F.; Lee, M. H.; Sung, H. W., *J. Appl. Polym. Sci.* **2004**, *91*, 4017.
30. Otani, Y.; Tabata, Y.; Ikada, Y., *Macromol. Symp.* **1998**, *130*, 169.
31. Lee, H.; Jeong, Y.; Park, T. G., *Biomacromolecules* **2007**, *8*, 3705.
32. Bodnar, M.; Daroczi, L.; Batta, G.; Bako, J.; Hartmann, J. F.; Borbely, J., *Colloid Polym. Sci.* **2009**, *287*, 991.
33. Rafat, M.; Li, F. F.; Fagerholm, P.; Lagali, N. S.; Watsky, M. A.; Munger, R.; Matsuura, T.; Griffith, M., *Biomaterials* **2008**, *29*, 3960.
34. Nam, K.; Kimura, T.; Kishida, A., *Biomaterials* **2007**, *28*, 1.
35. Bodnar, M.; Hartmann, J. F.; Borbely, J., *Biomacromolecules* **2006**, *7*, 3030.
36. Kunioka, M.; Furusawa, K., *J. Appl. Polym. Sci.* **1997**, *65*, 1889.
37. Murakami, S.; Aoki, N., *Biomacromolecules* **2006**, *7*, 2122.
38. Eiselt, P.; Lee, K. Y.; Mooney, D. J., *Macromolecules* **1999**, *32*, 5561.
39. Omidian, H.; Hashemi, S. A.; Sammes, P. G.; Meldrum, I., *Polymer* **1998**, *39*, 6697.
40. Valeur, E.; Bradley, M., *Chem. Soc. Rev.* **2009**, *38*, 606.
41. Debord, J. D.; Lyon, L. A., *Bioconjug. Chem.* **2007**, *18*, 601.
42. Appel, E. A.; Dyson, J.; del Barrio, J.; Walsh, Z.; Scherman, O. A., *Angew. Chem. Int. Ed. Engl.*, **2012**, *51*, 4185.
43. Greim, H.; Bury, D.; Klimisch, H. J.; Oeben-Negele, M.; Zeigler-Skylakakis, Z., *Chemosphere* **1998**, *36*, 271.
44. Abdurrahmanoglu, S.; Can, V.; Okay, O., *Polymer* **2009**, *50*, 5449.
45. Yang, B. D.; Lu, Y. C.; Luo, G. S., *Ind. Eng. Chem. Res. 51*, 9016.
46. Thiele, J.; Seiffert, S., *Lab Chip* **2011**, *11*, 3188.
47. Wan, J.; Bick, A.; Sullivan, M.; Stone, H. A., *Adv. Mater.* **2008**, *20*, 3314.
48. Kanai, T.; Lee, D.; Shum, H. C.; Weitz, D. A., *Small* **2010**, *6*, 807.
49. Kanai, T.; Lee, D.; Shum, H. C.; Shah, R. K.; Weitz, D. A., *Adv. Mater.* **2010**, *22*, 4998.
50. Shepherd, R. F.; Conrad, J. C.; Rhodes, S. K.; Link, D. R.; Marquez, M.; Weitz, D. A.; Lewis, J. A., *Langmuir* **2006**, *22*, 8618.
51. Chen, C. H.; Abate, A. R.; Lee, D. Y.; Terentjev, E. M.; Weitz, D. A., *Adv. Mater.* **2009**, *21*, 3201.
52. Kim, J. W.; Utada, A. S.; Fernandez-Nieves, A.; Hu, Z. B.; Weitz, D. A., *Angew. Chem. Int. Ed. Engl.*, **2007**, *46*, 1819.