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Nonvolatile copolymer compositions for fabricating gel element microarrays

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ABSTRACT

By modifying polymer compositions and cross-linking reagents, we have developed a simple yet effective manufacturing strategy for copolymerized three-dimensional gel element arrays. A new gel-forming monomer, 2-(hydroxyethyl) methacrylamide (HEMAA), was used. HEMAA possesses low volatility and improves the stability of copolymerized gel element arrays to on-chip thermal cycling procedures relative to previously used monomers. Probe immobilization efficiency within the new polymer was 55%, equivalent to that obtained with acrylamide (AA) and methacrylamide (MA) monomers. Nonspecific binding of single-stranded targets was equivalent for all monomers. Increasing cross-linker chain length improved hybridization kinetics and end-point signal intensities relative to *N,N*-methylenebisacrylamide (Bis). The new copolymer formulation was successfully applied to a model orthopox array. Because HEMAA greatly simplifies gel element array manufacture, we expect it (in combination with new cross-linkers described here) to find widespread application in microarray science.

Microarrays are widely used in scientific research and are now finding practical application in drug discovery, pathogen detection, and clinical diagnostics [1–7]. Typically, oligonucleotide or protein microarrays are manufactured on a two-dimensional surface such as glass, plastic, or polymeric films [8–11]. One of the limitations of two-dimensional arrays is the slow reaction kinetics and relatively low level of sensitivity, due in part to comparatively low immobilized probe density and probe proximity to the support surface that can affect the efficiency and kinetics of target capture [12–14]. For these reasons, three-dimensional microarrays using hydrogel matrices are attractive supports for nucleic acid and protein microarrays.

Gel element arrays formed via copolymerization [15,16] have proven to be effective in nucleic acid and protein applications, including on-chip enzymatic assays [17–20]. Copolymerized gel elements are formed by mixing biological probes with gel-forming reagents, depositing the mixture on a substrate, and simultaneously polymerizing the mixture to form the single gel elements. The resulting three-dimensional gel elements have a number of performance-enhancing properties relative to planar arrays, including higher probe immobilization capacity, sterically favorable spacing of immobilized molecules throughout the volume of the gel element, and an aqueous environment surrounding attached probes. The net result of these attributes is an increase in hybridization efficiency [21–23], with an early report claiming a 100-fold increase in detection sensitivity for gel element arrays compared with planar arrays [24].

The standard composition of copolymerized gel elements includes acrylamide (AA)[1] or methacrylamide (MA) as the gel-forming monomer and *N,N*-methylenebisacrylamide (Bis) as a cross-linking reagent [25–27]. A 5% AA gel is suitable for detecting and analyzing intact nucleic acid fragments of 100 to 150 nucleotides. However, efforts to increase the diffusion rate of large DNA fragments into gel elements by changing the pore size of the AA gel network lead to a decrease of gel mechanical stability, such that the resulting biochips are quite fragile and unsuitable for high-temperature experiments such as on-chip polymerase chain reaction (PCR) [20,25]. Several synthetic and naturally derived hydrogel polymer networks based on poly(ethylene) glycol, polypyrrol, chitosan, or polysaccharide have been proposed and tested over the past several years [28–31]. However, none of these compositions has proven to be effective in the range of microarray applications described above or made the transition to high-throughput manufacturing. Thus, there is still a need to develop gel compositions that are conducive

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[1] *Abbreviations used:* AA, acrylamide; MA, methacrylamide; Bis, *N,N*-methylenebisacrylamide; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; TR, Texas Red; TMR, tetramethyl rhodamine; NMR, nuclear magnetic resonance; NHS–MA, *N*-hydroxysuccinimide methacrylate; HEMAA, 2-(hydroxyethyl) methacrylamide; DMDAP, *N,N′*-dimethacryloyl-1,3-diaminopropan-2-ol; DMDAB, *N,N*-dimethacryloyl-1,4-diaminobutane-2,3-diol; RP, reverse-phase; UV, ultraviolet; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; SNR, signal-to-noise ratio; HEMA, 2-(hydroxyethyl) methacrylate; EDEMA, 2,2-(ethylenedioxy)bis (ethylenemethacrylamide).

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to high-throughput manufacture yet overcome some of the pore-size and thermostable limitations of AA or MA hydrogels used for microarray fabrication. The objective of this study, therefore, was to develop a new class of copolymers with enhanced thermomechanical stability and manufacturing properties relative to those based on AA or MA.

Materials and methods

AA, MA, Bis, methacryloyl chloride, sodium sulfate, glycerol, ethanolamine, 1,3-diamino-2-hydroxypropane, 1,3-butadiene diepoxide, 2,2′-(ethylenedioxy)bis(ethylamine), trimethoxysilylpropyl methacrylate, *N*-hydroxysuccinimide, Tween 20, methanole, triethylamine, acetonitrile, tetrahydrofuran, dichloromethane, chloroform, acetone, and ammonium hydroxide were obtained from Sigma–Aldrich Chemical (Milwaukee, WI, USA) and used without further purification. Sodium chloride, dibasic sodium phosphate, and monobasic sodium phosphate (monohydrate) were purchased from Fisher Scientific (Fair Lawn, NJ, USA), and 40% AA–Bis solution (19:1) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Ethanol was purchased from AAPER Alcohol and Chemicals (Shelbyville, KY), and 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0) and 20× SSPE (sodium chloride–sodium phosphate–EDTA) buffer solution were purchased from Ambion (Austin, TX, USA). Texas Red (TR) sulfonyl chloride (mixed isomers) and tetramethyl rhodamine (TMR)-5 (and -6) isothiocyanate (mixed isomers) were purchased from Molecular Probes (Eugene, OR, USA). Reagents for solid-phase oligonucleotide synthesis were purchased from Glen Research (Sterling, VA, USA). Acrylic glass slides for microarray fabrication were purchased from CEL Associates (Pearland, TX, USA). Mass spectra were recorded using a Bruker BiFlex III matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometer, and nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Advance 400-MHz NMR spectrometer (Bruker Daltonics, Billerica, MA, USA).

N-Hydroxysuccinimide methacrylate (NHS–MA)

Methacryloyl chloride (0.1 mol) in 25 ml of tetrahydrofuran was added dropwise with stirring and cooling on ice to a solution of *N*-hydroxysuccinimide (0.1 mol) and triethylamine (0.1 mol) in 50 ml of tetrahydrofuran. After 1 h of stirring at room temperature, the precipitate was removed and the filtrate was evaporated under vacuum. The residue obtained was crystallized from methanol to give NHS–MA with 75% yield and a melting point of 102 to 104 °C.

2-(Hydroxyethyl) methacrylamide (HEMAA)

A solution of NHS–MA (1.83 g, 10 mmol) in 25 ml of acetonitrile was added to a solution of ethanolamine (0.67 g, 11 mmol) in 50 ml of acetonitrile. The reaction mixture was stirred for 20 min at room temperature, filtered, and then concentrated under vacuum. The residue was purified by column chromatography on silica gel by applying a gradient of 0% to 5% acetone in dichloromethane. The final product was obtained with 81% yield (1.05 g). FAB–MS: 130.1 $(M+H)^+$. Calcd. for $C_6H_{11}NO_2$: MW 129.04. ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 6.54 (s, 1H, N**H**), 5.69 (s, 1H, $(H_3C)C{=}C\mathbf{H_a}H_b$), 5.31 (s, 1H, $(H_3C)C{=}CH_a\mathbf{H_b}$), 3.70 (t, *J* = 5.1 Hz, 2H, C$\mathbf{H_2}$OH), 3.43 (dt, *J* = 5.3 Hz, 5.1 Hz, 2H, NHC$\mathbf{H_2}$), and 3.33 (s, 1H, O**H**), 1.92 (s, 3H, C$\mathbf{H_3}$).

N,N′-Dimethacryloyl-1,3-diaminopropan-2-ol (DMDAP)

A mixture of 1,3-diamino-2-hydroxypropane (0.9 g, 10 mmol), NHS–MA (3.84 g, 21 mmol), and triethylamine (2.02 g, 20 mmol) was dissolved in 50 ml of acetonitrile and stirred at room temperature for 1.5 h. The solvent was removed under vacuum, and the crude material was partitioned between water and ether (1:2, 3 × 20 ml). Organic phases were combined, concentrated under vacuum, and purified by silica gel column chromatography by applying a gradient of 0% to 10% acetone in chloroform. The final product was obtained with 82% yield (1.87 g). EAB–MS: 228.1 $(M+2H)^+$. Calcd. for $C_{11}H_{18}N_2O_3$: MW 226.08.

N,N-Dimethacryloyl-1,4-diaminobutane-2,3-diol (DMDAB)

1,3-Butadiene diepoxide (4.3 g, 0.05 mol) was placed in a two-necked flask equipped with a condenser, dropping funnel, and magnetic stirrer. Ammonium hydroxide (15 ml of 32% water solution) was added, and the contents were stirred at room temperature. After 3 h, the reaction mixture was evaporated to dryness, and then the oil residue was coevaporated with ethanol. The product obtained was reconstituted in a solution of ethyl alcohol (150 ml), triethylamine (10.1 g, 0.1 mol), and methacrylic anhydride (16.0 g, 0.103 mol) and stirred at room temperature for 5 h. The solution was then concentrated to dryness under reduced pressure. Chloroform (200 ml) was added to the residue, and the resulting mixture was washed with water (50 ml), dried under sodium sulfate, concentrated under vacuum, and purified by silica gel column chromatography by applying a gradient of 0% to 10% methanol in chloroform to give DMDAB (as an oil) with a yield of 15% (3.9 g). FAB–MS: 252.1 $(M+H)^+$. Calcd. for $C_{12}H_{20}N_2O_4$: MW 256.08.

Synthesis of 3′-metacrylamido-modified oligonucleotides

Oligonucleotide synthesis was carried out on an ABI 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA) at a 0.5-μmol scale according to the manufacturer's recommended protocol and using commercial β-cyanoethyl phosphoramidites. Methacrylamido modifier CPG was used as a support for solid-phase oligonucleotide synthesis to prepare probes containing a methacrylic function at their 3′ ends. Cleavage from the solid support and deprotection of 3′-methacrylated oligonucleotides were accomplished with concentrated aqueous ammonium hydroxide for 8 to 12 h at 55 °C. 5′-DMTr-protected 3′-methacrylated oligonucleotides were purified by reverse-phase (RP)–HPLC. The 5′-DMTr-protective group was removed with 80% acetic acid (10 min), and 3′-methacrylated oligonucleotides were isolated by RP–HPLC. HPLC-purified oligonucleotides were evaporated to dryness (CentiVap concentrator, Labconco, Kansas City, MO, USA), reconstituted in 500 μl of Milli-Q water, and quantified by ultraviolet (UV) adsorption (UV/VIS [visible] Spectrophotometer Lambda Bio 10, PerkinElmer, Boston, MA, USA). Thereafter, oligonucleotides were normalized in Milli-Q water to a final concentration of 2 mM and stored at −4 °C until use. 5′-Cy-3-labeled and 3′-methacrylated oligonucleotides were synthesized by standard solid-phase phosphoramidite chemistry according to the manufacturer's recommendations using methacrylamido modifier CPG and commercial Cy3 phosphoramidite (Glen Research, Sterling, VA, USA) and were purified by reverse-phase HPLC after the deprotection procedure.

Preparation of copolymerization mixtures

Gel-forming monomers and cross-linking reagents used in this study are listed in Table 1. Standardized prepolymer mixtures contained gel-forming monomer (0.47 M), cross-linker reagent (0.0235 M), glycerol (65%, v/v), and 0.2 M sodium phosphate buffer (pH 7.2) and are modeled after recipes originally developed for 4% MA monomer. Aliquots of normalized oligonucleotide solutions were dried in vacuum and then redissolved in prepolymer mixtures to achieve a final concentration of 0.25 mM. The prepolymer solutions containing oligonucleotides were then placed in a