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Covalently controlled drug delivery via therapeutic methacrylic tissue adhesives

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Supporting Information

Supplemental Materials and Methods

Materials list with product numbers and origins

Methacryloyl chloride, 97% stabilized with 200 ppm 4-methoxyphenol (#L14511, Alfa Aesar, USA), methacrylic anhydride, 94% stabilized with 2,000 ppm topanol A as inhibitor (#276685, Aldrich Chemistry, USA), Acetaminophen, 98% (#A5000, Sigma Aldrich, USA), Benzocaine (#E1501, Sigma Life Sciences, USA), 4-methoxyphenol, ≥ 98.0% (MEHQ, #54050, Fluka, USA), triethylamine, ≥99.5% (TEA, #90335, Sigma Life Sciences, USA), dimethylaminopyridine (DMAP, #A13016, Alfa Aesar, USA), potassium carbonate (K_2CO_3, Alfa Aesar, USA), benzoyl peroxide (BPO, Sigma Aldrich), N,N-dimethyl-p-toluidene ≥ 98.0% (DMPT, #D0807, TCI America, USA), hydrochloric acid, ACS plus grade (HCl, #A144SI212, Fischer Scientific, USA), sodium bicarbonate ($NaHCO_3$, #S233-3, Fischer Scientific, USA), anhydrous magnesium sulfate ($MgSO_4$, #M7506, Sigma-Aldrich, USA) and Vetbond™ (BCA, #1469SB, 3M, USA) were used as received. Ibuprofen sodium salt (#I1892, Fluka Analytical, USA) was dried under vacuum at 90 °C for several hours prior to use and stored in a 120 °C oven between uses. Solvents including hexanes (#HX0299-5, EMD Millipore, USA), dichloromethane (DCM, #DX0831-1, EMD Millipore, USA), and chloroform ($CHCl_3$, #CX1058-1, EMD Millipore, USA) were dried over 3Å molecular sieves, degassed with argon, and passed through a column of activated neutral alumina to dry directly prior to use. $CHCl_3$ and DCM were purchased as HPLC-grade; hexanes was ACS-grade. NMR solvents chloroform-d (#DLM-7-100) and acetonitrile-d3 (#DLM-21-25) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Formaldehyde, 37% w/w aq. stabilized with methanol (#A16163, Alfa Aesar, USA), ammonium acetate, 98% (#A1542, Sigma, USA), acetoacetanilide, 98+% (#A17534, Alfa Aesar, USA), and dimethyl sulfoxide, ≥ 99.9% ACS reagent grade (DMSO, #472391, Sigma-Aldrich, USA) were used in the assay to determine formaldehyde release. Dulbecco's Modified Eagle Medium (#11995065, ThermoFisher Scientific), calf serum (#16010159, ThermoFisher Scientific), penicillin-streptomycin, 10,000 U/mL (#15140122, ThermoFisher Scientific), fetal bovine serum (#26140079, ThermoFisher Scientific), phosphate buffered saline (PBS, #10010049, ThermoFisher Scientific), Hoechst 33342 (#62249, ThermoFisher Scientific), calcein AM (#PK-CA707-80011-2, PromoKine), ethidium homodimer-1 (#L3224, ThermoFisher Scientific) were used in cell culture.

Synthesis

Acetaminophen Methacrylate (AceMA). Briefly: a flame-dried 50 mL roundbottom flask under argon was charged with dry chloroform (25 mL). Acetaminophen (1.51g, 10 mmol) and DMAP (0.098g) were added in one shot. TEA (1.66 mL, 12 mmol) was added by syringe, and the flask placed on ice. Methacrylic anhydride (1.49 mL, 10 mmol) was added dropwise by syringe over several minutes. The reaction was then allowed to warm to room temperature and stirred overnight before being washed with 3N HCl (4 x 25 mL) followed by saturated $NaHCO_3$ (1 x 25 mL). The organic layer was dried over $MgSO_4$ for several hours before being filtered. The solvent was removed under vacuum to yield a white powdery solid.

Covalent Controlled Release

20 mL scintillation vials were pre-washed (Section 2.5.1), then dried and pre-heated to 37 °C on a hot plate equipped with a thermocouple. 20 µL of an adhesive formulation was applied in the center bottom of each vial. Vials

were capped and sealed; adhesives were cured for 24h at 37 °C in a non-sterile incubator. At time t = 0 h, 7 mL of either pH 4.9 sodium acetate buffer or pH 7 deionized water was added to each vial to submerge the adhesive. At each time point, 200 µL of the supernatant was removed from each vial and placed in a Greiner UV-clear 96 well plate for analysis. Absorbance at 264 nm for IbuMA, 300 nm for AceMA and BenzMA was used to quantify the amount of drug released from each adhesive. In the case of IbuMA, since ibuprofen and BPO degradation products both absorb at 264 nm but ibuprofen does not absorb at 300 nm, absorbance from BPO degradation products was measured at 300 nm and used to correct the percent release of ibuprofen calculated for IbuMA. Each adhesive was tested in triplicate, i.e., three vials were prepared for each adhesive per pH condition. One measurement was taken from each vial for each time point. Vials were sealed thoroughly between measurements to prevent evaporation.

Quantification of formaldehyde release from cured adhesives

Sample preparation and collection of aliquots. 20 mL scintillation vials were pre-washed, then dried and pre-heated to 37 °C on a hot plate equipped with a thermocouple. 20 µL of an adhesive formulation was applied in the center bottom of each vial. Vials were capped and sealed; adhesives were cured for 24h at 37 °C in a non-sterile incubator. One vial was prepared for each adhesive. At time t = 0 h, 2 mL of deionized, degassed water was added to each vial to submerge the adhesive. At each time point, four 50 µL aliquots of the supernatant were removed from each vial and placed in a Greiner UV-clear 96 well plate for analysis.

Assay for the quantification of formaldehyde. Solutions of formaldehyde in water (eight standards ranging in concentration from 0 to 300 µM), ammonium acetate in water (1 M), and acetoacetanilide in a 1:1 DMSO water mixture (0.6 mM) were prepared fresh on the day each time point was assayed. A Costar polystyrene 96-well plate was divided into three sections: “Standard” wells, “Sample” wells, and “Sample Background” wells. Standard wells were loaded with 40 µL ammonium acetate solution and 50 µL of a formaldehyde standard of known concentration. Standards were performed in duplicate. Sample wells were loaded with 40 µL ammonium acetate and 50 µL of sample supernatant (described in S1.4.1). Sample Background wells were loaded with 40 µL ammonium acetate, 50 µL of sample supernatant, and 20 µL of deionized water. One well per sample was dedicated to sample background.

The reaction (which creates the fluorescent species) was then initiated by adding 20 µL of acetoacetanilide solution to every well except Sample Background wells. The well plate was then covered and placed in the dark to incubate at room temperature. After exactly 15 minutes from the addition of acetoacetanilide, the fluorescence intensity of each well was measured with a Tecan Safire^{2} ™ plate reader by exciting samples at 370/20 nm and reading emission at 470/20 nm.

Cell Culture

Cytotoxicity. Lactate dehydrogenase (LDH) is a cytosolic enzyme that becomes released into the cellular media due to plasma membrane damage and is routinely used to determine cytotoxicity. LDH release was assessed using a commercially available assay kit (Pierce LDH Cytotoxicity Assay Kit, #88953, ThermoFisher Scientific). The LDH assay was run according to manufacturer’s recommendations. Briefly, 50 µL of cell culture media was added to 50 µL of reaction mixture in a new 96-well plate and was allowed to incubate for 30 min at room temperature protected from

light. Then, 50 µL of stop solution was added to each well, the plate was centrifuged at 1,000×g for 1 min to break up any bubbles, and then the absorbance was acquired from 300–800 nm with a 10 nm step size. The background absorbance at 680 nm was subtracted from the signal at 490 nm. Data is reported as %, as calculated by dividing the experimental well absorbance minus the spontaneous LDH release control (addition of deionized water) by the maximum LDH activity control (lysis of cells) minus the spontaneous LDH release control multiplied by 100%.

To further quantify cytotoxicity, from the same cell culture wells another 50 µL of cell culture media from each was aspirated and added in a new 96-well plate to 100 µL of staining solution containing 20 µM of Hoechst 33342 and 1.5 µM of propidium iodide. The samples incubated for 30 min; then, the fluorescence was assessed using a fluorescence microplate reader. Data is presented as feature scaled to the positive and negative controls for the LDH assay.

Bulk Mechanical Properties

Rheology. Elastic moduli were determined using a TA Instruments Discovery HR-2 Rheometer, with a disposable plate geometry loaded with 8 mm aluminum plates outfitted with an environmental test chamber for temperature control. A total of three drops of adhesive were applied to the plates. Adhesives were spread thin to cover each plate so that mixing would be even, and the geometry was programmed to oscillate as the gap was set. Vetbond™-only controls were prepared by depositing Vetbond™ on the lower plate only. IbuMA-BCA adhesives were prepared by depositing an IbuMA-DMPT mixture on the upper plate and a Vetbond™-BPO mixture on the lower plate. AceMA-BCA and BenzMA-BCA adhesives were prepared by applying DMPT to the upper plate and a TMA-Vetbond™-BPO mixture to the lower plate. Data was recorded as soon as the gap was set and the sample quickly trimmed. Temperature was ramped from room temperature up to 37 °C. Storage and loss moduli were monitored at 1 Hz and 1% strain until the storage modulus superseded the loss modulus and both moduli had stabilized, approximately 35 minutes. Elastic moduli were then determined through a frequency sweep experiment (0.1 Hz – 100 Hz) performed at 37 °C. The elastic modulus was taken as the average of the storage modulus at 1 Hz for at least three trials. In all cases the gap was set to approximately 500 µm, in order to consistently assess the curing behavior and mechanical properties of a thin layer of adhesive.

Procedural details for the preparation of aluminum lap shear samples. Lap shear strength of adhesives was assessed using an Instron Tensile Tester with a 50 kN load cell. Samples were prepared in accordance with ASTM D1002-10.

Aluminum substrates 0.2 cm thick were trimmed to 2.5 x 10 cm strips, washed with isopropyl alcohol, and pre-treated (Section 2.5.1). Substrates were then dried and pre-heated to 37 °C on a hot plate equipped with a thermocouple, and arranged with the upper and lower substrates apposed along the 2.5 cm side. For the IbuMA-BCA adhesive, a mixture of IbuMA and DMPT was applied to the 'upper' substrates, and a mixture of Vetbond™ (BCA) and BPO was applied to the 'lower' substrates. For AceMA- and BenzMA-BCA adhesives, DMPT was applied to 'upper' substrates, and a mixture of TMA, Vetbond™, and BPO was applied to 'lower' substrates. Each upper

substrate was then immediately flipped and overlapped with a lower substrate by 2.5 cm. A weight was applied, and the substrates were allowed to cure overnight at 37 °C.

Preparation of porcine tissue samples for ex vivo wound closure ability. Ability of TMA-BCA adhesives to adhere apposed sections of porcine cutaneous tissue under simulated biological conditions was tested according to ASTM F2458 - 05(2015), modeling wound closure.

Freshly harvested porcine skin was washed with isopropyl alcohol and fresh phosphate buffered saline (PBS, #10010049, ThermoFisher Scientific), shaved, trimmed of subcutaneous fat and muscle, and cut into strips measuring 2.5 cm x 10 cm x 0.2 cm. At all times during preparation and experimentation, tissue was kept moist with PBS solution containing 5% v/v penicillin-streptomycin that had an initial concentration of 10,000 U/mL (#15140122, ThermoFisher Scientific).

Skin substrates (wrapped in PBS-soaked gauze and sealed in plastic bags) were primed by warming to 37 °C in a water bath for at least 15 minutes before use. To prepare one specimen, two strips of skin were removed from the water bath, blotted with sterile gauze, and placed on a hotplate equipped with a thermocouple set to 37 °C. The two strips were apposed along one 2.5 cm side. Except in Vetbond™ (- BPO, DMPT) controls, the "wound area" around the apposed ends of the strips was blotted lightly with DMPT. Adhesive was then applied according to the Vetbond™ manufacturer's instructions, in a swirling motion across the wound area and in several layers. Samples were misted with PBS and allowed to cure for ten minutes on the warm hotplate surface before being braced, wrapped in gauze, and repackaged into sealed bags containing PBS. Specimens were placed back in the warm water bath to cure for another two hours prior to mechanical testing.

Five specimens were prepared for each adhesive. Precise length, width, and thickness of each specimen's adhesive area were measured with calipers and recorded prior to testing. Total specimen length between grips was measured after samples were loaded into the instrument. Mechanical properties of prepared specimens were assessed using an Instron Tensile Tester with a 50 kN load cell. The force was measured as the crosshead was raised at a rate of 30 mm/min until adhesive failure occurred.

Supplemental figures

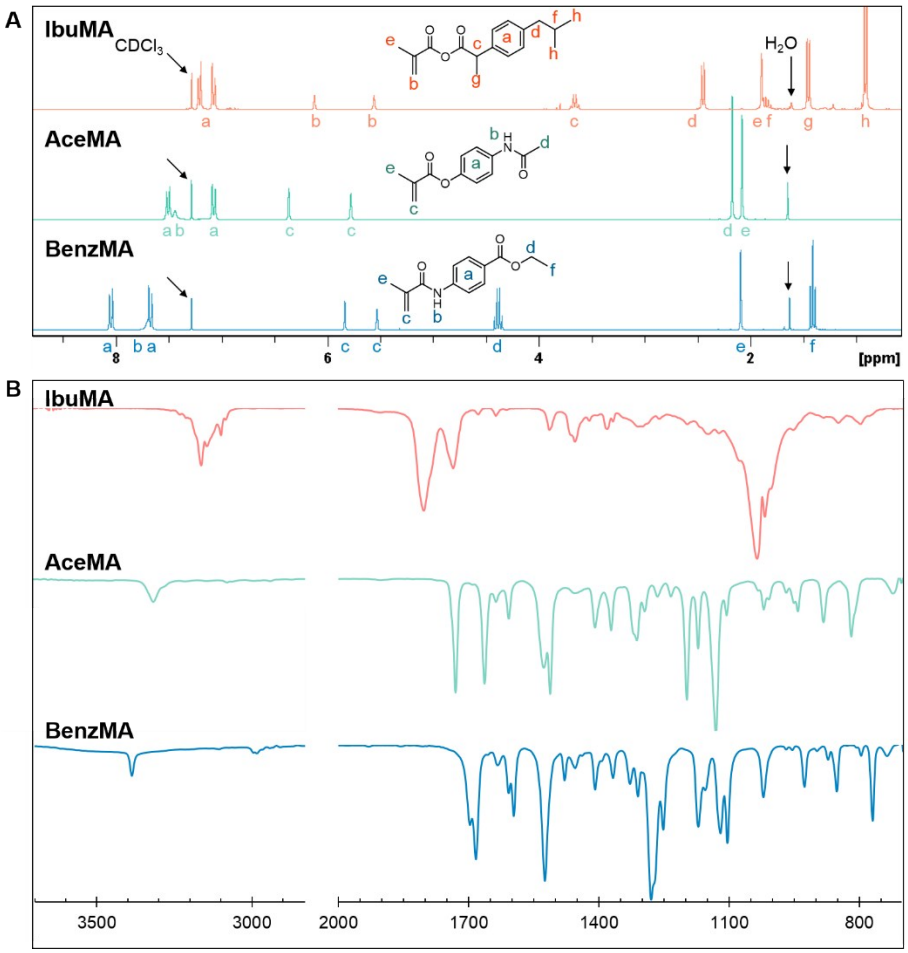

Figure S1. A) ^{1}H NMR (300 MHz) of TMA monomers in $CDCl_3$. Arrows indicate solvent peaks. B) FT-IR spectra of TMA monomers acquired neat using a Germanium ATR crystal.

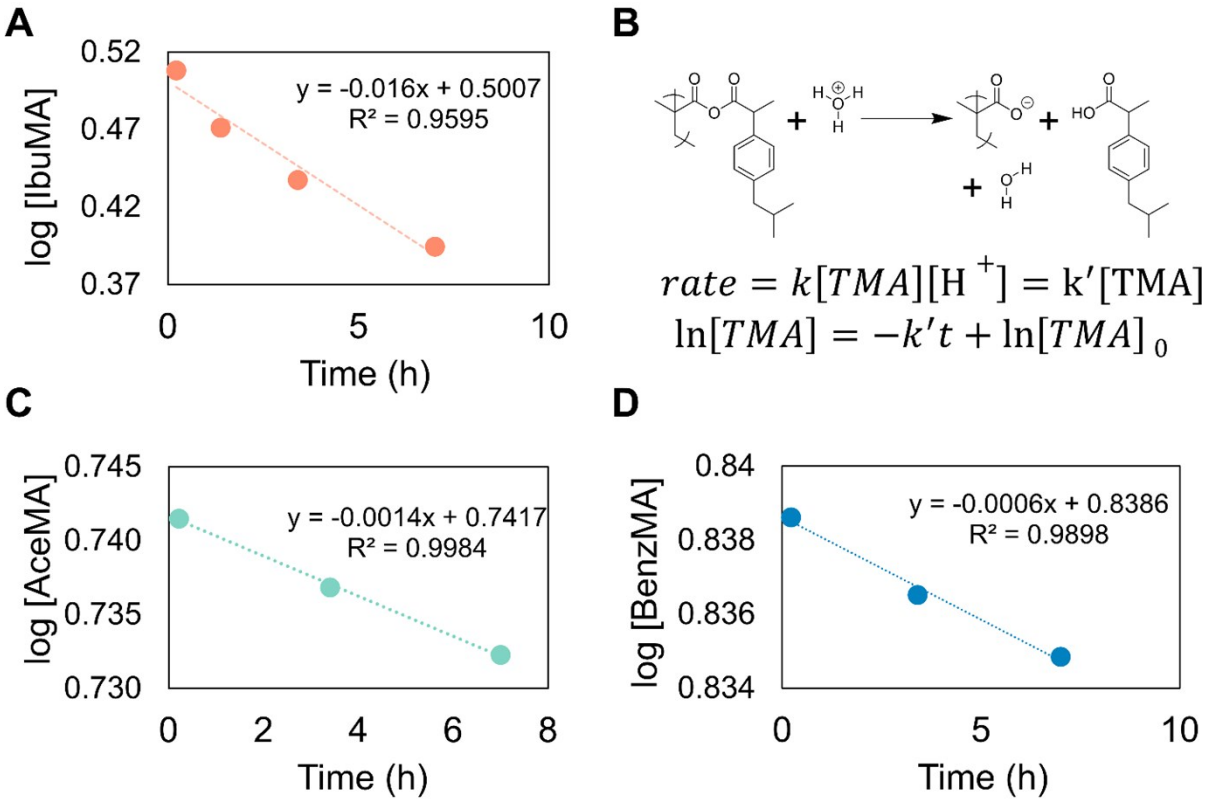

Figure S2. Plots of log [TMA] versus time for A) IbuMA, C) AceMA, and D) BenzMA used to determine the rate constants of hydrolysis/ therapeutic release from TMA-BCA adhesives in sodium acetate buffer (pH 4.9). Unhydrolyzed TMA ([TMA]) was calculated from the concentration of free drug and the mass of TMA monomer applied in the adhesive. A pseudo-first order reaction rate was assumed in all cases because the acidic buffer provides a constant excess of protons. B) A representative hydrolysis reaction scheme and the corresponding pseudo-first order rate equation (above) and integrated rate law (below) is shown for IbuMA-BCA.

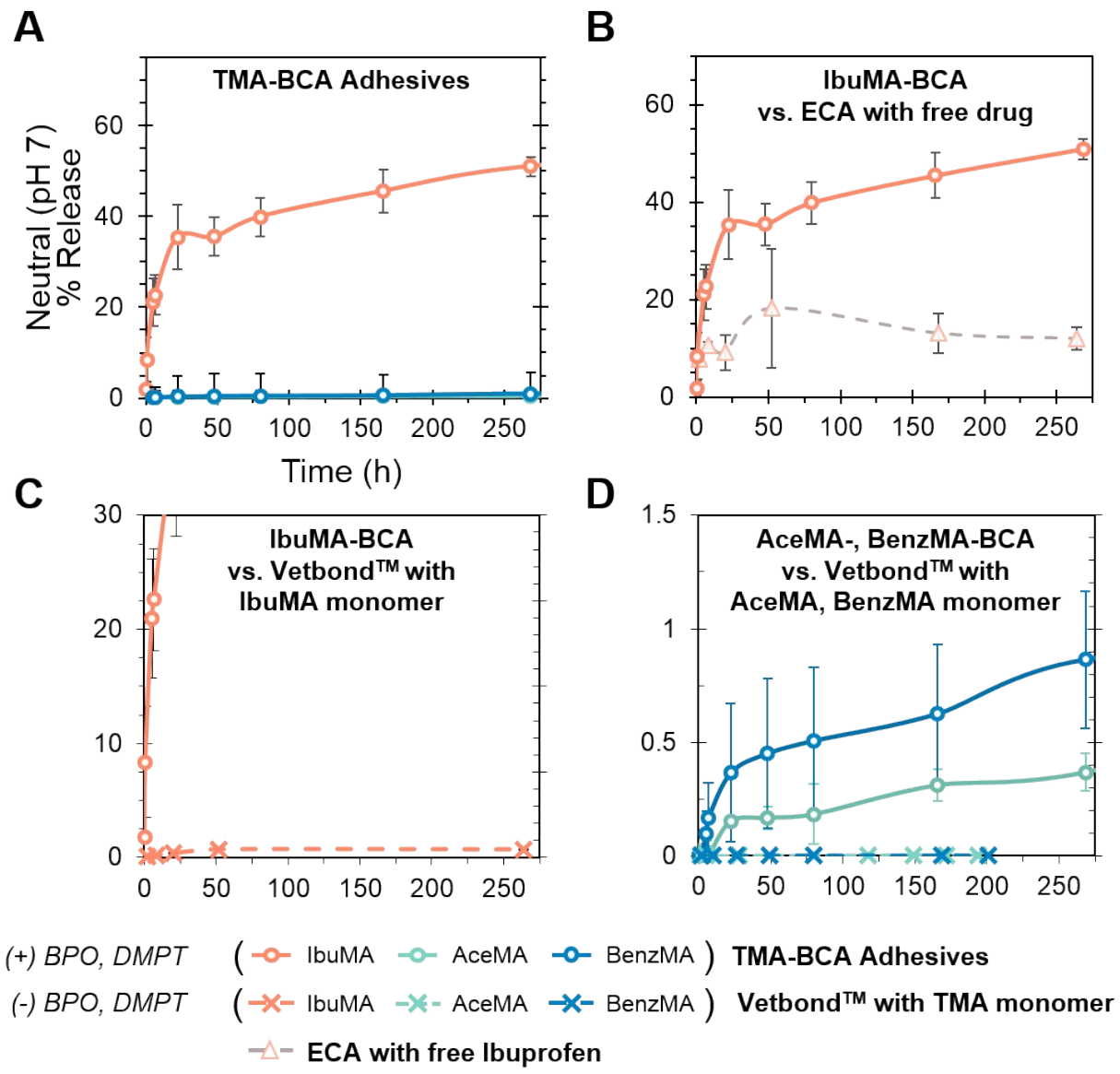

Figure S3. Comparison of therapeutic release from TMA-BCA adhesives cured using the BPO-DMPT radical initiator-accelerator system with B) release of free ibuprofen from an ethyl cyanoacrylate (ECA) matrix (- BPO, DMPT), and C, D) release of therapeutics and/or TMA monomer (which are not directly distinguishable by ultraviolet-visible light absorption) from a Vetbond™ (BCA) matrix (- BPO, DMPT). A) shows therapeutic release from TMA-BCA adhesives (+ BPO, DMPT) as presented in the main text in Figure 1, rescaled for ease of comparison with B – D. All experiments were performed in pH 7 deionized water.

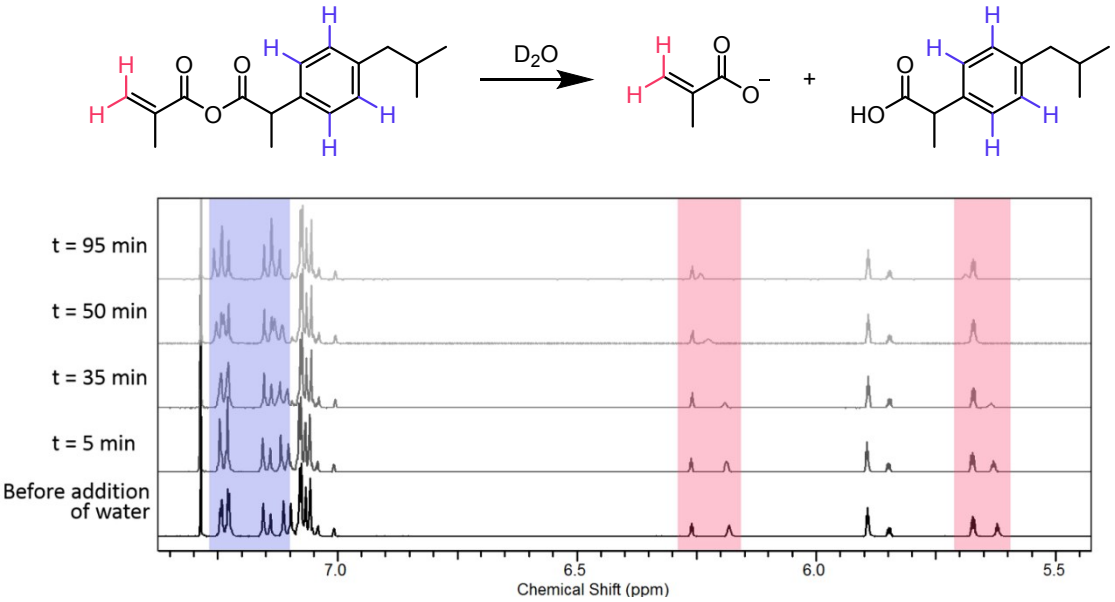

Figure S4. ^{1}H NMR (500 MHz) observation of reaction of oligo-IbuMA (dissolved in 90 µL $CDCl_3$) with D_2O (10 µL). "Oligo-IbuMA" represents the mixture of IbuMA monomer, short-chained IbuMA oligomers containing a variety of end groups, and partially hydrolyzed IbuMA that is obtained upon exposure of IbuMA monomer to moist/ ambient air. Over time following exposure to D_2O, the multiplets describing the mixture of IbuMA derivatives at t = 0 are seen to converge, especially in the aromatic (blue) and vinylic (red) regions, suggesting a homogenization of the IbuMA mixture through hydrolysis of the ibuprofen side group.

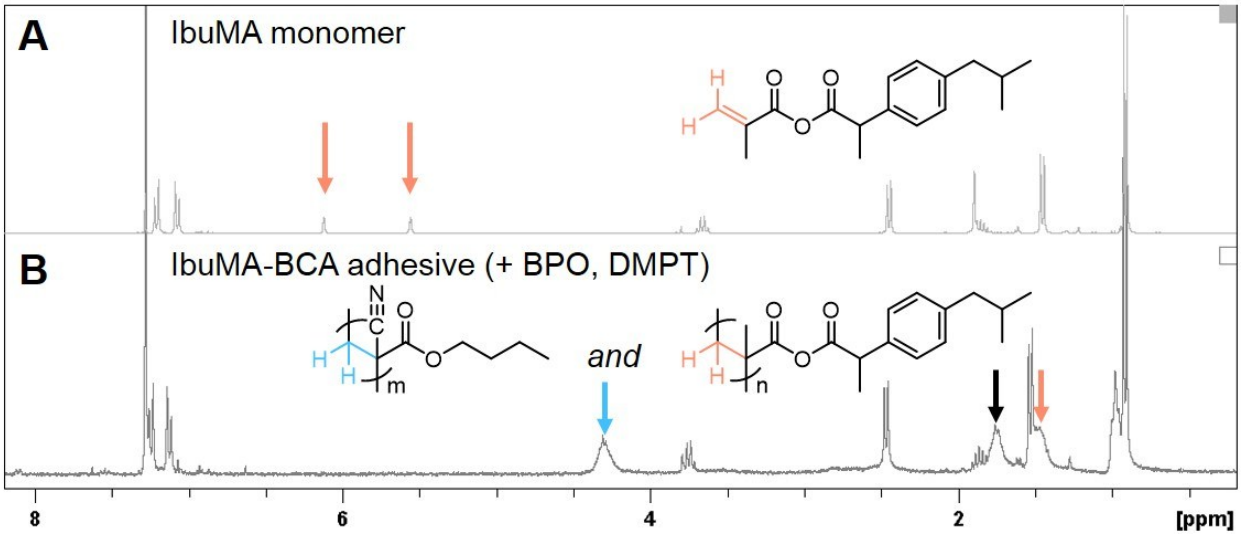

Figure S5. Comparison of ^{1}H NMR spectra (300 MHz, $CDCl_3$) of A) IbuMA monomer and B) cured IbuMA-BCA adhesive. (A) displays two signals (arrows at 5.57 and 6.14 ppm) that correspond to the vinyl protons of the IbuMA monomer (highlighted orange), which is typical of acrylic-type monomers including cyanoacrylates and methacrylates. Vinyl peaks are not present in (B), indicating polymerization has occurred to completion. Instead, several broad peaks are

visible, which are attributed to the same two protons when present in repeat units of polymers as opposed to vinyl-containing monomers. Signals labelled with blue, black, and orange arrows are attributed to protons neighbored only by cyanoacrylate repeat units, a mixture of cyanoacrylate and IbuMA units, and only IbuMA units, respectively. These signals suggest that copolymerization of BCA and IbuMA does occur during curing of the IbuMA-BCA adhesive.

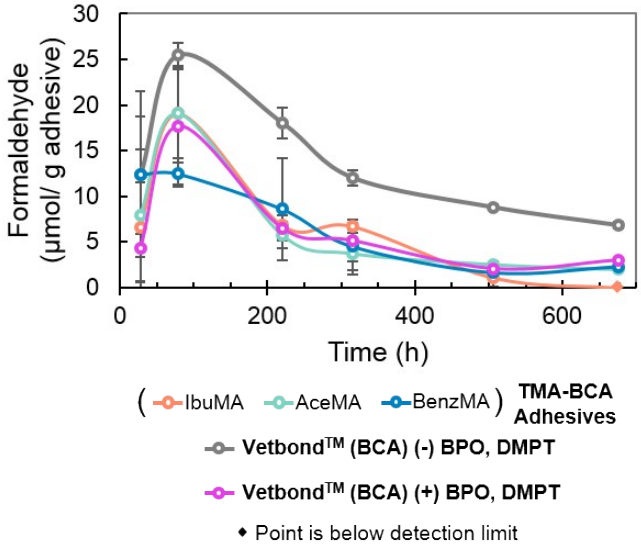

Figure S6. Concentration of formaldehyde that is present in the aqueous supernatant above cured adhesives that have been submerged in submerged in deionized, degassed water over the course of four weeks (675.5 h), normalized per gram of adhesive, detected via fluorometric assay utilizing the reaction between formaldehyde, acetoacetanlilide, and ammonia that produces fluorescence (excitation 370/20 nm, emission 470/20 nm), including the radically-cured Vetbond™ (+) BPO, DMPT. Vetbond™ (+) BPO, DMPT shows a release profile similar to TMA-BCA adhesives, suggesting the radical polymerization mechanism of curing plays a role in reducing formaldehyde release from TMA-BCA adhesives compared to the anionically cured Vetbond™ (-) BPO, DMPT.

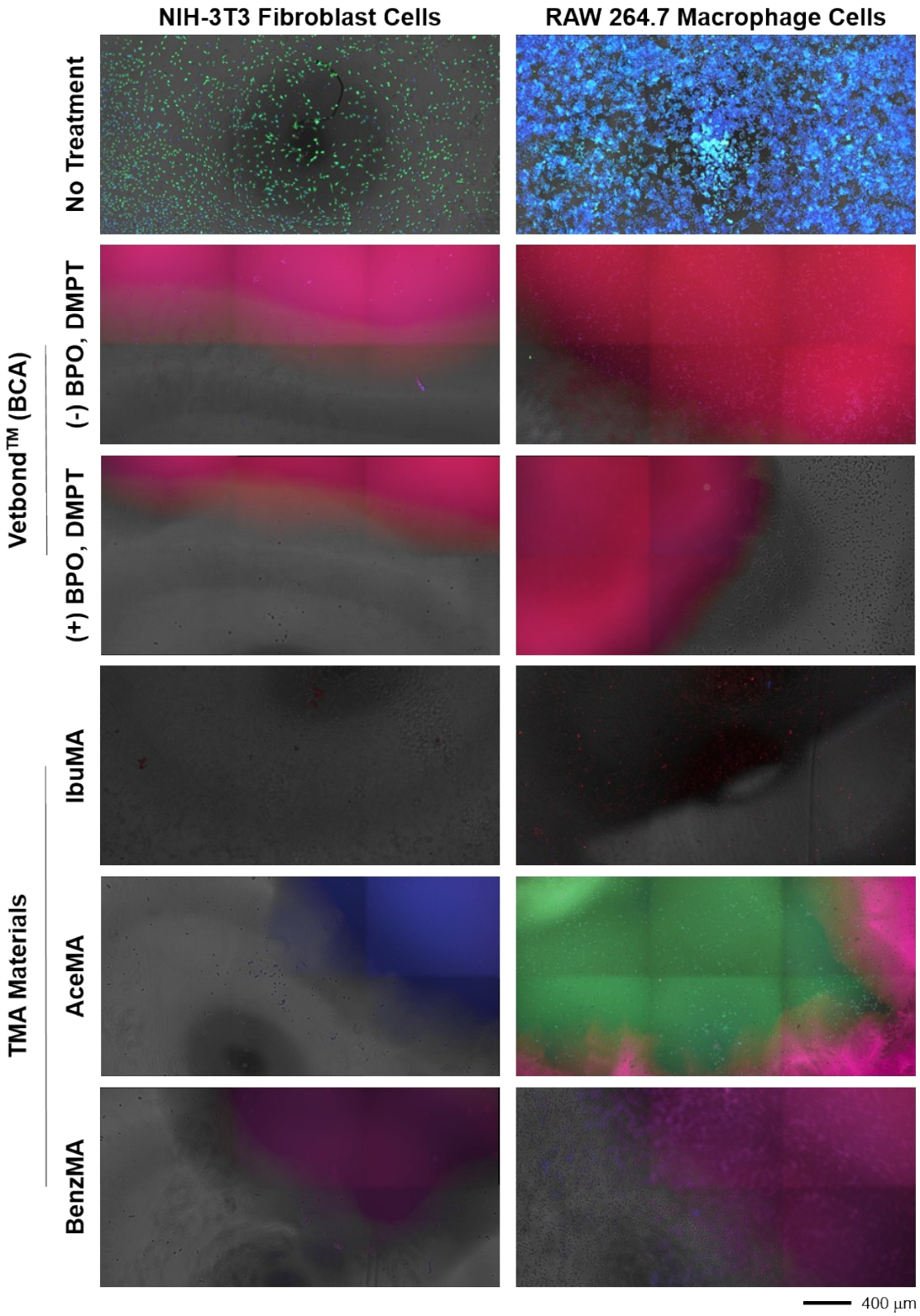

Figure S7. Fluorescence images showing that adhesives resulted in background fluorescence that prohibited an accurate determination of cellular vitality using fluorescence assays. After 24 h of exposure to adhesives that were

polymerized in the cell culture media, the cells were labeled with Hoechst 33342 to label cellular nuclei, Calcein AM to label actively metabolizing cells, and ethidium homodimer-1 to label the nuclei of dead cells. Fluorescence from live, healthy cells is clearly identified for cells receiving no treatment (no adhesive), but for cells exposed to adhesives, there is substantial background from the adhesive itself prohibiting quantification through imaging or plate reader analysis.

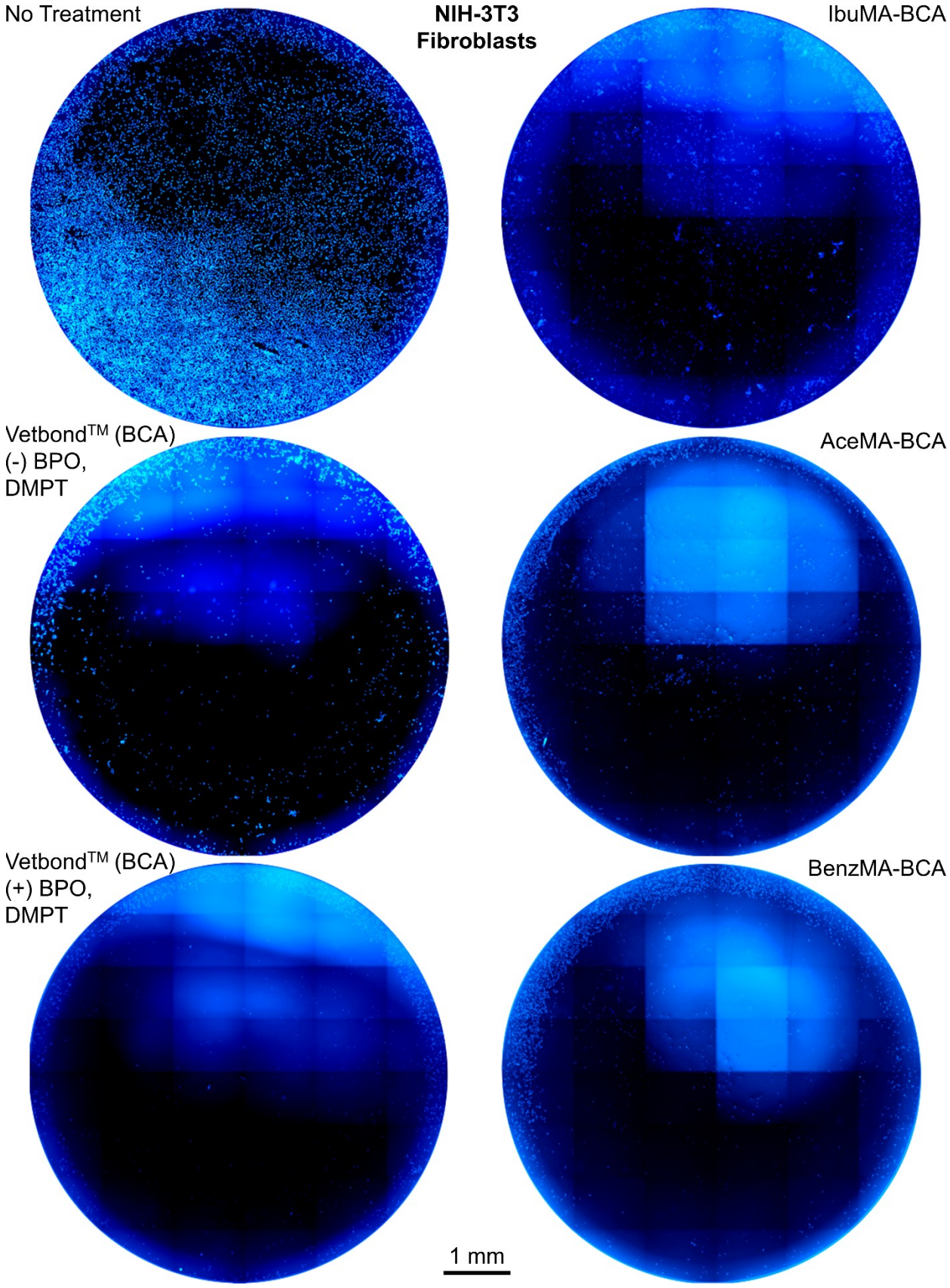
No Treatment
NIH-3T3
Fibroblasts
IbuMA-BCA
Vetbond™ (BCA)
(-) BPO,
DMPT
AceMA-BCA
Vetbond™ (BCA)
(+) BPO,
DMPT
BenzMA-BCA
1 mm

Figure S8. Multiple fields of view of NIH-3T3 fibroblast nuclei fluorescently labeled with Hoechst 33342 were automatically acquired and concatenated into an image from the whole well. Background signal from the adhesives is observed, but small, punctate dots that are cell nuclei can be observed, enabling a determination of % confluency.

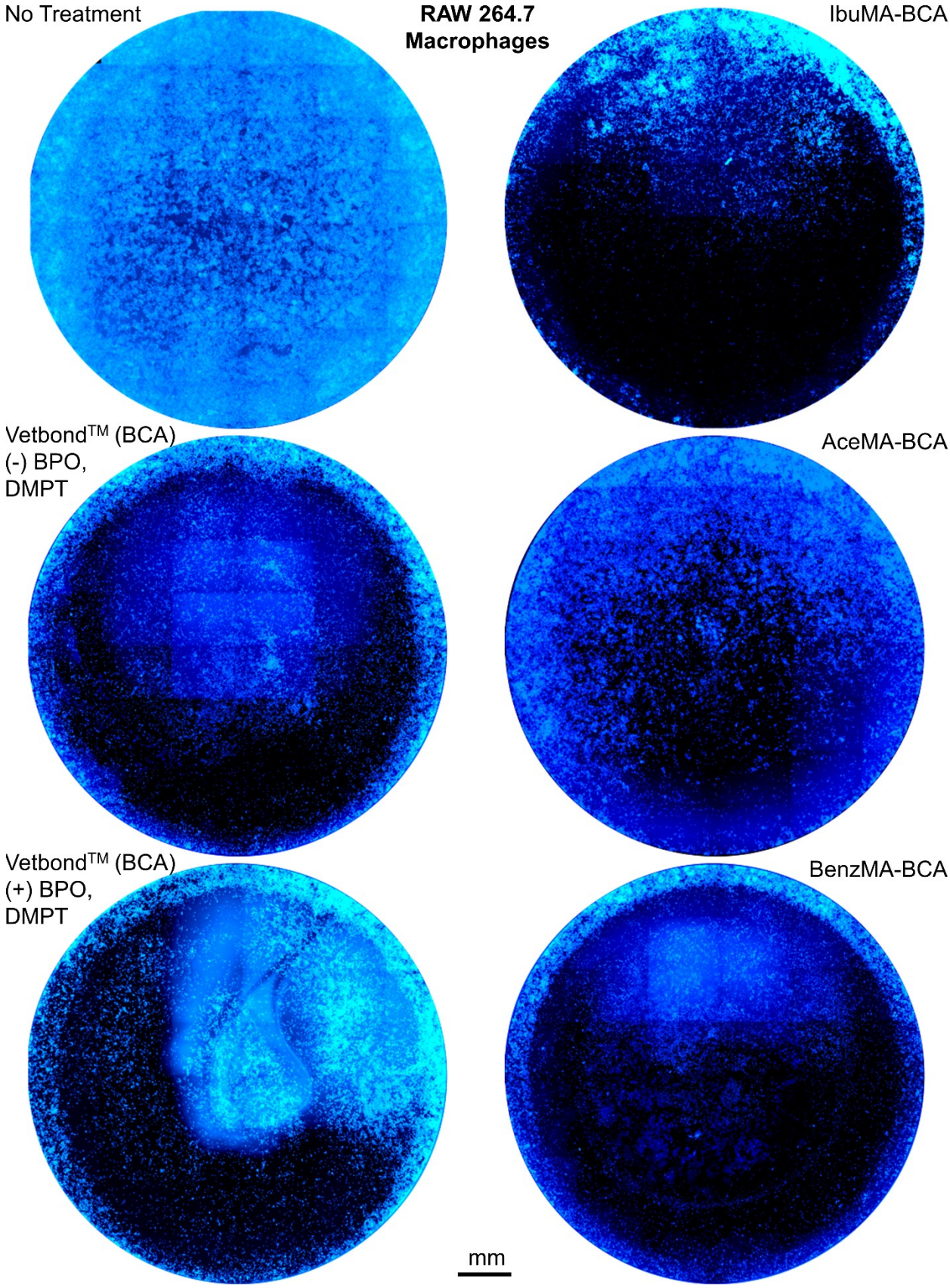
No Treatment
RAW 264.7
Macrophages
IbuMA-BCA
Vetbond™ (BCA)
(-) BPO,
DMPT
AceMA-BCA
Vetbond™ (BCA)
(+) BPO,
DMPT
BenzMA-BCA
mm

Figure S9. Multiple fields of view of RAW 264.7 macrophage nuclei fluorescently labeled with Hoechst 33342 were automatically acquired and concatenated into an image from the whole well. Background signal from the adhesives is observed, but small, punctate dots that are cell nuclei can be observed, enabling a determination of % confluency.