A novel biocompatible adhesive incorporating plant-derived monomers

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Abstract: We describe a new class of biomaterials with potential for a variety of applications in tissue engineering, wound healing, and transdermal drug delivery. These materials are based on oleic methyl ester (OME), which is derived from various plant oils including soybean oil. The OME was acrylated (AOME) and subsequently copolymerized with methyl methacrylate (MMA) and ethylene glycol dimethacrylate (EGDMA) to form pressure sensitive adhesives (PSAs). We assessed the cytocompatibility of each PSA product using Alamar Blue and Live/Dead assays. It was found that after 2 h, human fibroblast cells attached on all four of the PSA polymers tested. After 24 h, cell spreading was seen on all materials with the exception of the polymerized AOME product (PAOME). Cells attached to the copolymer PSA products continued to proliferate for up to 2 weeks, as shown by fluorescent confocal microscopy imaging. Finally, a mechanical analysis of each of the copolymers is presented demonstrating that they have a range of mechanical properties and cell adhesiveness depending on the formulation, making them attractive candidates for use as bioactive adhesives. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 91A: 378–384, 2009

Key words: copolymer; adhesive; fibroblasts; cytotoxocity

INTRODUCTION

Polyacrylates have long been used as adhesive materials in biomedicine as tapes, and dressings and as components of other medical devices. More recently, polyacrylates have been used in transdermal drug delivery devices as pressure sensitive adhesives,[1–3] drug-polymer matrices, or both because their relative biocompatibility and good adhesion to skin. In transdermal drug delivery applications, it is important that the matrix polymer and the adhesive not affect the drug in any adverse manner. It is also important that the incorporation of the drug and any necessary additives, such as skin penetration enhancers, do not negatively affect the mechanical properties of the polymer.[4] As a result, acrylate copolymers are commonly used and the cohesive and adhesive properties are tailored to a particular drug formulation and enhancer content. Comonomers are typically alkyl acrylates or methacrylates that are added in order to make the copolymer more flexible or improve "tack," or the adhesion to skin at short times and small pressures.[2] In addition, some polyacrylates have been shown to have permeation enhancement properties, making them even more attractive for this application.[5]

Here, we discuss a flexible new class of acrylate copolymers that may be suitable for a range of tissue engineering and adhesive applications. These copolymers are based on natural products that can be tailored at the molecular level to meet chemical, biological, and mechanical design requirements. The materials are based on monomers of modified triglycerides derived from plant sources. Triglyceride molecules contain many active sites available for modification with polymerizable groups, as reviewed in Ref. 6. Both ring opening and free radical polymerization of these monomers has been demonstrated.[7] Further, fatty acids have been used as skin permeation enhancers. They are thought to increase transcellular and paracelluar transport via the perturbation of phospholipid acyl chains in the cell membrane.[5]

In this study, we used an acrylated oleic methyl ester (AOME) derived from oleic methyl ester

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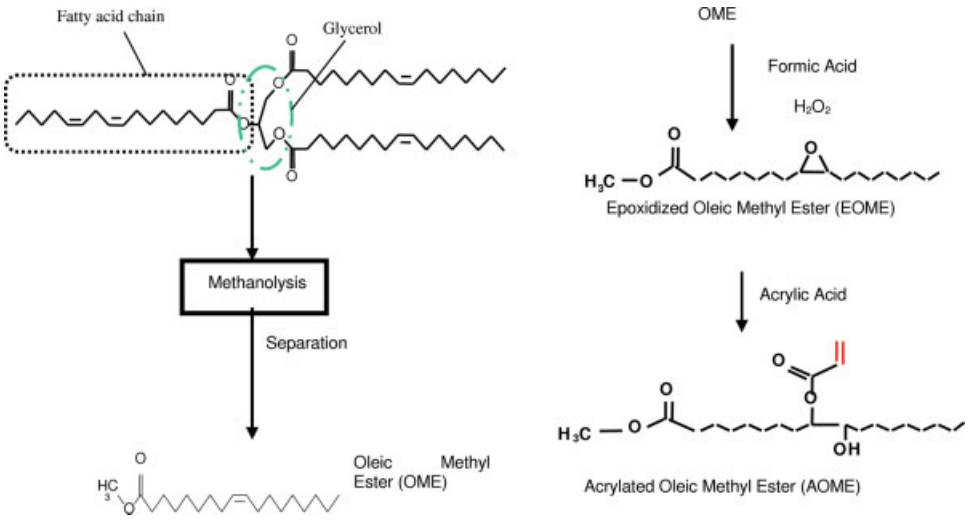

Figure 1. Acrylated oleic methyl ester (AOME) monomer synthesis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

copolymerized with methyl methacrylate (MMA) and ethylene glycol dimethacrylate (EDGMA) to make pressure sensitive adhesives (PSAs)[8,9] When polymerized, the AOME forms linear polymer chains (PAOME) with some branching; and this architecture is suited to PSAs.[8,9] Because these materials are derived from plant sources and some of them are copolymerized with MMA, which is known to form biocompatible polymers, we hypothesized that the resulting polymers would be cytocompatible and thus suitable as a new class of medical adhesives.

MATERIALS AND METHODS

Monomer synthesis and polymerization

Methyl oleate (99.9+% purity), formic acid (98% aqueous), hydrogen peroxide (30% aqueous), acrylic acid, and hydroquinone were used as received without further purification (Aldrich Chemical Co., Milwaukee, WI). AOME was synthesized via the methods discussed in Bunker et al.[9] and shown schematically in Figure 1. Briefly, the unsaturated methyl oleate starting material was epoxidized using formic acid and hydrogen peroxide. The resulting product was washed extensively with basic and neutral salt solutions, and dried over sodium sulfate to remove any residual water, leaving the pure epoxidized methyl oleate (EMO) product. The EMO was reacted with acrylic acid in the presence of a chromium-based catalyst, AMC-2 (Aerojet Chemicals, Rancho Cordova, CA), which is a mixture of 50% trivalent organic chromium complexes and 50% phthalate esters, to form the AOME monomer.

Pressure sensitive adhesives

EGDMA and MMA (Sigma-Aldrich, St. Louis, MO), sodium dodecyl sulfate (Aldrich Chemical Co.), and 4,4′-azobis(cyanopentanoic acid) (Sigma-Aldrich) were used as received. A set of PSAs was prepared by mini-emulsion polymerization according to methods in Bunker et al.[8] In brief, samples were synthesized by combining AOME with the appropriate comonomers, water, and detergent in the presence of the catalyst and the solution was refluxed for 1 h at 85°C under nitrogen. Increasing ratios of the comonomers MMA and EGDMA were combined with AOME to generate PSAs with different mechanical and thermal properties. The compositions are listed in Table I. Methanol was used to precipitate the product out of the resulting mixture. Samples are named according to the relative weight percents of AOME, MMA, and EGDMA and are written as whole numbers separated by slashes: (%AOME)/(%MMA)/(%EDGMA). So the 100% AOME sample is written 100/0/0. The samples are summarized in Table I.

Mechanical testing

PSA samples were subjected to a probe tack test using an Instron 5848 (Instron, Norwood, MA) equipped with a 10 N load cell. Tests were performed with the instrument in the horizontal position. Polyethylene probes with a diameter of 7 mm were attached to the instrument clamps. Approximately 85 μL of each copolymer sample (neat) was applied to one probe using a micropipettor and the second probe zeroed after it was in contact with the polymer.

TABLE I
Compositions of Pressure Sensitive Adhesive (PSA) Samples. Percentage by weight

Sample	%AOME	%MMA	% EGDMA
100/0/0	100	0	0
95/5/0	95	5	0
79/20/1	79	20	1
59/40/1	59	40	1

After a 1–2 min incubation time at room temperature, the probes were pulled apart at a rate of 0.3 mm/s until the copolymer was pulled off of the second probe. Samples were not incubated longer to avoid excessive flow of the material before the test. The incubation time was the time needed to get the test started after application of the sample. All samples were tested on the same day to maintain relatively constant room temperature and humidity values for each test. Because of the relatively large amount of material used in each test, this test cannot be compared directly to a peel test, but does provide more information about the PSA mechanical behavior. Tests were repeated 10 times for each PSA sample, and the mean value and standard deviation of the area under the curve, a measure of tack energy and an composite curve composed of the average of the 10 tests for each sample are reported.

Cell culture test vessel preparation

The test materials were dissolved in $CHCl_3$ to allow a thin coating to be uniformly deposited on the bottom of the cell culture wells. The $CHCl_3$ was allowed to evaporate for 48 h leaving the copolymer coated glass wells to be rinsed thoroughly with 70% ethanol, distilled deionized H_2O, sterile 1 × PBS, and sterile media before commencing with the live cell culture experiment. Traditional polystyrene tissue culture (PSTC) plates would have been damaged by the effects of the chlorinated solvent, and so the glass wells were prepared as an alternative. Glass sample vials (Fisher Scientific, 10 mL volume) were cut to the same height as PSTC plates using a Dremmel cutting tool fitted with a diamond studded cutting disk. The glass vials were chosen as alternative sample wells because of their similar diameter to the wells in a 24-well PSTC plate (16 and 15 mm, respectively). The same number of cells was added to each test and control well. Duplicate identical sample coatings were prepared for the express purpose of measuring the thickness of each copolymer. For the wells used in the current experiment, the thickness of polymers 100/0/0, 95/5/0, 79/20/1, and 59/40/1 was measured with digital calipers and determined to be 150, 50, 330, and 300 μm, respectively.

Cell viability/proliferation assay and optical imaging

Primary human foreskin dermal fibroblasts (ATCC, Cat # CRL-2522, Manassas, Virginia) were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing fetal calf serum (10%) and penicillin/streptomycin (1%). Cells were incubated at 37°C in the presence of 5% CO_2 balance air at 100% humidity. Media was changed every 48 h, and cells were subpassaged as they approached 80% confluence. Cells used in these studies had been previously cultured in our lab and the population doublings at the start of the assay ranged from 20 to 30. Cells were trypsinized and triturated and resuspended in media before they were applied to the different copolymer materials.

Cell viability on the PSAs was determined by performing an Alamar Blue *in vitro* cell viability and proliferation assay[10] (Invitrogen, Carlsbad, CA). The Alamar Blue assay incorporates a fluorometric/colormetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation/reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth.

Cell attachment and spreading was monitored on all of the materials by optical microscopy using a standard inverted light microscope. Live/Dead assays (Invitrogen) were performed at $t = 24$ h and $t = 2$ weeks using a confocal microscope (Olympus FV1000, Center Valley, PA). Each well was seeded with ~50,000 cells in 2.0 mL of the DMEM media solution. Positive controls were created by seeding the same number of cells into both empty glass wells and empty polystyrene wells. Material negative controls were created by performing the entire assay in the presence of each material (Table I) without cells present to ensure that the material itself was not inducing false positives. Acellular wells served as blank negative controls.

After 24-h of incubation, cells were examined using the optical microscope. Images of cells attached to the materials were recorded (10× magnification). Duplicate samples of coated glass bottom culture plates were then subjected to the Live/Dead assay and fluorescent imaging on a confocal microscope (Olympus FV1000). After optical images were recorded at 24 h, the appropriate volume of Alamar Blue solution was added to the wells and the cells were incubated for an additional 4 h. The concentration of viable cells on each sample material after 28 h was determined using a standard curve measuring absorbance at 570 and 600 nm.

RESULTS

Pressure sensitive adhesive cytocompatibility

After 2 h in culture, cells began to attach to all of the PSAs. Some materials were more cell adhesive than others, and this effect was seen both at the 2 h (images not shown) and 24-h time points (Fig. 2). At 2 h, large numbers of rounded cells were observed beginning to attach to all of the PSA materials. After 24 h, the attached cells began to spread on all of the PSAs, with the exception of sample 100/0/0. On samples 95/5/0, 79/20/1, and 59/40/1, most of the attached cells elongated taking a typical fibroblast phenotype, which was still observed after 2 weeks in culture (Fig. 3), whereas on sample 100/0/0, only single, rounded cells were observed.

The quantitative data from the Alamar Blue assay at the 24-h time point, indicated that neither the blank media wells nor the material coated control wells showed a significant color change as expected, because no cells were present. The cells in the polystyrene positive control wells grew at a normal rate, doubling every 1–2 days,[11] thus exhibiting more than the number of seeded cells after the 24-h incubation, whereas the cells in the glass positive control wells indicated that a portion of the initial 50,000 cells

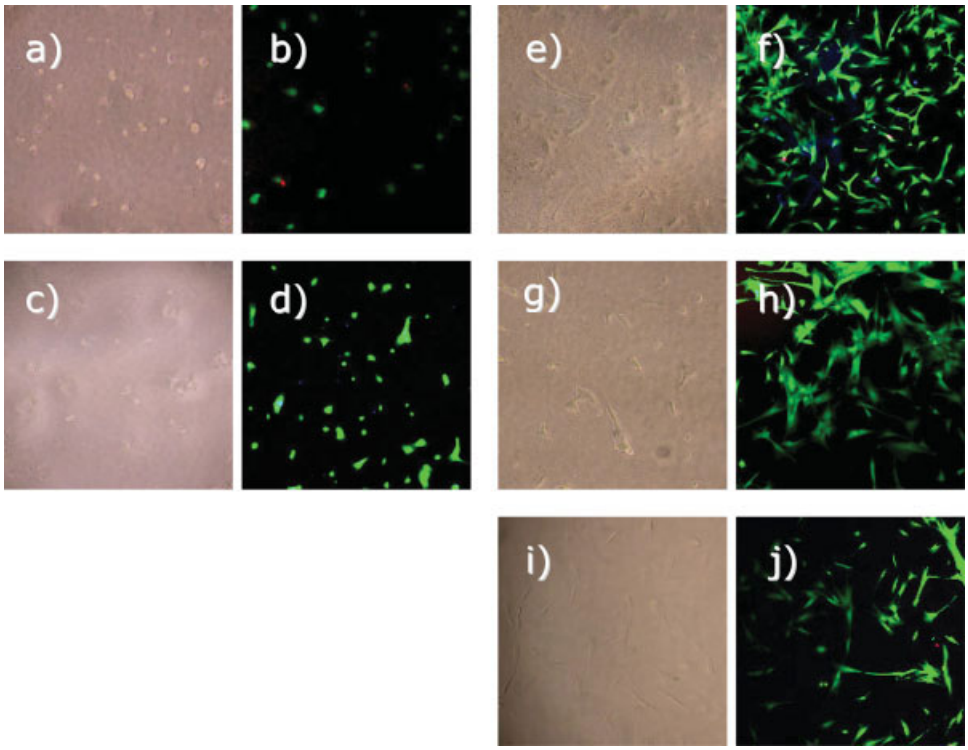

Figure 2. Primary human foreskin dermal fibroblasts on PSAs at ×10 magnification, t = 24 h. On sample 100/0/0: Optical (a) and Live/Dead (green/red) with nuclear DAPI stain (blue)—fluorescent (b); sample 95/5/0 optical (c) and fluorescent (d); sample 79/20/1 optical (e) and fluorescent (f); sample 59/40/1 optical (g) and fluorescent (h); and polystyrene positive control optical (i) and fluorescent (j).

seeded did not attach (Fig. 4). Data for sample 100/0/0 showed a statistically significant decrease in the number of cells present from the initial seeding to the 24-h time point, also reflected by fluorescent imaging (Fig. 2). Cells did not survive on sample 100/0/0 until imaging at the 2-week time point (Fig. 3).

Quantitative data for sample 95/5/0 showed an increase in cell attachment in comparison to sample 100/0/0, but still had a statistically significantly lower number of attached cells in comparison to the positive control wells at the 24-h time point (Fig. 4). Fluorescent imaging indicated the attached cells

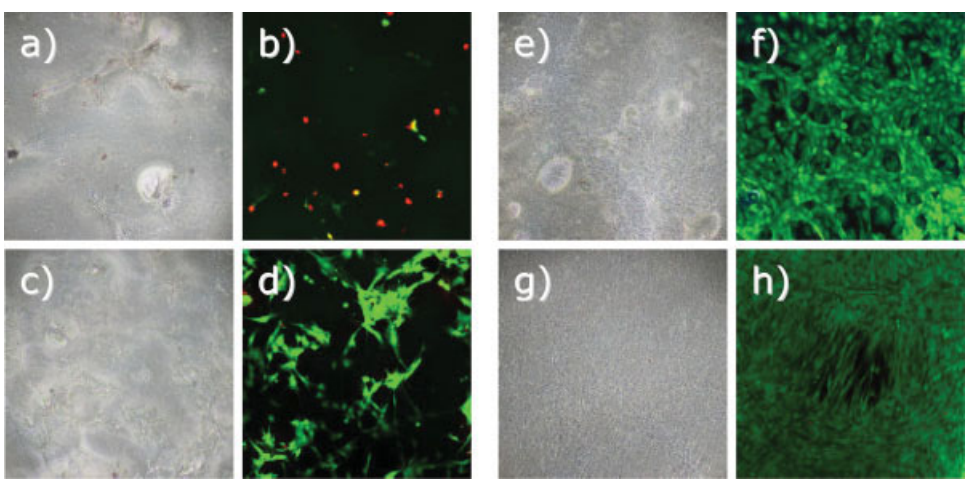

Figure 3. Primary human foreskin dermal fibroblasts on PSAs at ×10 magnification, t = 2 weeks. On sample 100/0/0: optical (a) and Live/Dead fluorescent (b); sample 95/5/0 optical (c) and fluorescent (d); sample 79/20/1 optical (e) and fluorescent (f); sample 59/40/1 optical (g) and fluorescent (h); and polystyrene positive control optical (i) and fluorescent (j).

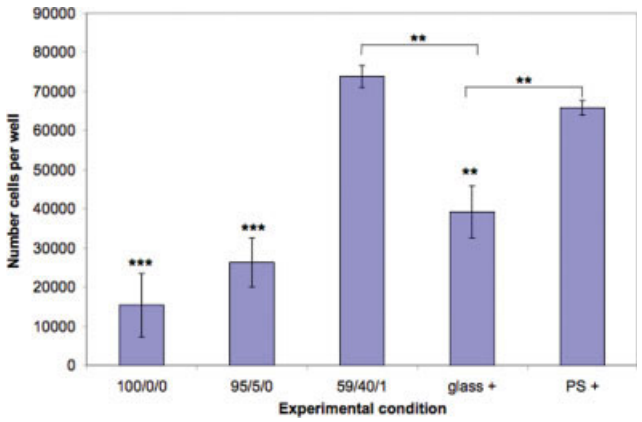

Figure 4. Quantitative Alamar Blue assay results 28 h after seeding. All samples have been zeroed by subtracting the appropriate negative control. The PS + (polystyrene positive) sample served as a positive control with 50 000 cells seeded per well, as did the glass + sample. The results indicate that sample 59/40/1 showed cell growth from $t = 0$. Samples 100/0/0 and 95/5/0 supported cell attachment and growth, but at lower rates than the polystyrene or glass controls, $^{*}p < 0.005$, $^{**}p < 0.0005$. It should be noted that the values for sample 79/20/1 were unreadable and therefore omitted due to the physical detachment of the polymer from the glass sample vial. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

were beginning to elongate on the material at 24 h (Fig. 2) and continued to proliferate up to 2 weeks (Fig. 3).

Sample 59/40/1 showed a high number of cells attached and proliferating at 24 h. The fluorescent images support the quantitative data, and the cells proliferated up to 2 weeks.

The sample 79/20/1 coating detached from all glass wells within 24 h of seeding with fibroblasts, and so we were unable to run the Alamar Blue Assay on these samples at 24 h. The duplicate samples prepared for fluorescent imaging did not fully detach from the flat, glass bottomed plates and showed increased cell growth similar to that of sample 59/40/1, continuing to grow over a 2-week period. Samples 79/20/1 and 59/40/1 both had 1% EGDMA crosslinker added to the copolymers, whereas samples 100/0/0 and 95/5/0 did not. Of the two crosslinked samples, polymer 79/20/1 exhibited slightly less adhesive properties than sample 59/40/1, as evidenced by the detachment of the polymer from the glass well plates during the Alamar Blue proliferation assay and confirmed by the mechanical test data (Figs. 5 and 6).

Probe tack tests

All of the PSAs failed adhesively by pulling off completely from one of the probes. The work of adhesion was calculated for each sample using the following formula,

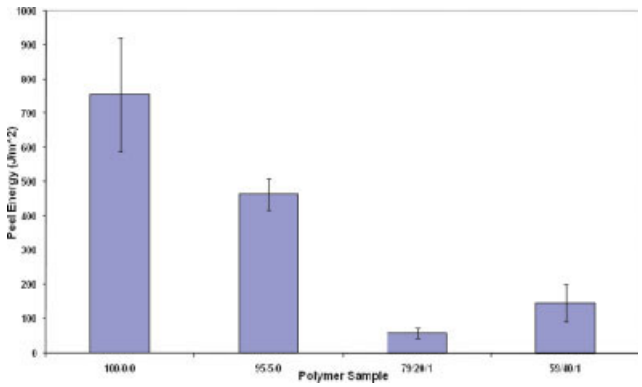

Figure 5. Work of adhesion, or peel energy, for each of the pressure sensitive adhesives. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

$$W = \frac{W'}{\pi - a_{\max}^2},$$

where W' is the area under the force displacement curve and $a_{\max}$ is the maximum contact radius. The data are presented in Figure 5. Each bar represents the average work of adhesion measured from 10 independent tests. Figure 6 is a plot showing four composite curves, one for each PSA, to demonstrate the average shape of the full force displacement curve. Together Figures 5 and 6 show that the polyAOME (sample 100/0/0) was the most tacky, but did not support the highest load before pull-off. For the 95/5/0, 79/20/1, and 59/40/1 copolymers, the

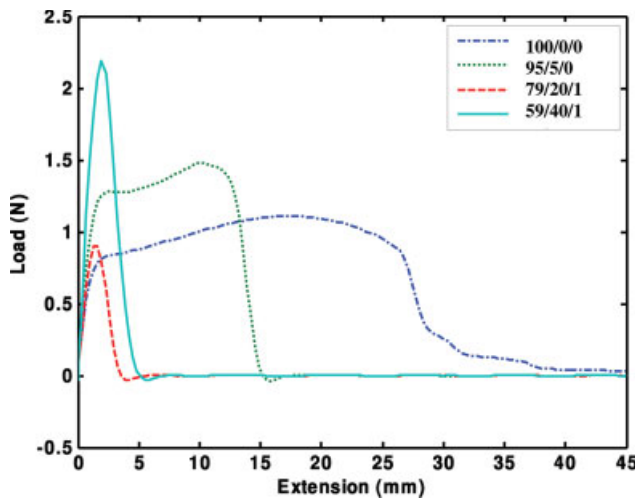

Figure 6. Average force-displacement curves ($n = 10$) for each of the pressure sensitive adhesives tested. Error bars are omitted for clarity. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

10 stress–strain curves were all very similar in shape and peak load (as can be seen by the standard deviation error bars in the peel energy curve shown in Fig. 5). There was somewhat more variation in the 10 tests for the 100/0/0 sample, but the overall shape is consistent with the shape of the curve shown in the averaged plot (Fig. 6). This discussion has been added to the article. Adding MMA to form a 5% copolymer (sample 95/5/0) increased the force to pull-off, but reduced the overall work of adhesion (tackiness). Adding more MMA (samples 79/20/1 and 59/40/1) also further reduced the work of adhesion. Sample 79/20/1 had a higher amount of the EDGMA crosslinker than sample 59/40/1 as a percentage of the amount of MMA present in the copolymer. Sample 79/20/1 also had a much lower force to pull-off and a somewhat lower work of adhesion than sample 59/40/1, suggesting that sample 79/20/1 was more highly crosslinked, and a less effective PSA.

DISCUSSION

Before discussing the potential utility of these copolymers as bioadhesives, it is important to look back on past applications of triglycerides and triglyceride derivatives in the body. First, it is important to note that these acrylate copolymers are not meant to biodegrade because the backbone of the polymer consists of saturated carbon–carbon bonds, and that any significant degradation would be considered a disadvantage in a medical application. The most prominent example is the Trilucent breast implant, which was implanted in roughly 5000 women in Europe from 1995–1999.[12] The implants were removed from the market after many patients complained of discomfort, and upon having the implants removed evidence of implant bleed, leakage, and rupture were seen.[13] Animal studies performed in rabbits looked at bleed of the implants over a period of 6 months.[14] This work indicated that any oil leaking from the implants was readily metabolized by the surrounding tissues. The histology found fibrous capsules, but these were thin and flexible. No other hallmarks of foreign body response were noted.

It is known that unsaturated fatty acids are susceptible to autooxidation, which can eventually lead to the formation of aldehydes. It was suspected that aldehyde breakdown products from the implants were causing toxicity in women with the implants. A multicenter follow-up study completed in 2004 that included 47 women with Trilucent implants and 34 women with other types of implants concluded that there was no risk to the health of women from these breakdown products.[15] The copolymers presented here, although based on soy oil extracts, are processed in such a way that the unsaturated carbon–carbon bonds are converted to acrylate groups before copolymerization (Fig. 1). The acrylate carbon–carbon double bonds then participate in the polymerization reaction. It is thus unlikely that a fully polymerized and purified PSA (to remove unreacted monomers) would suffer from these degradation issues. However, with the lessons of the Trilucent implants as a guide, we know it is necessary for the degradation products and their toxicities, both due to active biological (cell metabolism) and chemical pathways must be explored.

It is also important to mention the potential cytotoxicity of the catalyst used in PSA synthesis. In preliminary work (data not shown), we grew cells directly onto and near (in tissue culture polystyrene dishes) the neat PSAs before any washing steps. All of the PSAs allowed cells to attach to some extent on the PSA and to full confluence on the uncovered TCPS dish. Although it does not appear to be a critical issue in these *in vitro* tests, we are aware that the catalyst may be problematic in the future, and we are currently looking at more biocompatible alternatives.

In summary, we have presented a class of materials derived from plant oils that have properties suitable for a wide range of biomedical applications. The copolymers are cytocompatible and encourage fibroblast attachment and growth as indicated by the Alamar Blue assay and fluorescent imaging. The homopolymer sample 100/0/0 (polyAOME) did not support cell growth. This result could be due to the mechanical properties of the sample, because anchorage dependent fibroblasts have been shown to prefer stiffer substrates to attach to and migrate over.[16] It is also possible that the MMA monomers are driving the ability of cells to adhere and spread on the substrate, perhaps by reducing the mechanical tackiness of the PSA. Polymer 100/0/0 exhibited much stronger cohesive and adhesive properties than samples 95/5/0, 79/20/1, and 59/40/1 (Figs. 5 and 6). We speculate that the addition of more comonomer MMA to sample 59/40/1 (40% by weight) when compared with the less adhesive sample 79/20/1 (20% by weight) appears to have affected the overall degree of crosslinking, with sample 59/40/1 remaining slightly more adhesive. If sample 79/20/1 has a higher degree of crosslinking, then it would follow that sample 79/20/1 would make a "worse" adhesive material than sample 59/40/1. The modified tack tests support this hypothesis (Figs. 5 and 6).

Samples 95/5/0 and 59/40/1 also appeared to lose some adhesive properties following submersion in media over time and began to peel away from the glass wells after 48 h of incubation. The tackiest polymer, sample 100/0/0 started to peel away after ~5 days. Submersion in media over time appeared

to lower the attachment properties of the PSAs to glass. Data was taken from all samples except those that full detached from the plate. Previous experiments using a spatula to thickly apply nonuniform layers (~0.5–1 mm) (data not shown) of each polymer did not detach after submersion in media for several days and did not require the initial solvation in $CHCl_3$.

The copolymer adhesives described here, in thin film form, may be appropriate for transdermal drug delivery applications, as they are both adhesive to skin and can be tailored to incorporate both water and fat soluble drugs. It is also possible that the addition of the fatty acid-based copolymer may enhance skin permeation of certain drugs. Again, it is critical to fully assess the degradation pathways and products of these materials before they are used as bioadhesives. We are currently synthesizing AOME-pHEMA copolymers that have more tunable hydrophilicity with composition for the transdermal delivery application. Now that the basic cytocompatibility has been demonstrated for a range of copolymers incorporating these plant-derived monomers, continuing work is focused on tissue specific applications and molecular level cell-biomaterial interactions.

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