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Cross-Linked, Heterogeneous Colloidosomes Exhibit pH-Induced Morphogenesis

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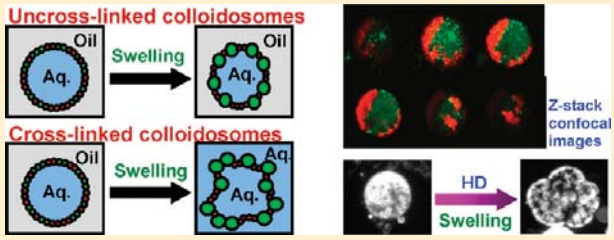
ABSTRACT: Inspired by morphogenesis in biology, we present a strategy for developing functional 3D materials with the capacity to morph based on environmental cues. We utilized local mechanical stresses to cause global shape changes in colloidosomes. Colloidosomes were assembled from pH-sensitive calcium alginate particles (CAPs) with high and low swelling ratios. Colloidosomes were subsequently cross-linked via diamine compounds with varying carbon chain lengths. New colloidosome isoforms were generated from heterogeneous mixtures of CAPs, which resulted in nonuniform stresses. Our study demonstrated that coordinated networks of heterogeneous subunits may be used to design programmable materials.



INTRODUCTION

Morphogenesis is the process by which an organism changes shape, where networks of individual units collectively respond to chemical or mechanical cues to alter their form.[1,2] At the molecular level, integrins undergo dramatic conformational changes to achieve a high-affinity configuration during the process of integrin activation. Integrin activation can be regulated by intracellular and extracellular signals, including the extracellular pH, which becomes acidic in several biological contexts. Whereas normal physiological pH is 7.4, the average extracellular pH in the tumor environment ranges between 6.2 and 6.9.[3−6] During the early stages of wound healing, the extracellular pH falls within the range of 5.7−6.1.[7] Recently, Paradise et al. has shown that pH-induced structural changes in integrins may be used to control the binding affinity.[8] In parallel to this theme, we have developed a colloidosome that morphs in response to pH, which may be used to control distances between two individual units. Such changes in the 3D environment may be useful for controlling the adhesion of colloidosomes to cells or other substrates.

Here, we present a biologically inspired strategy for preparing functional materials with the capacity to morph. Using colloids as the unit, we generated local mechanical stresses, from the pH-responsive swelling of calcium alginate particles (CAPs), to induce global shape changes in colloidosomes. Colloidosomes are hollow spheres with elastic shells composed of colloids that self-assemble at the oil/water interface.[9,10]

Spherical colloidosomes are prepared via self-assembly at oil/water interfaces followed by sintering, chemical cross-linking, or particle packing.[11−14] Assembling polystyrene particles over poly(*N*-isopropyl acrylamide) microgels generated colloidosomes that increased the size of voids between individual colloids on the basis of temperature.[15] In addition, colloidosomes were investigated as a platform for controlling chemical modifications (e.g., Janus particles[16,17]). Despite the range of applications in which colloidosomes have been used, most colloidosomes have been synthesized from homogeneous particles. In this report, we generated heterogeneous colloidosomes from particles that differed only in their swelling character to induce mechanical stresses within the colloidosome.

We utilized colloidosomes as a platform to study the morphogenesis of a network of cross-linked, closely packed spheres (Figure 1). Colloidosomes were prepared from pH-responsive CAPs, which exhibit swelling as a function of the environmental pH and the extent of ionic cross-linking.[18] Subsequent cross-linking of particles was achieved using a series of linear diamines with varying carbon chain lengths.[19] The synthesis of morphogenic colloidosomes may be employed in the design of programmable materials that utilize mechanical stresses to control buckling.

EXPERIMENTAL SECTION

Materials. The alginic acid sodium salt from brown algae and calcium chloride ($CaCl_2$) as a cross-linker was purchased from Sigma (St. Louis, MO). Toluene, sorbitan monooleate (span 80), acetone, putrescine (butane-1,4-diamine), hexamethylene diamine (hexane-1, 6-diamine), *N*-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Sigma. The Pluronic 31R1 surfactant was kindly provided by BASF Corporation (Mount Olive, NJ). Ethylene diamine (1,2-diaminoethane) and hexadecane were obtained from Aldrich (St. Louis, MO). Mineral oil was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). To prepare phosphate buffer, monobasic and dibasic sodium phosphates were purchased from Sigma. Deionized water used in all experiments was obtained from a Milli-Q reagent water-purification system (Millipore Corp., Billerica, MA).

Preparation of Calcium Alginate Particles (CAPs). CAPs were prepared by a water-in-oil (w/o) emulsification method. Sodium

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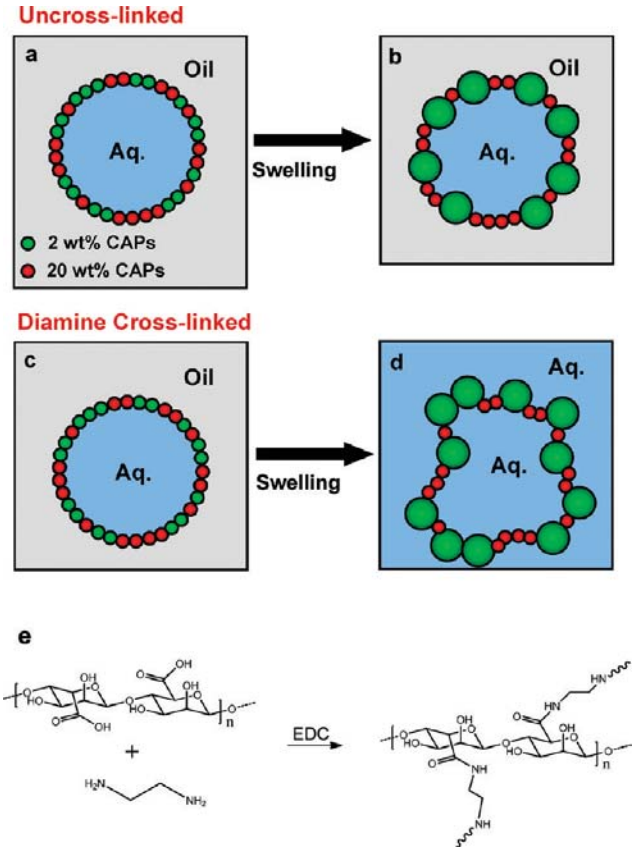

Figure 1. Schematic illustration of colloidosomes composed of particles with low and high swelling ratios. The swelling of colloidosomes is depicted (a, b) without and (c, d) with diamine cross-linking. (e) Carbodiimide coupling reaction between CAPs and diamine compounds.

alginate was dissolved in deionized water at a 2 wt % concentration. Pluronic 31R1 (0.5 g) was dissolved in 30 mL of toluene, and then sodium alginate solution was emulsified into the oil phase using a sonicator (200 W, 20 kHz; digital sonifier 250, Branson Ultrasonics Corp., Danbury, CT) in a laminar flow hood over an ice bath for 10 min. After the sodium alginate emulsion was obtained, 20 mL of $CaCl_2$ (2 or 20% w/v) was added with vigorous stirring to form CAPs. After 10 min, 20 mL of acetone was mixed into the solution to dehydrate and harden CAPs. CAPs were collected by centrifugation at 14 800*g* for 20 min (Microfuge 16, Beckman Coulter, Inc., Brea, CA). The pellet was redispersed in 4:1 deionized water/acetone followed by centrifugation and resuspension in deionized water three times to remove residual toluene and surfactant.

Characterization of CAPs. Dynamic light scattering (ZetaPALS, Brookhaven Instrument, Holtsville, NY) and scanning electron microscopy (SEM Ultra55, Zeiss, Thornwood, NY) were used to determine the size and morphology of CAPs. Particles (0.1 mg) were suspended in 1 mL of deionized water. A drop of particle-containing solution was placed on an SEM specimen stub (TED PELLA Inc., Redding, CA) and dried in a laminar flow hood for 6 h. Dried particles were coated with platinum−palladium for 90 s at 40 mA using a sputter coater (Cressington 208HR, Watford, U.K.). Mass swelling studies of calcium alginate microbeads cross-linked with 2 and 20 wt % w/v $CaCl_2$ were carried out in phosphate-buffered saline at pH 7.4 for 2, 4, and 6 h. Calcium alginate microbeads were placed into scintillation vials containing 5 mL of the buffered medium. The mass swelling ratio was calculated as the average particle mass after swelling divided by the dry mass of the particles.

pH-Sensitive Colloidosome Formation. CAPs (2 mg) were dispersed in 1 mL of deionized water or a pH-buffered medium. Meanwhile, 10 mL of mineral oil and 0.1 mL of span 80 surfactant were also mixed together. The two solutions were then mixed and gently stirred for 10 min to form calcium alginate colloidosomes.

Diamine Conjugation with Colloidosomes. Colloidosomes stabilized on the w/o interface were treated with various diamines in order to modify the carboxyl groups exposed to the aqueous phase to amine groups. Ethylene diamine, butane-1,4-diamine, and hexane-1, 6-diamine were used to determine the effect of the carbon chain length on the morphing capabilities of the colloidosomes. Prior to the formation of colloidosomes, EDC (8.4 mg) and the diamines (0.15 μL) were added to the aqueous phase containing alginate particles, the pH was decreased to 6.5 using HCl, and the mixture was added to mineral oil. Once colloidosomes were formed, the mixture was stirred at room temperature for 2 h to allow for amine modification. The oil phase was removed by washing multiple times with hexadecane, filtered with sieves that have 180 and 212 μm pores (USA Standard Testing Sieve, VWR), and finally resuspended in phosphate buffer (Figure 1d). The modified colloidosomes were visualized under bright-field microscopy (Zeiss Axiovert 200M, Carl Zeiss, Inc., Thornwood, NY). Colloidosomes were also visualized using confocal microscopy (Zeiss LSM 510 META, Carl Zeiss, Inc., Thornwood, NY).

Shape Deformation under pH Change. The pH response of CAPs was investigated using bright-field and fluorescence microscopy in buffered media at pH 7.4. Images of colloidosome shape changes were taken 0, 4, and 6 h after swelling. We measured the area of 2D projections (n = 5 projections) per colloidosome (n = 12 colloidosomes) using ImageJ software (NIH). The data is presented in the form of the area ratio, which may be described as the ratio of the area after a shape change at a specific swelling time (A_t) to the area at 0 h swelling time (A_0).

$$A = \frac{A_t}{A_0}$$

RESULTS AND DISCUSSION

CAPs were fabricated in a water/oil (w/o) emulsion.[20] A continuous phase, composed of toluene and Pluronic 31R1, was used to disperse a 2% w/v sodium alginate solution after sonication. Alginate micelles were cross-linked with either 2% or 20% w/v $CaCl_2$ to produce monodisperse 1.0 ± 0.1 μm diameter particles with low and high swelling characteristics (Figure 2a). The mass swelling ratios of CAPs were evaluated over 6 h for each particle formulation (Figure 2b). The mass swelling ratio is related to changes in the particle radius by the relationship

$$\frac{m_f}{m_0} = \left[\frac{r_f}{r_0}\right]^3$$

where m_f is the final particle mass, m_0 is the initial particle mass, r_f is the final particle radius, and r_0 is the initial particle radius. CAPs made with 2% w/v $CaCl_2$ exhibited uniform swelling, approximately twice the swelling ratio of CAPs ionically cross-linked with 20% w/v $CaCl_2$. This resulted in a 28% difference in radii after swelling.

Colloidosomes were formed by the assembly of 1 μm CAPs at an oil/water interface. CAPs, ionically cross-linked with either 2 or 20% w/v $CaCl_2$, were mixed in a 1:1 ratio and dispersed into the aqueous phase. Mineral oil containing 1% sorbitan monooleate (Span80) surfactant was added to the aqueous dispersion, resulting in the formation of colloidosomes after gentle agitation using a magnetic stir bar. Figure 1 illustrates the formation of

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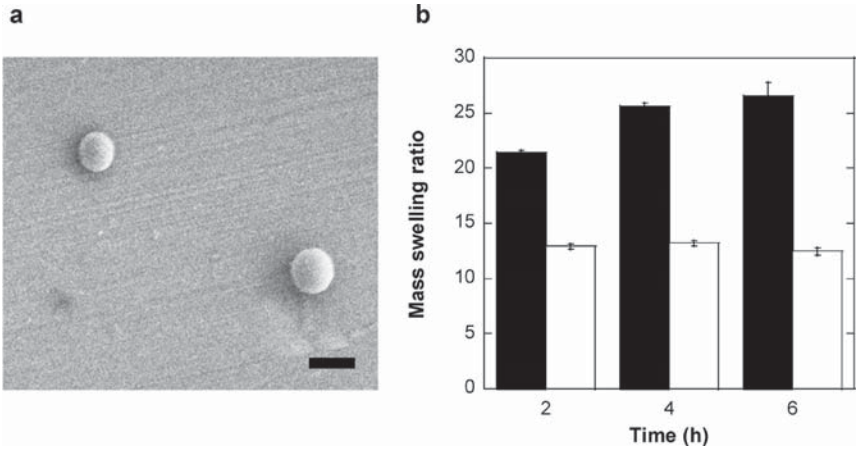

Figure 2. (a) Scanning electron microscopy image of CAPs cross-linked with 2% w/v $CaCl_2$. The scale bar is 1 μm. (b) Mass swelling ratio of CAPs cross-linked with 2 (black) and 20% (white) w/v $CaCl_2$ in pH 7.4 phosphate-buffered saline.

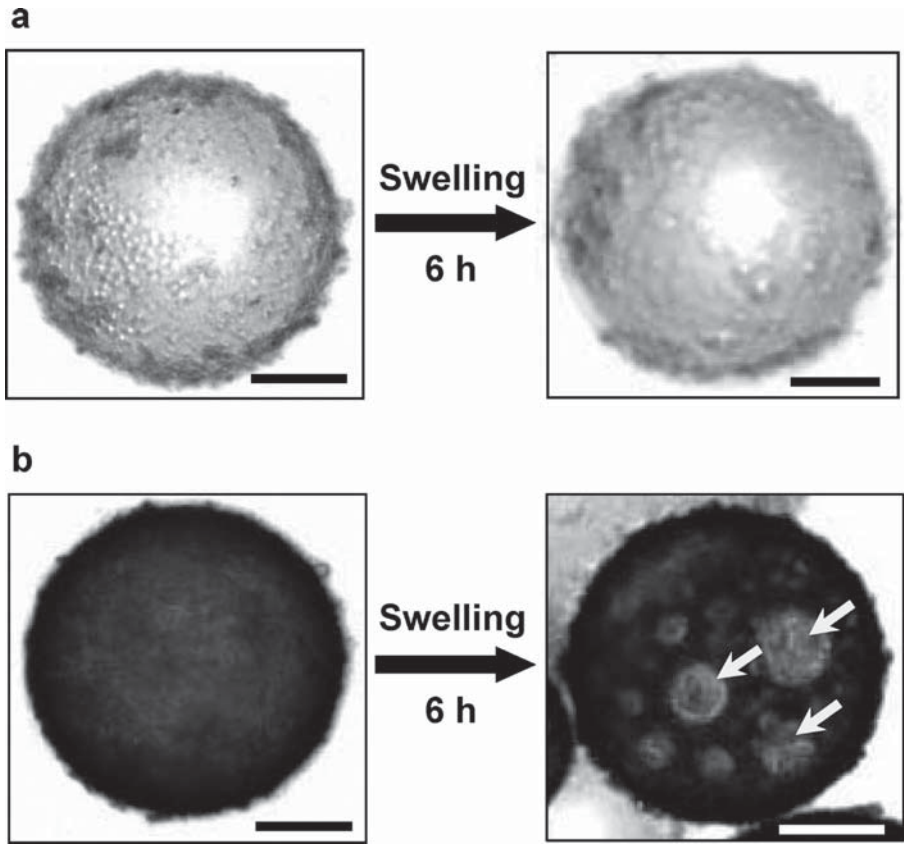

Figure 3. Formation and swelling of colloidosomes. (a) Bright-field microscopy images of un-cross-linked homogeneous colloidosomes using CAPs with 20% w/v $CaCl_2$ before and after swelling in 20 mM pH 7.4 phosphate buffer for 6 h. (b) Phase microscopy images of un-cross-linked heterogeneous colloidosomes using a 1:1 mixture of CAPs with 2 and 20% w/v $CaCl_2$ before and after swelling in 20 mM pH 7.4 phosphate buffer for 6 h. White arrows indicate swollen CAPs with 2% w/v $CaCl_2$. The scale bar is 100 μm.

colloidosomes and the resultant action of swelling of pH-responsive CAPs for un-cross-linked and diamine cross-linked colloidosomes.

Un-cross-linked colloidosomes do not exhibit morphing; however, particle swelling is observed. Figure 3 depicts bright-field microscopy images of spherical colloidosomes before and after 6 h of swelling in 20 mM pH 7.4 phosphate buffer. Homogeneous colloidosomes made from only 20% w/v CAPs do not show changes in individual particle sizes. In contrast, Figure 3b illustrates heterogeneous colloidosomes with a 1:1 mixture of 2 and 20% w/v $CaCl_2$ cross-linked CAPs; clear differences in particle sizes were evident after swelling. Colloidosome diameters were quantified by optical microscopy; they remain 196 ± 16 μm in all images. In the absence of colloidosome

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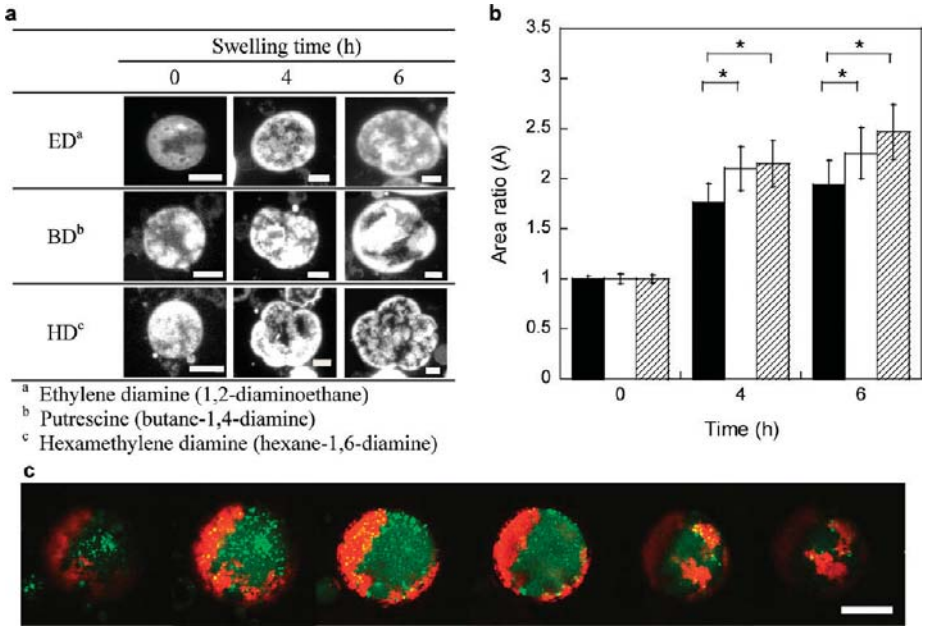

Figure 4. (a) Fluorescence micrograph images of cross-linked colloidosomes after shape changes in 20 mM pH 7.4 phosphate buffer for 0, 4, and 6 h of swelling. Colloidosomes were composed of unlabeled particles cross-linked with 2% w/v $CaCl_2$ (dark) and rhodamine-labeled particles cross-linked with 20% w/v $CaCl_2$ (bright) (1:1 ratio). The scale bar is 100 μm. (b) Area ratio of colloidosomes after shape changes for ED (black)-, BD (white)-, and HD (diagonal)-modified colloidosomes. The error is the standard deviation of the mean, where $n = 12$. Statistical significances are calculated with * $p < 0.05$. (c) z-stack confocal microscopy analysis of colloidosomes with a 1:1 ratio of 2 (green FITC-labeled) and 20% (red rhodamine-labeled) w/v $CaCl_2$ CAPs. Images were taken in 17.51 μm sections. The scale bar is 100 μm.

cross-linking, CAPs were able to rearrange on the surface to retain a spherical shape.

To constrain particles and observe the effects of mechanical stress, colloids were cross-linked to one another using diamine compounds with an increasing number of carbon−carbon bonds. Colloidosomes were selectively modified on the interior surface in the aqueous phase.[16,17,21,22] Exposed carboxyl groups on CAPs were covalently linked via primary amines using a carbodiimide coupling reaction. Briefly, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and ethylene diamine (ED), butane-1,4-diamine (BD), or hexane-1,6-diamine (HD) were added to the aqueous phase at pH 6.5 for 2 h (Figure 1c).[22] Prepared colloidosomes were robust and could be centrifuged, washed in hexadecane to remove mineral oil, and resuspended in buffer after diamine cross-linking, unlike un-cross-linked colloidosomes that would dissociate after centrifugation.

Colloidosomes cross-linked with ED, BD, and HD are shown in Figure 4a. Increasing the diamine chain length did not affect the initial colloidosome stability or size. The shape of the colloidosome after swelling was dependent on the cross-linker. Cross-linking with ED or BD resulted in a significant increase in the colloidosome diameter after swelling. Colloidosomes cross-linked with ED became elliptical whereas colloidosomes cross-linked with BD exhibited evidence of budding. Significant colloidosome shape changes occurred when HD was used to tether CAPs together. HD cross-linked colloidosomes exhibited multiple areas of budding. Increasing the carbon chain length between cross-linked particles resulted in more deformation; the larger distance between CAPs may give colloidosomes greater flexibility, allowing for more pronounced shape changes. Approximately 80% of the intact colloidosome population tethered with HD deviated from a spherical structure and exhibited a large shape deformation; the remaining 20% was spherical.

We quantified the extent of global shape deformation by calculating the ratio of the colloidosome surface area after swelling at time t (A_t) to the initial surface area at 0 h (A_0), $A = (A_t/A_0)$ (Figure 4b). Colloidosomes cross-linked with HD exhibited the largest surface area ratio after 6 h of swelling. The area ratio between colloidosomes cross-linked with ED was statistically significant from that of colloidosomes cross-linked with BD and HD after swelling. This suggested that longer cross-links between CAPs increased the extent that cross-linked colloidosomes could swell and deform.

Colloidosome morphing may be approximated by the deformation resulting from the contact between two spheres as described by Hertzian theory.[23] The contact area between two spheres of radii R_1 and R_2 is a circle of radius a. The distribution of normal traction in the contact area as a function of distance $p(r)$ from the center of the circle is described as

$$p(r) = p_o\left(1 - \frac{r^2}{a^2}\right)^{1/2}$$

where p_0 is the maximum contact pressure given by

$$p_o = \frac{3F}{2\pi a^2} = \frac{1}{\pi}\left(\frac{6FE^{*2}}{R^2}\right)^{1/3}$$

and the effective radius R is defined as

$$\frac{1}{R} = \frac{1}{R_1} + \frac{1}{R_2}$$

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The area of contact is related to the applied load F by the equation

$$a^3 = \frac{3FR}{4E^*}$$

where the depth of indentation d is related to the maximum contact pressure by

$$d = \frac{a^2}{R} = \left(\frac{9F^2}{16RE^{*2}}\right)^{1/3}$$

and E^* is defined as

$$\frac{1}{E^*} = \frac{1-\upsilon_1{}^2}{E_1} - \frac{1-\upsilon_2{}^2}{E_2}$$

where E_1 and E_2 are the elastic moduli and υ_1 and υ_2 are the Poisson ratios for the spheres.

For Hertzian theory to apply, we assume that the load does not exceed the material's elastic limit, the area of contact is much smaller than the characteristic radius of the body, the surfaces are continuous and nonconforming, and the surfaces are frictionless.

Colloidosomes were analyzed by confocal microscopy (Figure 4c). Colloidosomes did not have colloids cross-linked in the interior. We also observed that the CAPs were not homogeneously mixed. The inhomogeneity in particle mixing is a result of our protocol; the two alginate particle solutions were prepared, added simultaneously to mineral oil (high viscosity), and gently stirred for 10 min. Vigorous mixing or a low-viscosity oil phase resulted in homogeneous mixing. The localization of 2% w/v $CaCl_2$ cross-linked CAPs on the colloidosome surface yields more pronounced shape changes than if CAPs were homogeneously dispersed, as observed visually from images before and after swelling.

CONCLUSIONS

This study demonstrated how local mechanical stresses can be used to generate new colloidosome isoforms. We described the synthesis of heterogeneous cross-linked colloidosomes from pH-responsive colloids that exhibit low and high swelling. Colloidosome morphogenesis resulted in nonuniform deformations; greater deformation arose in areas where highly responsive particles were more densely packed. Constraining particles via chemical cross-linking enabled control over mechanical stresses arising from volumetric swelling; longer cross-linkers exhibited greater deformation. This biologically inspired strategy utilizes mechanical forces to direct global shape change through the organized growth and shrinkage of individual cells. Coordinated networks composed of a mixture of heterogeneous subunits may be used to develop programmable materials. Hybrid materials composed of colloids with very different properties (e.g., responsive vs nonresponsive) may be useful in creating new approaches to sensing, tissue engineering, and drug delivery. Novel materials constructed from individual units may be used to modulate structure and chemistry that could be useful for the on-time manipulation of 3D scaffolding, the recruitment or activation of cells, the regulation of adhesion, and the delivery of molecules.

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