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Article

Chelator Regulation of *In Situ* Calcium Availability to Enable Spray-Dry Microencapsulation in Cross-Linked Alginates

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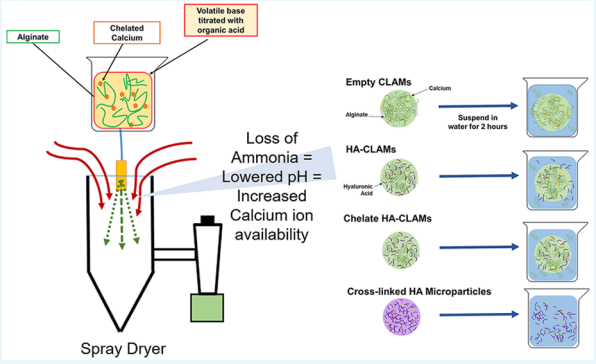
ABSTRACT: A recently patented one-step *in situ* cross-linked alginate microencapsulation (CLAM) by spray-drying (i.e., the UC Davis CLAMs technology) can overcome the high cost of scale-up that limits commercial applications. While increasing calcium loading in the CLAMs process can increase the extent of cross-linking and improve retention and protection of the encapsulated cargo, the potential for residual undissolved calcium salt crystals in the final product can be a concern for some applications. Here, we demonstrate an alternate one-step spray-dry CLAMs process using pH-responsive chelation of calcium. The "Chelate CLAMs" process is an improvement over the patented process that controls ion availability based on pH-responsive solubility of the calcium salt. Hyaluronic acid was encapsulated in CLAMs to minimize swelling and release in aqueous formulations. CLAMs with 61% (d.b.) hyaluronic acid (HA-CLAMs) demonstrated restricted plumping, limited water absorption capacity, and reduced leaching, retaining up to 49% hyaluronic acid after 2 h in water. Alternatively, "Chelate HA-CLAMs" formed by the improved process exhibited nearly full retention of hyaluronic acid over 2 h in water and remained visibly insoluble after 1 year of storage in water at 4 °C. Successful hyaluronic acid retention in CLAMs is likely due in part to its ability to cross-link with calcium.



1. INTRODUCTION

Cross-linked alginate microcapsules (CLAMs) provide protection and long-term storage of ingredients (herein referred to as "cargo") with controlled release capabilities. Conventional methods for forming CLAMs, e.g., by external gelation, require multiple time-consuming steps that are prohibitively costly to scale up.[1] In contrast, CLAMs produced by a patented, one-step spray-drying method (UC Davis CLAMs technology) is industrially scalable.[2] *In situ* cross-linking of alginates during spray-drying is pH mediated; the calcium salt in the formulation only dissolves after atomization in a spray dryer, where volatilization of the base drops the pH, where the environment becomes favorable for calcium solubility (Figure 1). Relying on the pH-mediated dissolution of calcium during spray-dried production of CLAMs, however, has the potential to leave residual undissolved calcium salt crystals in the final product, which can be undesirable, detrimental, and unwanted for food, pharmaceuticals, and cosmetic applications.[3] Alternatively, a new process explored in this study utilizes pH-responsive chelation to control the availability of calcium ions during spray-drying.[4] The "chelate-CLAMs" process prevents ion-mediated cross-linking of alginates in the spray dryer feed by chelating soluble calcium—pH reduction of the environment beyond the pK_a of the chelator in the atomized droplets in the spray dryer releases calcium ions to facilitate alginate cross-linking. Both spray-dry CLAMs processes yield

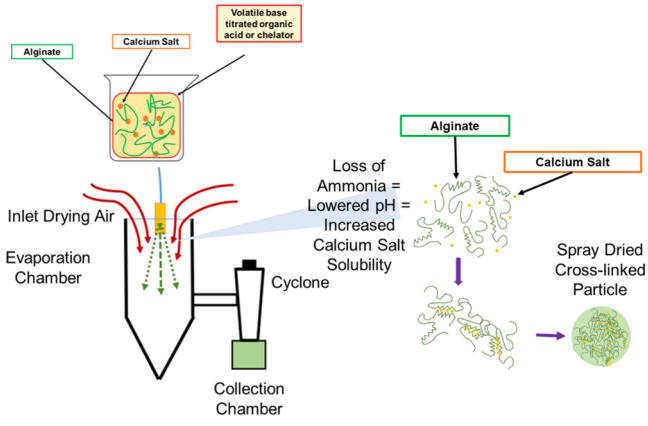


Figure 1. Schematic of patented technology for pH-mediated electrostatic cross-linking during spray-drying to produce dry, cross-linked microparticles. Schematic depicts the formation of unloaded, empty CLAMs.

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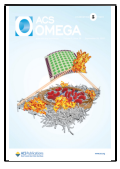

Table 1. Dry Basis Composition of the Samples Tested in This Study[a]

sample	alginate[b]	alginate (%)	hyaluronic acid (%)	calcium salt (%)	succinic acid (%)	phytic acid (%)
LV empty CLAMs	LV	57.1	0	14.3	28.6	
LV HA-CLAMs		22.1	61	5.6	11.1	
HV empty CLAMs	HV	61.5	0	0.8	30.7	
HV HA-CLAMs		24	61	3	12	
chelate empty CLAMs	HV	57.1	0	7.2		35.7
chelate HA-CLAMs		22.3	61	2.8		13.9
cross-linked HA microparticles		0	61.5	7.7	30.8	

[a]Final product for all formulations was collected in the form of a dry, white powder after water had been driven off during spray-drying. [b]LV = low-viscosity alginates; HV = high-viscosity alginates (Hydagen 558P).

uniform, dry, cross-linked powder particles in a single-unit operation; however, differences in calcium distribution and mass transfer kinetics will likely impact cross-linking and cargo retention/release. In this study, using hyaluronic acid as a cargo, we examined how the two CLAMs processes impacted the extent of cross-linking, physical properties, and controlled release of cargo in water.

Hyaluronic acid or hyaluronan (HA) is an important naturally occurring anionic glycosaminoglycan biopolymer found in cell membranes, as synovial fluid lubricant between joints, as the main component in ocular fills, and is a major component of the skin.[5] Previously, limited to surgical fillers and semipermanent injections,[6,7] hyaluronic acid use is expanding into over-the-counter personal care and cosmetics products.[8,9] Hyaluronic acid is composed of regularly ordered β-D-glucuronic acid and *N*-acetyl-β-D-glucosamine linked by alternating $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ glycosidic bonds. The proximity of the amide and carboxylic acid groups within each repeating unit facilitates tight water associations with the polymer such that hyaluronic acid is a widely documented humectant.[8,10] Few have attempted to spray-dry hyaluronic acid, all with final applications in pharmaceuticals.[11,12] In this study, hyaluronic acid was encapsulated in CLAMs (HA-CLAMs) by spray-drying, with the goal of producing dry particles with limited swelling and release during aqueous storage.

The alginate type and source can impact properties such as particle size and the extent of cross-linking.[13,14] As a process parameter, spray dryer feed viscosities modulated by the alginate source have been shown to impact CLAMs properties.[13,14] In this study, two commercially sourced alginates were compared on the basis of the resulting viscosities when dissolved in water (high-viscosity (HV) and low-viscosity (LV) alginates). The HA-CLAMs were water insoluble and exhibited unique release characteristics. Encapsulation of hyaluronic acid by the chelate CLAMs process resulted in significant improvements in preventing leaching of the cosmetic polymer in water over the patented spray-drying CLAMs process. The HA-CLAMs formed by the two processes were characterized and evaluated on the effectiveness in preventing leaching, swelling, and water absorption.

2. RESULTS AND DISCUSSION

2.1. Microencapsulation of Hyaluronic Acid in Cross-Linked Alginate Microcapsules (CLAMs). Microcapsules in this study were formed by a patented, one-step, industrially scalable spray-drying method of *in situ* ion-mediated cross-linking of acidic polymers to form dry, cross-linked alginate microcapsules (CLAMs).[2] The CLAMs formulation was varied (Table 1) to examine the role of alginates and the process to control the availability of calcium ions on the physical properties and hyaluronic acid retention during the storage of the hyaluronic acid-loaded CLAMs (HA-CLAMs) in water. For all variations, CLAMs without cargo ("empty CLAMs") and containing 61% (d.b.) hyaluronic acid as cargo ("HA-CLAMs") were formulated with the same calcium content in the final product. Additionally, cross-linked HA microparticles formed by the CLAMs process with no alginates were produced to test the ability of hyaluronic acid alone to effectively form calcium cross-links. The seven variations of microcapsules/particles generated in this study are summarized in Tables 1 and 2. Microencapsulation of HA by both traditional pH-mediated UC Davis CLAMs technology and chelated formulations of calcium chloride CLAMs were successful and characterized physically and chemically.

Table 2. Final Moisture Content and the Extent of Alginate Cross-Linking Determined for Each Hyaluronic Acid Microencapsulation Formulation

sample	feed pH[a]	final pH[b]	calcium salt[c]	moisture content (%)[d]	extent of (alginate) cross-linking (%)[e]
LV empty CLAMs	5.6	5.1	$CaHPO_4$ (i)	21 ± 2	75 ± 3^{A}
LV HA-CLAMs		5.4		11 ± 0.1	80 ± 7^{A,B}
HV empty CLAMs	7.0	4.8	$CaHPO_4$ (i)	18 ± 0.8	87 ± 1^{B}
HV HA-CLAMs		5.4		7 ± 0.1	71 ± 13^{A,C}
chelate empty CLAMs	8.4	6.0	$CaCl_2$ (s)	19 ± 0.3	82 ± 4^{A,B}
chelate HA-CLAMs		6.3		12 ± 0.1	55 ± 9^{C}
cross-linked HA microparticles	7.0	4.9	$CaHPO_4$ (i)	26 ± 0.1	n.a.[f]

[a]The pH of the feed formulation prior to spray-drying is adjusted by titrating ammonium hydroxide into the solution containing succinic acid. [b]Final pH of the sample after spray-drying, determined by measuring the pH of the water into which the particles are suspended [1% (w/v)]. [c](i) = insoluble at feed pH; (s) = soluble at feed pH. [d]The moisture content of the spray-dried CLAMs determined gravimetrically. [e]The extent of cross-linking of alginates in CLAMs is defined in eq 3. Statistical differences are noted by superscript upper case letters. [f]Cross-linked HA microparticles do not contain alginates; thus, the extent of alginate cross-linking is not applicable.

2.2. Physical Characterization of Dry Cross-Linked Microcapsules. Spray-dried CLAMs containing no cargo (i.e., empty CLAMs, Table 2) exhibit a "bowl" morphology, as shown in Figure 2a–c, consistent with previous observations.[15]

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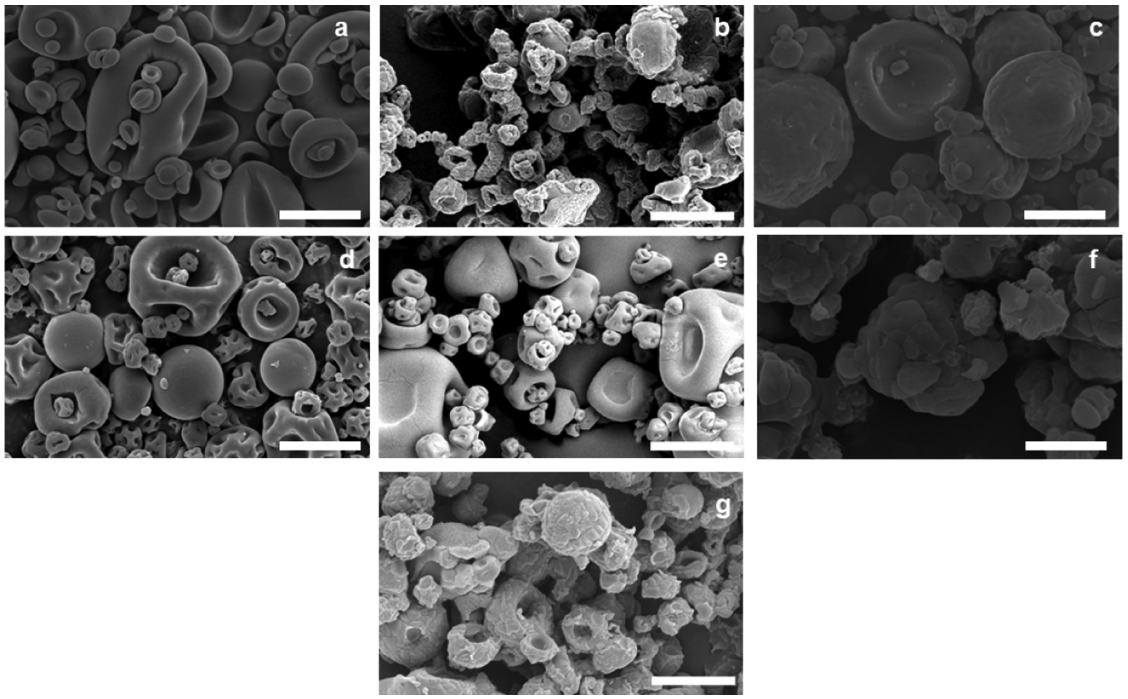

Figure 2. Scanning electron micrographs of empty CLAMs. (a) LV empty CLAMs, (b) HV empty CLAMs, (c) chelate empty CLAMs, and hyaluronic acid-loaded CLAMs, (d) LV HA-CLAMs, (e) HV HA-CLAMs, (f) chelate HA-CLAMs, and (g) cross-linked HA microparticles. Micrographs were captured at 5 kV and 5000× magnification; the scale bars represent 5 μm. The images shown are representative of the entire sample.

Empty CLAMs formed using low-viscosity alginates (LV empty CLAMs, Figure 2a) had smooth surfaces, while those formed using high-viscosity alginates (HV empty CLAMs and chelate empty CLAMs, Figure 2b,c, respectively) had rougher surface topography. Including hyaluronic acid as cargo (HA-CLAMs, Figure 2d–f) resulted in particles that appear more filled-out than the empty CLAMs. While the surfaces of HV HA-CLAMs are significantly smoother than the HV empty CLAMs, the surfaces of chelate HA-CLAMs remained rough. The cross-linked HA microparticles exhibited rough surface characteristics and topography and generally appear similar to chelate HA-CLAMs. No holes or broken particles were observed in any of the samples. There were no differences of note of the physical appearance of CLAMs produced by either formulation except for the tendency to build up excess undissolved calcium salt crystals on the surface of the original CLAMs.

The micrographs suggested that the particles in all of the samples range between ~1 and 10 μm in size. The measured size distributions, however, show broad distributions centered at ~10–80 μm, reflecting the tendency of the particles to aggregate in isopropanol (Figure 3). Despite efforts to break up aggregates by vigorous mixing and sonication, particle aggregation in isopropanol persisted. Sizing in water was not attempted because of the potential for swelling and dissolution discussed in Sections 2.3 and 2.4.

2.3. Characterization of Cross-Linked Microcapsules in Water. In addition to being biocompatible and non-immunogenic, hyaluronic acid has a high affinity for water; water uptake and retention are useful in many medical and dermal applications. The water absorption capacity (WAC) of water-absorptive polymers such as hyaluronic acid is commonly used to evaluate the ingredient efficacy by the dermal cosmetics industry.[16−19] Additionally, "plumping ratio,"

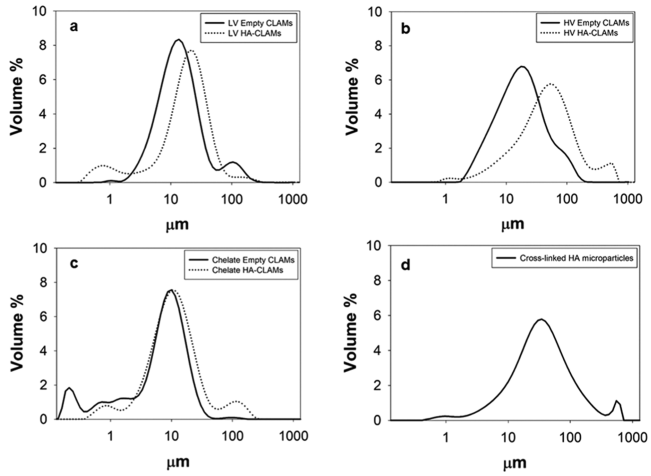

Figure 3. Size distributions obtained from the light scattering of (a) LV empty CLAMs and LV HA-CLAMs, (b) HV empty CLAMs and HV HA-CLAMs, (c) chelate empty CLAMs and chelate HA-CLAMs, and (d) cross-linked HA microparticles. Averages of 10 measurements are shown. The samples were measured in isopropanol to prevent particle swelling.

an *in tubo* measurement of product volume change, indicates the extent to which hydrogels swell with water uptake, a favorable attribute of hyaluronic acid. In this study, however, hyaluronic acid was microencapsulated with the goal of minimizing water uptake and swelling during aqueous storage to extend its shelf-stability in water.

Empty CLAMs in water (LV empty CLAMs, HV empty CLAMs, and chelate empty CLAMs) absorbed 10–15 times its original mass and exhibited ~2-fold increase in volume (Figure 4). All formulations containing hyaluronic acid as a cargo significantly decreased both water uptake and swelling of the

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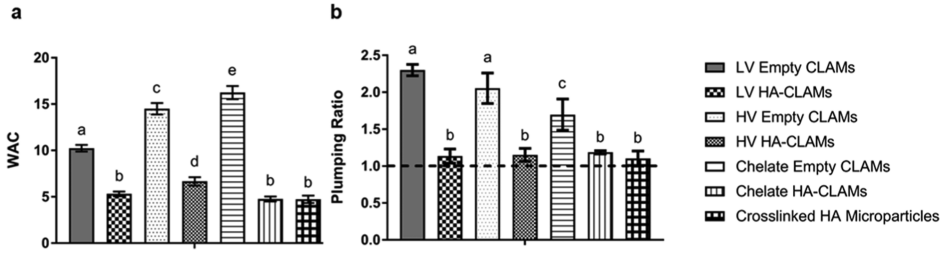

Figure 4. (a) WAC (eq 4) and (b) plumping ratio (eq 5) of all cross-linked microcapsules incubated in water for 45 min at room temperature. The plumping ratio of 1 (dashed line in (b)) indicates no swelling of the hydrated sample. Descriptions of each sample are provided in Table 1. Lower case letters representing statistical differences for either WAC or plumping, $n = 4$.

CLAMs; the WAC and plumping ratio of LV HA-CLAMs, HV HA-CLAMs, and chelate HA-CLAMs were ∼5 and ∼1, respectively. A plumping ratio of close to 1, exhibited by the hyaluronic acid-loaded CLAMs, suggests minimal swelling of the particles in water. In other words, the data in Figure 4 suggest that the addition of hyaluronic acid in the CLAMs prevented product swelling. The cross-linked HA microparticles behaved similarly in water, with a WAC of 4.7 ± 0.4 and a plumping ratio of 1.1 ± 0.1. We could not compare these results to water uptake and swelling of pure hyaluronic acid because the polymer fully dissolved in water.

The method of CLAMs formation also had some influence on the water interaction properties of the CLAMs. Chelate empty CLAMs that were formed by releasing chelated calcium during spray-drying had a higher WAC but lower plumping ratio than HV empty CLAMs formed by solubilizing calcium hydrogen phosphate during spray-drying (Figure 4). Including the hyaluronic acid cargo to form chelate HA-CLAMs resulted in a 2.4-fold decrease in WAC and a ∼0.4-fold decrease in the plumping ratio of chelate CLAMs.

Some differences were noted when either LV or HV alginates were used in the CLAMs. CLAMs formed with the higher viscosity alginates (HV empty CLAMs and HV HA-CLAMs) had higher WACs than those formed with the lower viscosity alginates (LV empty CLAMs and LV HA-CLAMs) (Figure 4a). Further characterization of these alginates and their influence on CLAMs properties is on-going. The type of alginates, however, had no significant influence on the plumping ratios (Figure 4b).

The results in Figure 4 suggest that microencapsulation of hyaluronic acid in CLAMs can limit water uptake and swelling of the product; however, these measurements did not account for any dissolution of the microcapsules and release of hyaluronic acid during incubation in water. Although the hyaluronic acid content was higher than alginate in these loaded CLAMs, it was categorized as cargo as the intent of the microcapsules was to sequester and protect hyaluronic acid. An overall successful strategy for aqueous storage stability of hyaluronic acid requires indefinite retention of hyaluronic acid in microcapsules during aqueous storage.

2.4. Alginate and Hyaluronic Acid Release During the Storage of Microparticles in Water. In the formation of CLAMs, a fraction of the alginates may not end up cross-linking within the matrix, thus will solubilize in water.[20] During spray-drying, microparticles are formed within seconds from atomized droplets produced at the spray nozzle that evaporate water rapidly. While all of the alginates in the feed become incorporated within the dried particles, not all may become cross-linked. When the dried powder sample is suspended in an aqueous solution, non-cross-linked alginates will release into the solution over time. The extent of cross-linking of the CLAMs (eq 3 and Table 2), a metric indicating the extent to which the particles will remain undissolved when suspended in water, is influenced by formulation (e.g., calcium content in the feed[3,20]) and spray-dry process conditions (e.g., solids loading and inlet temperature[13]). In this study, higher viscosity alginates resulted in more extensively cross-linked CLAMs than the lower viscosity alginates; HV empty CLAMs were 87 ± 1% cross-linked, while LV empty CLAMs were 75 ± 3% cross-linked (Table 2). Loading hyaluronic acid as cargo did not significantly impact cross-linking in the CLAMs; HV HA-CLAMs were 71 ± 13% cross-linked compared to 80 ± 7% cross-linked LV HA-CLAMs. For HA-CLAMs, choosing the higher viscosity alginates did not improve cross-linking. In contrast, hyaluronic acid as cargo appeared to significantly affect cross-linking in the CLAMs formed with chelated calcium, where chelate empty CLAMs were 82 ± 4% cross-linked, while chelate HA-CLAMs were only 55 ± 9% cross-linked.

The extent of cross-linking of CLAMs was previously shown to influence the retention of cargo in water.[3] To assess the kinetics of alginate and hyaluronic acid release during storage, the cross-linked microparticles were suspended in water and monitored over 120 min (Figure 5). Non-cross-linked alginates

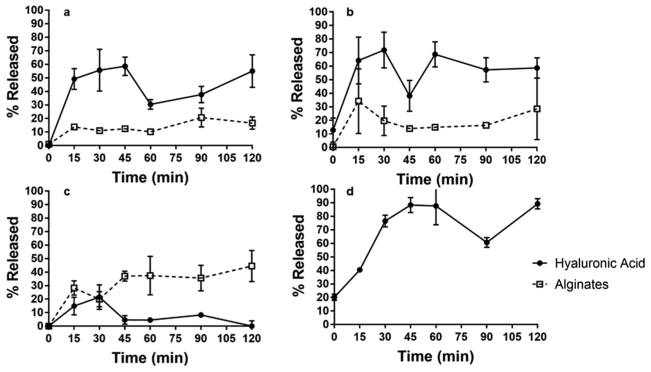

Figure 5. HA and alginate release from (a) LV HA-CLAMs, (b) HV HA-CLAMs, (c) chelate HA-CLAMs, and (d) cross-linked HA microparticles, $n = 4$.

in all CLAMs formulation dissolved within the first 15 min, and the extent of dissolution was consistent with measured extents of cross-linking (Table 2). The alginate type used in the HA-CLAMs formation influenced the initial retention of hyaluronic acid in water. At time = 0 min, 12.8% of the total hyaluronic acid released from HV HA-CLAMs (Figure 5b) while 0% released from LV HA-CLAMs (Figure 5a). By 15 min, 64 ± 17 and 49 ± 8% hyaluronic acid released from HV

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HA-CLAMs and LV HA-CLAMs, respectively. The influence of alginate type on long-term retention was less distinct. Over the course of 2 h, the HA-CLAMs formed with the higher viscosity alginates released slightly more hyaluronic acid than those formed with the lower viscosity alginates (~60−72 and ~50−59% hyaluronic acid release for HV HA-CLAMS and LV HA-CLAMS, respectively); however, the differences were not significant. Overall, microencapsulation in CLAMs successfully retained ~28−49% of the hyaluronic acid when stored in water, and this retention was minimally influenced by the viscosity of the alginates used in the microcapsules.

In contrast to previous observations with dextran-loaded CLAMs,[3] CLAMs containing hyaluronic acid did not fully release the cargo after 2 h. One reason for this difference may be because hyaluronic acid could form calcium-mediated cross-links within the CLAMs matrix, whereas dextrans lack charged groups along the polymer backbone for electrostatic interactions. The potential for hyaluronic acid alone to form insoluble, cross-linked microparticles was examined by forming cross-linked HA microparticles (Table 2 and Figure 5d). In water, ~20% of the cross-linked HA microparticles dissolved immediately, indicating that this fraction was not cross-linked within the microparticles. A nearly linear dissolution of hyaluronic acid was observed in the first 45 min in water, and only 11% remained insoluble after 2 h. Hyaluronic acid alone dissolves completely and immediately upon addition to water. Thus, the relatively slow release of hyaluronic acid from the cross-linked HA microparticles suggest that the polymer was likely cross-linked within the microparticles. Ultimately, the near complete dissolution, however, indicates that the cross-linking was weak and could be disrupted in water. Comparing the gradual but nearly complete release kinetics of hyaluronic acid from the cross-linked HA microparticles to that of rapid but incomplete release from CLAMs (Figure 5a,b) supports the hypothesis that cross-linking in the CLAM matrix facilitated long-term retention of a fraction of the hyaluronic acid. Figure 6 illustrates the release of alginate and HA from the spray-dried microparticles quantified in Figure 5. A relatively low molecular weight (MW) form of hyaluronic acid (36 kDa) was used in this study. It remains to be seen if larger MW hyaluronic acid can be more effective at forming cross-linked HA microparticles and cross-link more extensively in CLAMs.

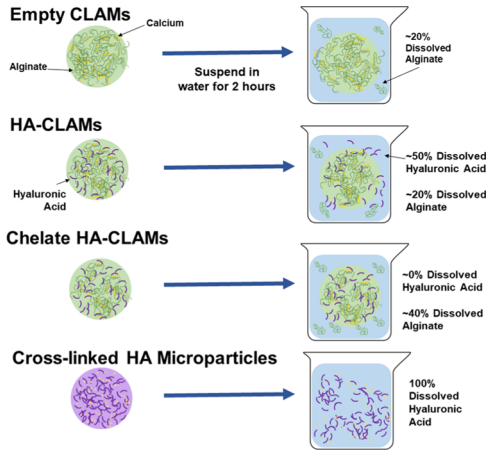

Figure 6. Illustrated schematic of microparticle alginate and/or HA release in water after 2 h.

The measurements of hyaluronic acid release over time have large error bars and fluctuate significantly because of clumping of the microparticles and gelation of released polymers in the supernatant during agitation. Nevertheless, Figure 5 demonstrates unambiguously that hyaluronic acid retention in HA-CLAMs formed with chelated soluble calcium significantly improved over HA-CLAMs formed with insoluble calcium phosphate. Chelate HA-CLAMs limited hyaluronic acid release to less than 22 ± 9% within the 2 h period, in contrast to up to ~72% from HV HA-CLAMs. Contrary to previous findings, the extent of alginate cross-linking in the chelate CLAMs did not correlate with cargo retention as the alginates in chelate HA-CLAMs were significantly less cross-linked than in chelate empty CLAMs (Table 2).

CLAMs were stored for 12 months at 1% (w/v) in deionized water at 4 °C. The samples were refrigerated for long-term storage to prevent microbial and fungal growth common with biopolymer formulations in aqueous environments containing no preservatives. Hydrated particles were visible to the eye, could be suspended upon physical disturbance, and settled out of solution over time (Figure 7). There were clear differences

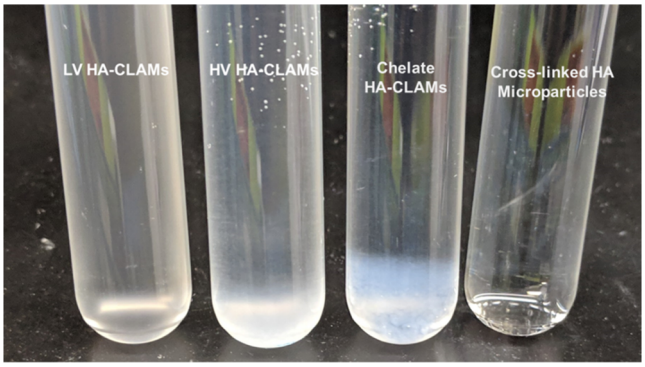

Figure 7. HA-CLAMs after storage in water at 4 °C for 12 months (1% w/v).

in the persistence of insoluble particles between the different formulations. Turbidity in the LV and HV HA-CLAM suspensions indicated residual insoluble particles. Individual, hydrated particles could be observed in the HV HA-CLAMs and chelate HA-CLAMs suspensions after the extended storage period. Thus, CLAMs show the potential to remain cross-linked during storage in aqueous formulations like those of cosmetics until release is triggered by the addition of chelators that will dissociate the calcium cross-links.

3. CONCLUSIONS

To address the problem of residual undissolved calcium salt crystals in alginate encapsulated products, a new chelate CLAMs formulation was evaluated in comparison to the state-of-the-art CLAMs. Chelate CLAMs had similar physical attributes, but far outperformed in retaining hyaluronic acid in water during storage. Hyaluronic acid likely participates in calcium-mediated cross-linking in microparticles. The polyuronic acid molecular structure avails repeating carboxylic acid groups along the biopolymer that allows for extensive electrostatic interactions with ions. This expands the technology capability and possible applications due to increased potential for using polyuronic acids as reinforcement in CLAMs while maintaining characteristic-triggered release capabilities.

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Physical characterization and release data support the notion that biopolymer active cargo can contribute to the tailored application of microencapsulation methods to improve release and barrier properties. Hyaluronic acid incorporation is not limited to CLAMs and can reasonably be tested for hydrogels, foams, and many material dispersions. Further implications of enhanced release control with a mixed CLAM matrix include improved toggling of storage and release for pharmaceutical, medical, food, and whole host of applications where fine-tuning is imperative. CLAMs successfully carried hyaluronic acid after *in situ* cross-linking with calcium during spray-drying. HA-CLAMs in water retained up to half the cargo even after 2 h, suggesting that hyaluronic acid participates in calcium cross-linking within the CLAM particles. Further, hyaluronic acid in CLAMs significantly decreased particle swelling and water uptake in aqueous environments, an advantage for the incorporation of these microparticles in water-based formulations.

4. EXPERIMENTAL SECTION

4.1. Materials. High-viscosity (HV) sodium alginate (Hydagen 558P) and sodium hyaluronate/hyaluronan (36 kDa) were provided by BASF SE. Calcium phosphate, succinic acid, sodium citrate, glacial acetic acid, sodium carbonate, β-D-glucose, sodium hydroxide, hydrochloric acid, calcium carbonate, sodium citrate, phytic acid (inositol hexakisphosphate), sodium tetraborate, sulfuric acid, and ammonium hydroxide were purchased from Thermo Fisher. Schiff's fuchsin sulfite reagent, sodium metabisulfate, periodic acid, calcium chloride, citric acid, low-viscosity (LV) sodium alginate from brown algae (catalog #A1112), and carbazole were purchased from Millipore Sigma. Ethanol (200 proof) was purchased from Koptek. Carbon tape and microscopy stands were purchased from Ted Pella. Ultrapure deionized water was sourced from a MilliQ 85/15 system (Millipore Sigma).

4.2. Methods. *4.2.1. Spray-Dried Cross-linked Alginate Microcapsules (CLAMs).* CLAMs were formed with and without hyaluronic acid as cargo following previously published methods with some adjustments.[2,3,13,21] Spray dryer feed formulations using either low-viscosity (LV) at 2% (w/w) or high-viscosity (HV) alginates at 0.5% (w/w) were adjusted to pH 5.4 and 7, respectively, by titrating succinic acid with ammonium hydroxide to maintain insoluble calcium hydrogen phosphate (as 0.5% of the feed for LV and 0.0625% of the feed for HV). Succinic acid was included at one half the concentration of alginate, or 1% for LV and 0.25% (w/w) for HV of the inlet feed. Hyaluronic acid-loaded CLAMs (HA-CLAMs) were prepared by introducing sodium hyaluronate to the feed solution during alginate hydration. HA-CLAMs formulations were prepared to result in 61% dry basis final hyaluronic acid content. Final particle compositions calculated on a dry weight basis are provided in Table 1.

Chelate CLAMs were also formed with and without hyaluronic acid cargo. This variation on the methods outlined above used HV alginate at 0.5% (w/w) in the spray dryer feed formulation. The feed solution was prepared by titrating a solution of phytic acid (inositol hexakisphosphate) to pH 8.4 with ammonium hydroxide. Phytic acid was included in the feed formulation at 5 times the concentration of calcium chloride (0.625% of solution). Hyaluronic acid was incorporated to the chelate solution to achieve 61% dry basis final hyaluronic acid content to be directly compared to previous formulations.

Spray-dried hyaluronic acid particles were formed with calcium hydrogen phosphate to assess the ability of hyaluronic acid to participate in ion-mediated cross-linking. Solutions were prepared with 2% (w/w) hyaluronic acid at twice the concentration of succinic acid (1% w/w). Succinic acid was titrated to pH 5.6 with ammonium hydroxide to prevent the solubility of calcium hydrogen phosphate, which was included at 0.25% (w/w) in the solution. In summary, all formulations were prepared at a 2:1 ratio of alginate or hyaluronic acid to succinic acid and an 8:1 ratio of polymer to calcium. These formulation ratios were determined to achieve maximum alginate−calcium cross-linking of empty CLAMs with the lowest concentration of excess calcium salt in the feed. Previous work with X-ray diffractometry showed residual unbound calcium salt was available in CLAMs above 0.25% (w/w).[3] All of the variations of the CLAMs tested in this study are summarized in Tables 1 and 2.

CLAMs were produced in a benchtop spray dryer (B-290, Büchi, New Castle, DE) at an inlet temperature of 150 °C, aspirator air flow of 35 m^3/h (maximum), feed pump at 20% of the maximum, and air nozzle flow at 40 mm. The resulting dried powders were stored in desiccators until analysis.

4.2.2. Monitoring Hyaluronic Acid Release in Water. Hyaluronic acid release from HA-CLAMs in water was monitored over 120 min at room temperature. Both empty CLAMs and HA-CLAMs were suspended at 1% (w/v) in water in separate tubes for each timed point. Samples were analyzed for hyaluronic acid release at 0, 15, 30, 45, 60, 90, and 120 min after a continuous rotation at 25 rpm. Two minutes prior to the predetermined time, samples were centrifuged at 5000 rpm for 2 min to separate residual solid CLAMs from the supernatant. The supernatant was diluted 50 times in water and measured for soluble alginate and total uronic acid (alginates + hyaluronic acid) concentrations by the periodic Schiff's fuchsin assay and the carbazole assay, respectively. Hyaluronic acid released into the solution at each time was calculated as

$$[\mathrm{HA}] = [\mathrm{UA}] - [\mathrm{SA}] \quad (1)$$

where [HA] is the concentration of hyaluronic acid (mg/mL), [UA] is the total concentration of uronic acids (mg/mL), and [SA] is the concentration of soluble alginate (mg/mL) released in the solution at the given time.

Hyaluronic acid released as a percentage of the total concentration of hyaluronic acid upon full release ($[\mathrm{HA}]_{\text{full release}}$) from the microcapsules at each time point was calculated as

$$\text{hyaluronic acid released (\%)} = 100 \times \frac{[\mathrm{HA}]}{[\mathrm{HA}]_{\text{full release}}} \quad (2)$$

Respective empty CLAMs served as controls in all release measurements.

4.2.3. Determining the Extent of Cross-Linking of CLAMs—Periodic Schiff's Fuchsin Assay. CLAMs fully dissolve in chelating solutions that sequester ions and disrupt cross-links but remain insoluble in nonchelating, aqueous solutions. Thus, the extent of alginate cross-linking in CLAMs is defined as the fraction of insoluble alginates when suspended in a nonchelating, aqueous solution.[3] To measure the extent of cross-linking, alginates solubilized from CLAMs suspended in DI water (nonchelating solution) or 100 mM sodium citrate

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buffer (chelating solution) are compared. CLAMs at 1% (w/w) were incubated for 2 h with end over end mixing in the respective solutions at room temperature, then centrifuged for 5 min at 200 rpm to separate the supernatant from any remaining insoluble CLAMs. The supernatant was diluted in water 50 times, from which 200 μL was mixed with 30 μL of a solution of periodic acid (4.67% w/v) and acetic acid (0.67% w/v) in microtiter plates and incubated at 37 °C for 1 h. The Schiff's reagent was prepared as 66.67% (v/v) solution of Schiff's fuchsin sulfite in water with 100.2 mg sodium metabisulfate and also incubated at 37 °C for 1 h. After incubation of both the samples and the reagent, 30 μL of the Schiff's reagent was added to sample wells, the microtiter plate was wrapped in foil and held at room temperature for 45 min to develop color. Sample absorbances were measured at 550 nm in a plate reader (BioTek Synergy 4) and compared to a standard curve of alginates ranging between 0 and 0.25 mg/mL to determine the concentration of alginates in each solution. Separate standard curves were generated for the low- and high-viscosity alginates. The extent of cross-linking of the CLAMs was calculated as follows

$$\text{extent of crosslinking (\%)} = 100 \times \left(1 - \frac{[\text{SA}]_{\text{water}}}{[\text{SA}]_{\text{citrate buffer}}}\right) \quad (3)$$

where $[\text{SA}]_{\text{water}}$ is the concentration of solubilized alginates in DI water (mg/mL) and $[\text{SA}]_{\text{citrate buffer}}$ is the concentration of solubilized alginates in the sodium citrate buffer (mg/mL).

The Schiff's reagent does not react with hyaluronic acid and is therefore specific to alginate quantitation when both alginates and hyaluronic acids are present in the solution.

4.2.4. Carbazole Assay for Uronic Acid Quantitation. The carbazole assay measures total uronic acids in a solution.[12,22,23] The 50 times diluted samples (50 μL) of supernatant from the hyaluronic acid release experiments were combined with 200 μL of 25 mM sodium tetraborate in 72% sulfuric acid in a polymerase chain reaction (PCR) plate and heated at 100 °C for 10 min in a thermocycler (Bio-Rad MyCycler 1709703). After cooling to room temperature, 50 μL of 0.125 mM carbazole in 200 proof ethanol was added to each well and incubated for an additional 10 min at 100 °C. Sample absorbances were read at 550 nm in a plate reader (Biotek Synergy 4) when cooled to room temperature and compared to a standard curve of equal parts alginate and sodium hyaluronate a total concentration of 0−100 μg/mL in water.

4.2.5. Scanning Electron Microscopy (SEM). Samples were adhered to stands with carbon tape and coated with 15 nm gold using a Cressington 108 Auto Coating System (Watford, U.K.). All micrographs of CLAMs were produced by a FEI/Philips XL 30 SFEG SEM or a FE-SEM Hitachi S-4100T. Unless indicated, micrographs were produced with an operating voltage of 5 kV. Representative micrographs from numerous captures at varying magnifications are presented.

4.2.6. Quantifying the Water Absorption Capacity (WAC) and the Plumping Ratio of the Cross-Linked Microparticles in Water. Water absorption capacity (WAC), the mass ratio of a hydrated product compared to its dry counterpart, indicates the extent to which the cross-linked microparticles (with and without hyaluronic acid) uptake water. The plumping ratio, i.e., the volume ratio of hydrated product compared to its dry counterpart, is a measure of the product volume change due to absorption of water. Plumping indicates the extent to which the cross-linked microparticles (with and without hyaluronic acid) swell upon water absorption.

Dry powder samples (Table 2) (0.1 g) were added to 2 g of DI water in 13 × 100 flat bottom glass test tubes and allowed to sit for 45 min at room temperature. After 45 min, the test tubes were centrifuged at 190*g* for 3 min in a swinging bucket centrifuge. The separated supernatant was removed and weighed. The mass of the hydrated powder was determined from the difference between the mass of the dry powder and added water (2.1 g) and the mass of the supernatant. The hydrated product height was measured using calipers.

The WAC was calculated as follows

$$\text{WAC} = \frac{\text{mass of hydrated powder (g)}}{\text{mass of dry powder (g)}} \quad (4)$$

The height difference between dry and hydrated product was used to determine the plumping ratio in water.

$$\text{plumping ratio} = \frac{\text{height of hydrated CLAMs (mm)}}{\text{height of dry CLAMs (mm)}} \quad (5)$$

4.2.7. Determination of Particle Size Distribution. Average particle diameter was measured using a Mastersizer 3000 (Malvern, U.K.) with isopropanol diluent to prevent swelling. Particles were submerged in isopropanol and sonicated for 10 min before measurement. The refractive index of the samples was compared to that of sugar (0.1). Data were collected as an average of 10 measurements.

4.2.8. Statistics. Statistical analyses were conducted using GraphPad Prism software (v. 7.03, GraphPad Software, La Jolla, CA). Determination of statistical differences between sample means with a 95% confidence interval (CI, $p < 0.05$) was done by one-way ANOVA with Tukey's pairwise comparisons when noted. All error values are reported as standard deviation calculated from a specified $n \geq 3$ when noted.

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Notes

The authors declare the following competing financial interest(s): Dana Wong, Herbert Scher and Tina Jeoh are co-inventors on a pending patent (UC-2017-123-1) for the

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Chelate-CLAMs technology described herein. Herbert Scher and Tina Jeoh are also co-inventors on the original CLAMs patent (US9700519B2).

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■ REFERENCES

(1) Strobel, S. A.; Knowles, L.; Nitin, N.; Scher, H. B.; Jeoh, T. Comparative Technoeconomic Process Analysis of Industrial-Scale Microencapsulation of Bioactives in Cross-Linked Alginate. *J. Food Eng.* **2020**, *266*, No. 109695.

(2) Jeoh-Zicari, T.; Scher, H.; Santa-Maria, M.; Strobel, S. Spray Dry Method for Encapsulation of Biological Moieties and Chemicals in Polymers Cross-Linked by Multivalent Ions for Controlled Release Applications. U.S. Patent US9700519B22017.

(3) Strobel, S. A.; Scher, H. B.; Nitin, N.; Jeoh, T. Control of Physicochemical and Cargo Release Properties of Cross-Linked Alginate Microcapsules Formed by Spray-Drying. *J. Drug Delivery Sci. Technol.* **2019**, *49*, 440−447.

(4) Scher, H. B.; Strobel, S. A.; Jeoh-Zicari, T.; Wong, D. E. Encapsulation by Cross-Linking of Anionic Polymers by pH Induced Dissociation of Cation-Chelate Compexes. WIPO Patent WO2020/1178732020.

(5) Collins, M. N.; Birkinshaw, C. Hyaluronic Acid Based Scaffolds for Tissue Engineering—A Review. *Carbohydr. Polym.* **2013**, *92*, 1262−1279.

(6) Pilkington, S. J.; Belden, S.; Miller, R. A. The Tricky Tear Trough: A Review of Topical Cosmeceuticals for Periorbital Skin Rejuvenation. *J. Clin. Aesthet. Dermatol.* **2015**, *8*, 39−47.

(7) Simulescu, V.; Kalina, M.; Mondek, J.; Pekař, M. Long-Term Degradation Study of Hyaluronic Acid in Aqueous Solutions without Protection against Microorganisms. *Carbohydr. Polym.* **2016**, *137*, 664−668.

(8) Jou, C.-H.; Yuan, L.; Lin, S.-M.; Hwang, M.-C.; Chou, W.-L.; Yu, D.-G.; Yang, M.-C. Biocompatibility and Antibacterial Activity of Chitosan and Hyaluronic Acid Immobilized Polyester Fibers. *J. Appl. Polym. Sci.* **2007**, *104*, 220−225.

(9) Huh, Y.; Cho, H.-J.; Yoon, I.-S.; Choi, M.-K.; Kim, J. S.; Oh, E.; Chung, S.-J.; Shim, C.-K.; Kim, D.-D. Preparation and Evaluation of Spray-Dried Hyaluronic Acid Microspheres for Intranasal Delivery of Fexofenadine Hydrochloride. *Eur. J. Pharm. Sci.* **2010**, *40*, 9−15.

(10) Iskandar, F.; Nandiyanto, A. B. D.; Widiyastuti, W.; Young, L. S.; Okuyama, K.; Gradon, L. Production of Morphology-Controllable Porous Hyaluronic Acid Particles Using a Spray-Drying Method. *Acta Biomater.* **2009**, *5*, 1027−1034.

(11) Rohrich, R. J.; Ghavami, A.; Crosby, M. A. The Role of Hyaluronic Acid Fillers (Restylane) in Facial Cosmetic Surgery: Review and Technical Considerations. *Plast. Reconstr. Surg.* **2007**, *120*, 41S−54S.

(12) Cesaretti, M.; Luppi, E.; Maccari, F.; Volpi, N. A 96-Well Assay for Uronic Acid Carbazole Reaction. *Carbohydr. Polym.* **2003**, *54*, 59−61.

(13) Strobel, S. A. *Microencapsulation of Actives in Cross-Linked Alginates Prepared by Spray-Drying*; University of California: Davis, 2017.

(14) Jeoh, T.; Wong, D. E.; Strobel, S.; Hudnall, K.; Pereira, N.; Williams, K.; Arbaugh, B.; Cunniffe, J.; Scher, H. How Alginate Properties Influence in Situ Internal Gelation in Crosslinked Alginate Microcapsules (CLAMs) Formed by Spray Drying, 2020, DOI: 10.26434/chemrxiv.12611057.v1.

(15) Strobel, S. A.; Scher, H. B.; Nitin, N.; Jeoh, T. In Situ Cross-Linking of Alginate during Spray-Drying to Microencapsulate Lipids in Powder. *Food Hydrocolloids* **2016**, *58*, 141−149.

(16) Liu, Z. S.; Rempel, G. L. Preparation of Superabsorbent Polymers by Crosslinking Acrylic Acid and Acrylamide Copolymers. *J. Appl. Polym. Sci.* **1997**, *64*, 1345−1353.

(17) Zhao, X. Synthesis and Characterization of a Novel Hyaluronic Acid Hydrogel. *J. Biomater. Sci., Polym. Ed.* **2006**, *17*, 419−433.

(18) Kiatkamjornwong, S. Superabsorpent Polymers and Superabsorbent Polymer Composites. *Sci. Asia* **2007**, *33*, 39−43.

(19) Bencherif, S. A.; Srinivasan, A.; Horkay, F.; Hollinger, J. O.; Matyjaszewski, K.; Washburn, N. R. Influence of the Degree of Methacrylation on Hyaluronic Acid Hydrogels Properties. *Biomaterials* **2008**, *29*, 1739−1749.

(20) Santa-Maria, M.; Scher, H.; Jeoh, T. Microencapsulation of Bioactives in Cross-Linked Alginate Matrices by Spray-Drying. *J. Microencapsulation* **2012**, *29*, 286−295.

(21) Strobel, S. A.; Allen, K.; Roberts, C.; Jimenez, D.; Scher, H. B.; Jeoh, T. Industrially-Scalable Microencapsulation of Plant Beneficial Bacteria in Dry Cross-Linked Alginate Matrix. *Ind. Biotechnol.* **2018**, *14*, 138−147.

(22) Bitter, T.; Muir, H. M. A Modified Uronic Acid Carbazole Reaction. *Anal. Biochem.* **1962**, *4*, 330−334.

(23) Blumenkrantz, N.; Asboe-Hansen, G. New Method for Quantitative Determination of Uronic Acids. *Anal. Biochem.* **1973**, *54*, 484−489.

https://dx.doi.org/10.1021/acsomega.0c02030
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