ABSTRACT: Through charge-driven interfacial complexation, we produced millimeter-sized spheroidal hydrogels (SH) with a core–shell structure allowing long-term stability in aqueous media. The SH were fabricated by extruding, dropwise, a cationic cellulose nanofibril (CCNF) dispersion into an oppositely charged poly(acrylic acid) (PAA) bath. The SH have a solid-like CCNF–PAA shell, acting as a semipermeable membrane, and a liquid-like CCNF suspension in the core. Swelling behavior of the SH was dependent on the osmotic pressure of the aging media. Swelling could be suppressed by increasing the ionic strength of the media as this enhanced interfibrillar interactions and thus strengthened the outer gel membrane. We further validated a potential application of SH as reusable matrices for glucose oxidase (GOx) entrapment, where the SH work as microreactors from which substrate and product are freely able to migrate through the SH shell while avoiding enzyme leakage.

KEYWORDS: complex precipitate, cellulose, enzyme entrapment, glucose oxidase, biocatalysis

INTRODUCTION

Segregative phase separation is a common phenomenon observed upon mixing of oppositely charged polyelectrolytes. When two oppositely charged polyelectrolytes are mixed, they spontaneously form a polyelectrolyte-rich segregated phase, named a complex coacervate or complex precipitate, and a polyelectrolyte-poor coexisting phase containing mainly uncomplexed polyelectrolytes. This behavior is the consequence of the favorable mixing enthalpy and increased entropy due to counterion release. Since the first theoretical description of charge-driven polyelectrolyte complexation by Voorn and Overbeek, many studies focused on the applicability of the theory for well-defined polyelectrolyte-based systems. Nevertheless, complex coacervates and complex precipitates produced by mixing of soluble polyelectrolytes with dispersed colloidal mixtures have also been reported. The formation of complex coacervates or precipitates depends on the molar ratio, $f = c^+/c^−$, between the positively ($c^+$) and negatively ($c^−$) charged moieties, where $f = 0.5$ corresponds to a 1:1 stoichiometric ratio while $f < 0.5$ correspond to the excess of polycation or polyanion, respectively. Spruijt et al. showed that independent of the polycation/polyanion molar ratio, the coacervate maintains an equimolar ratio ($f \simeq 0.5$), and the excess polyelectrolyte stays in the coexisting phase. Ionic strength and pH are also crucial factors in the formation and stability of coacervates or precipitates with the coexisting phase. For instance, Hamad et al. demonstrated that by increasing the ionic strength, the coacervate exhibited a more liquid-like behavior; they attributed this to the lowering of the electrostatic attraction between oppositely charged moieties, thus facilitating chain motion.

The charge-driven complexation of polyelectrolytes and colloids has been broadly employed for the fabrication of capsules and drug carriers. This method exploits the labile nature of the physical cross-links to fabricate materials with mechanical properties that depend on external stimuli (e.g., pH and ionic strength). Charge-driven complexation has been successfully extended to two immiscible phases, often termed interfacial complexation. In this case, one charged species is solubilized or dispersed in one phase (e.g., water), with an oppositely charged species in the second immiscible phase (e.g., oil); the two species then complex across the liquid–liquid interface. This method has been shown to form strong interfaces, resulting in the formation of stable emulsions and microcapsules.

Analogously to the interfacial complexation method, we utilize the charge-driven complexation of cationic cellulose nanofibrils (CCNF) and poly(acrylic acid) (PAA) as a simple
methodology to fabricate millimeter-sized spheroidal hydrogels (SH) with long-term stability in aqueous and saline media; this method relies on the formation of a complex precipitate at the SH/water interface, with a solid-like shell (SH-shell), and a liquid-like core (SH-core) composed of uncomplexed particles. Similar core–shell structures have been previously reported, for instance, by coextruding an alginate solution around a second liquid phase and precipitating the core–shell droplet into a divalent cation bath (sometimes termed ionotropic gelation), leading to the gelation of the alginate around a liquid core. Although these structures have long-term stability in water, they have a limited life span in the range of just a few minutes when stored in a monovalent electrolyte solution, even at concentrations as low as 10 mM, due to alginate dissolution. The use of charge-driven complexation for the production of core–shell structures at the micro- and nanoscale has been broadly reported, while to our knowledge, stable macroscopic core–shell porous hydrogels have only been reported using a combination of ionotropic gelation and charge-driven complexation of oppositely charged species. Macroscopic core–shell hydrogels offer applications in tissue engineering, cell culture, and controlled delivery of active excipients. Core–shell hydrogels are potential microreactors for biocatalysis from which substrate and product are freely able to migrate through the shell while avoiding enzyme leakage. In contrast to common microreactors containing covalently immobilized enzymes on a substrate, the physical enzyme entrapment in the liquid-like core enables high-performance biocatalysis due to the fast diffusion of targeted species (e.g., product and substrate) from and/or into the hydrogel while overcoming drawbacks related to costs, laborious purification procedures, and preservation of the native enzymatic conformation. Driven by the potential applications of SH in biotechnology applications, we investigate the structure–property relationship of cellulose-based SH in relevant conditions and provide a proof-of-concept application of SH as matrices for enzyme entrapment.

**MATERIALS AND METHODS**

**Fabrication of Spheroidal Hydrogels.** Preparation of the CCNF via grafting of the glycidyltrimethyloxonium chloride (GTMAC) (≥90%, Sigma-Aldrich) was conducted as previously described using α-cellulose (product number C8002, Sigma-Aldrich) following the semidry protocol of Zaman et al., resulting in a degree of substitution of 26% as measured by conductometric titration (presented in the Supporting Information, Figure S1). To fabricate the SH, a 2.0 wt % CCNF dispersion (pH 7) was extruded, dropwise (17.8 ± 0.2 μL), into 50 mL of 0.1 wt % PAA (Sigma-Aldrich, Mw ~ 450 kDa) aqueous solution by using a syringe equipped with a blunt end polypropylene dispensing tip (Fisnar Inc., gauge 21, inner diameter 0.513 mm, the tip positioned at ca. 2 cm from the PAA solution–air interface), and the SH was aged for 5 min except where stated otherwise. A maximum of 50 SH were produced using the same PAA bath to ensure a large excess of the polyanion. To study the effective osmotic pressure of the continuous media (Ωcw) on the swelling properties of the SH, the swelling ratio (SR) of the SH aged in different media was obtained gravimetrically as SR = WSH/Wdroplet where WSH is the weight of a single SH and Wdroplet is the weight of a droplet of the CCNF dispersion used to fabricate SH. To obtain Wdroplet, the SH were removed from the aqueous media and placed in a weighing boat, and the excess of water was removed with the aid of a filter paper prior to measurement. A SR > 1, < 1, and = 1 corresponds to swelling, deswelling, and equilibrium, respectively. SH were separately aged in different media containing various concentrations of either NaCl or poly(ethylene glycol) (PEG) (Mw = 0.4 kDa, Sigma-Aldrich, Lot #BCBN5570 V) for 3 days at room temperature. Concentrations of NaCl and PEG were chosen to generate similar osmotic pressures, measured by using the van’t Hoff equation, Ωcw = MR, where M is the molarity (mol/L), R is the gas constant (8.31 × 10⁻³ Pa L/(mol K)), and T is the temperature (K).

![Figure 1](https://dx.doi.org/10.1021/acsapm.9b01086) **Figure 1.** Schematic representation of the SH fabrication and their structure.

**Imaging.** To image the SH-shell, the SH were fractured on a glass slide, and images were acquired by using an optical microscope (Brelu Ltd. SP200 equipped with a Canon EOS 1300D). The intensity profile (in arbitrary units) as a function of distance was measured by using the "plot profile" tool in Imagej.

**Leakage and Retention of Surface-Active Molecules.** The retention of amphiphilic nonionic species within the SH was probed via surface tension (Du Noiý ring method). A series of 2.0 wt % CCNF dispersions were loaded with 500 μM Pluronic F-127 (Mw = 12.6 kDa) (F-127), 5 μM 2-hydroxyethylcellulose (Mw ~ 380 kDa, 2HEC-380, Sigma-Aldrich), or 0.5 μM 2-hydroxyethylcellulose (Mw ~ 1300 kDa, 2HEC-1300, Sigma-Aldrich). The SH were produced as previously described, and, a specific number of SH aged for 3 days in 50 mL of 100 mM NaCl solution (respectively 1, 3, and 3 SH for the F-127, 2HEC-380, and 2HEC-1300). The concentration of the amphiphilic species in the CCNF dispersion, as well as the number of SH aged in the same vial, was chosen to (i) allow detection at the water–air (W/A) interface, (ii) avoid saturation of the W/A interface, and (iii) be as low as possible to avoid significant change in the SH structure. Then, the W/A surface tension (γ) of the aged media was measured before and after SH breakage by using the Du Noiý ring method (Sigma 701 instrument, Attension, Sweden, equipped with a 9.58 mm platinum ring and a vessel of 66 mm in diameter). Breakage of the SH was conducted by employing a homogenizer (Ultra Turrax, IKA T25 digital, 30 s at 6500 rpm). Unloaded SH were employed as controls to ensure that the SH on their own did not affect γ before or after breakage. The data presented were obtained from two separate repeat experiments.

**Swelling Rate.** Immediately after the fabrication of the SH, their swelling was monitored by placing them in quartz cuvettes (1 x 1 cm²) containing 1 mL of different continuous phases (DI water, 0.1 wt % PAA, and 1000 mM NaCl solution) and pictured over time (by using a drop shape analyzer, DSA30R). From the pictures, the area of the SH (A, in pixels) was obtained through image analysis conducted using the “analyze particles” tool in Imagej and normalized as A/A0 where A is the area at time = 1 and A0 the area at time = 0. In the case of the SH rupture, the expelled aggregates were not considered as part of A; only the shell remnants were measured, giving large variation in A after rupture due to irregular changes in osmotic pressures between the SH-core and the media. The presented data were obtained from three separate experiments.

**Osmotic Pressure.** To study the effect of the osmotic pressure of the continuous media (Ωcw) on the swelling properties of the SH, the swelling ratio (SR) of the SH aged in different media was obtained gravimetrically as SR = WSH/Wdroplet where WSH is the weight of a single SH and Wdroplet is the weight of a droplet of the CCNF dispersion used to fabricate SH. To obtain Wdroplet the SH were removed from the aqueous media and placed in a weighing boat, and the excess of water was removed with the aid of a filter paper prior to measurement. A SR > 1, < 1, and = 1 corresponds to swelling, deswelling, and equilibrium, respectively. SH were separately aged in 2 mL solutions containing various concentrations of either NaCl or poly(ethylene glycol) (PEG) (Mw = 0.4 kDa, Sigma-Aldrich, Lot #BCBN5570 V) for 3 days at room temperature.
Oscillatory Rheology. The rheological measurements of 2.0 wt % CCNF hydrogels (prepared by diluting the CCNF stock dispersion with DI water and a 2.5 M NaCl solution) were performed by using a stress-controlled rheometer (Discovery HR3, TA Instruments) equipped with a sandblasted stainless-steel plate–plate geometry (40 mm). Strain sweep experiments were conducted at 1 rad s\(^{-1}\) angular frequency, and the tan δ (\(\tan \delta = G''/G'\), \(G'\) being the storage modulus and \(G''\) the loss modulus) was obtained from the linear viscoelastic region. The reported values are obtained from duplicate samples.

\(\zeta\)-Potential. The \(\zeta\)-potential measurements were performed using a Malvern Zetasizer Nano ZSP (Malvern, UK), and the \(\zeta\)-potential was evaluated via the Smoluchowski approximation. The Smoluchowski approximation is independent from the particle shape for the case of \(ka > 10\), where \(1/k\) is the Debye length and \(a\) is the radius of the rod. This means that for particles with a small double layer and large \(a\) the Smoluchowski equation can be used independently from the particle shape. For the present case of CCNF at different NaCl concentrations, the Smoluchowski equation is expected to be in its limits of validity only in the absence of NaCl (where \(1/k\) would be on the order of a few nanometers) while gaining reliability upon NaCl addition where the Debye length thins and the case of \(ka > 10\) is satisfied.\(^{30,31}\)

CCNF dispersions were prepared at 0.02 wt % at different NaCl concentrations and placed in the folded capillary electrode cell, and the \(\zeta\)-potentials measured as an average of four measurements from 100 scans each. The \(\zeta\)-potential values reported here were obtained from the average of three separate samples.

Small-Angle X-ray Scattering (SAXS). SAXS measurements on films of CCNF–PAA complexes that mimic the droplet interface were performed at Diamond Light Source (Didcot, Oxfordshire, UK), on the I22 beamline (beam energy \(E = 12.4\) keV and wavelength \(\lambda = 1\) Å). The data were collected by using a Pilatus P3-2M (silicon hybrid pixel detector, DECTRIS) with a total acquisition time of 7 s (as an average of multiple frames of 100 ms). The CCNF–PAA film was prepared by pouring, dropwise, 50 mL of 0.1 wt % PAA solution into 25 mL of 0.5 wt % CCNF dispersion under continuous stirring, allowing complete complexation of the oppositely charged moieties (and excess of uncomplexed PAA (\(f \approx 0.19\))). The complex precipitate was compressed between two stainless-steel plates with the aid of a rheometer (Discovery HR3, TA Instruments) to form a film of \(\approx\)1 mm thickness and thoroughly rinsed with DI water to remove any excess of uncomplexed PAA, prior to aging in a concentration series of NaCl solutions. After 48 h aging SAXS measurements were performed. The wet films were removed from their media, placed in a film holder, and measured in air. The time between sample loading and measurement was kept below 5 min (allowing for hutch search and motor movement), avoiding significant drying of the sample. The SAXS pattern of the 2.0 wt % CCNF dispersion was obtained after loading the suspension into glass capillary tubes (Capillary Tube Supplies Ltd.) of 1.5 mm external diameter. All the scattering data were background subtracted and transmission corrected; for the CCNF dispersion the SAXS pattern was corrected for the capillary and solvent contribution. CCNF–PAA films were modeled as fractal aggregates composed by cylindrical building blocks of radius \(R\), length \(L\) corresponding to the characteristic node-to-node distance between cylinders, mass fractal dimension \(D\), and a correlation length of the aggregate \(\xi\), which defines the fractal length scale (i.e., aggregate size). This model couples the form factor of randomly oriented homogeneous cylinders, \(P_{oc}(q) = P_{oc}(q,R,L)\), with a structure factor, \(S(q) = S(q,\xi,D)\), describing a fractal structure as derived by Teixeira,\(^{32}\) to compute the scattering intensity \(I(q)\):\(^{33,34}\)

\[
I(q) \propto P_{oc}(q)S(q) \quad (1)
\]

To reduce the number of fitting parameters, \(R\) was extracted from the fitting of the scattering pattern of CCNF dispersion at 2.0 wt % in the high-\(q\) region by using the \(P_{oc}(q)\) model (scattering pattern of CCNF in Figure S2). It was then fixed to 2.0 nm for the study of the CCNF–PAA films—this length scale being in close agreement with our
Figure 3. Aging of SH in (a) DI water, (b) 1000 mM NaCl, and (c) 0.1 wt % PAA. SH images are shown on the top of the graphs in line with the time evolution indicated by the x-axis. Dotted lines indicated the region where the linear fittings were adopted to calculate the swelling rate, \( k = \frac{d(A/A_0)}{dt} \). Image of the 24 h SH aged in 0.1 wt % PAA solution shown as an inset in (c).

Previous study\(^\text{23}\) Background subtraction and data treatment were performed using the Irena package\(^\text{23}\) while data analysis was done using the NIST SANS Analysis package\(^\text{36}\) from which the described model was used without further modification, both within IGOR Pro (Wavemetrics, Inc.).

Enzyme Entrapment and Activity. The enzyme retention within the SH matrix was studied by using SH loaded with glucose oxidase (GOx) (EC 1.1.3.4, from Aspergillus niger, 100–250 units/mg, \( M_w \sim 160 \) kDa). The SH were fabricated as previously described, employing 2.0 wt % CCNF dispersions containing GOx concentrations of 3.1, 6.2, 12.5, 25.0, 50.0, and 75.0 \( \mu \)g/mL. A GOx stock solution, prepared in phosphate buffer (100 mM, pH 6), was used to reach the specific concentrations required. The GOx-loaded SH were thoroughly rinsed in DI water and immersed in a reaction well (Costar 48-well plate) containing 125 \( \mu \)L of phosphate buffer (100 mM, pH 6). Afterward, the well plate was placed on an orbital shaker, and an aqueous glucose solution (D-(+)-glucose, 99%, Sigma-Aldrich) was added to reach a final concentration of 44 mM in 500 \( \mu \)L. To quantify the produced \( \text{H}_2\text{O}_2 \), aliquots of the continuous media (20 \( \mu \)L) were withdrawn every 5 min (for 25 min) and mixed with horseradish peroxidase, HRP (EC Type II, EC 1.11.1.7, from horseradish, 150–250 units/mg solid), phosphate buffer, and Amplex Red (ThermoFisher Scientific) to reach final concentrations of 7.5 U/mL, 20 mM, and 5 \( \mu \)g/mL, respectively. The product of Amplex Red oxidation, resoruftin, produced in a 1:1 stoichiometric ratio with \( \text{H}_2\text{O}_2 \), was quantified by using fluorescence spectroscopy; the samples measured in a black 96-well plate by using a plate reader (FLUOstar Omega, BMG-LABTECH, excitation and emission of 540 and 590 nm, respectively). The \( \text{H}_2\text{O}_2 \) concentration was calculated by using a standard curve employing known concentrations (provided by Sigma-Aldrich, 100 mM, pH 6). The GOx activity was determined by the conversion rate of the substrate (glucose) into the reaction product (\( \text{H}_2\text{O}_2 \)) per unit time and per unit volume (U/mL).  

The reusability experiment was performed by cyclically monitoring the activity of the GOx-loaded SH upon repetitive catalytic cycles (four cycles) over a time span of 8 days. Freshly prepared SH, utilizing the GOx-loaded CCNF dispersion made on day 1, were employed as control samples. All the samples were stored at 4 °C, and the GOx-loaded SH were thoroughly rinsed before and after each catalytic cycle and separately stored in 1.5 mL phosphate buffer (100 mM, pH 6). The reported values were obtained from the average of three independent samples.

### RESULTS AND DISCUSSION

Structure and Stability. The stability of the SH was found to be strongly dependent on the aging media. The SH swell in DI water while acquiring long-term stability (several months) in a media containing concentrations of NaCl greater than 10 mM. Even when tested at 80 °C in 100 mM NaCl solution, the SH did not undergo significant changes. On this ground, the structure-to-stability relationship of the SH was evaluated as a function of aging media at different osmotic pressures, \( \Pi_m \).

At first, the morphological evidence of the core–shell structure of the SH was obtained upon breakage of the SH in DI water (Figure 2a). The SH-shell did not redisperse over the probed time due to the strong CCNF–PAA complexation. Contrarily, the structure within the SH-core slowly dispersed over time, indicating the presence of uncomplexed CCNF. The presence of a core–shell structure suggests that at the moment in which the CCNF dispersion is extruded, dropwise, into a PAA aqueous solution the CCNF–PAA complexation at the interface of the droplet-shaped CCNF dispersion (see Figure 1) is fast, and the resulting SH-shell hinders further migration of the PAA into the SH-core. Moreover, because of the liquid-like properties of the extruded CCNF dispersion, the uncomplexed CCNF in the SH-core also preserved the liquid-like feature. Microscope imaging of the SH-shell (Figure 2b) displayed a thickness of ca. 1 \( \mu \)m, confirming that the SH could be well depicted by a core–shell structure, containing uncomplexed CCNF in the core and a CCNF–PAA complexation across the SH/water interface, composing the SH-shell (depicted in Figure 1). On the basis of the phenomenological characterization of Chollakup et al., we define the CCNF–PAA complexation across the SH/water interface as a complex precipitate.\(^\text{7}\)

Solute can pass through the SH-shell if their size is similar or smaller than the typical mesh size of the SH-shell; this threshold is herein termed the cutoff size of the SH-shell. To qualitatively assess the cutoff size, the SH were loaded with surface-active molecules, and their leakage was investigated by probing \( \gamma \) of the media containing unbroken or broken SH (Figure 2c). Although the amphiphilic molecules were chosen to avoid strong interactions (e.g., electrostatic) with the building blocks of the SH, to highlight the effect of their \( M_w \) on their entrapment/release, selective interactions cannot be ruled out. In addition, it must be noticed that this technique does not probe the release kinetics of the loaded amphiphilic species; rather, it solely enables evaluation of their leakage or entrapment. Adsorption of the surface active molecules in these systems to the W/A interface will be influenced by several factors such as their concentration, amphiphilicity, and \( M_w \), and so this study does not provide a means to evaluate release kinetics. F-127, having a \( M_w \) of 12.6 kDa, displayed...
complete release, as shown by almost superimposable γ curves before and after breakage, with, in both cases, a strong decrease of the air−water surface tension due to the presence of F-127 at the interface. Contrarily, the probed amphiphilic molecules with \( M_w \geq 380 \text{ kDa} \) (2HEC-380 and 2HEC-1300) did not leak from the SH matrix, as indicated by the unchanged \( \gamma \approx 72 \text{ mN/m} \) before breakage, while a sudden drop is observed after breakage due to adsorption of the released amphiphilic molecules to the W/A interface. This experiment revealed the semipermeable nature of the SH-shell and the ability of the SH to encapsulate large macromolecules while allowing small molecules to permeate through. Moreover, the unreleased molecules with \( M_w \geq 380 \text{ kDa} \) from the SH further suggest that the PAA (\( M_v \sim 450 \text{ kDa} \)) used to create the complex precipitate cannot migrate through the SH-shell after it has been formed.

The SH swelling rate in media containing DI water, 1000 mM NaCl, and 0.1 wt % PAA was monitored to gain information regarding the SH stability over time. The SH aged in DI water showed remarkable swelling over time (\( t \)) up to fracture, occurring at ca. 2400 s, clearly noticeable by the spillage of the CCNF dispersion from the SH-core, as well as scattered \( A_t/A_0 \) data points over time (Figure 3a). This phenomenon suggests that the SH swelling occurs by stretching and thinning of the SH-shell up to the formation of localized fracture points, from which the liquid-like CCNF dispersion present in the SH-core spills out. Moreover, the presence of a liquid-like SH-core supports the hypothesis that the CCNF−PAA complexation solely occurs across the SH−water interface, with the resulting SH-shell shielding the PAA penetration into the SH-core.

The SH aged in 1000 mM NaCl solution showed minimal swelling up to ca. 1200 s, after which a steady state was reached (Figure 3b). When SH were aged in the 0.1 wt % PAA solution, the swelling rate was constant over the experimental time, and no fracture of the SH-shell was observed up to 24 h (Figure 3c, inset). It is expected that upon swelling the increase in stress along the SH-shell would induce fractures as previously described for DI water. However, the suppressed fracture of the SH-shell upon continuous SH swelling in 0.1 wt % PAA indicates that upon generation of new surface area the uncomplexed CCNF of the SH-core is exposed to the SH/water interface and quickly complexes with PAA in the aging media, the PAA acting as a "patch". Comparison of the three aging media showed that the swelling rate of the SH, as captured by \( k = \frac{d(A_t/A_0)}{dt} \), was substantially greater for DI water compared to the 0.1 wt % PAA and 1000 mM NaCl media, respectively (values of \( k \) (s\(^{-1}\)) shown in Figure 3). In principle, these dissimilarities could be

Figure 4. (a) Swelling ratio (SR) as a function of \( \Pi_m \) for NaCl (top) and PEG (bottom) solutions along with images of SH with PEG. Data points are labeled with concentration of NaCl (mM) and PEG (wt %). Data points for the SH at PEG concentrations \( \leq 1.74 \text{ wt %} \) (including SH in DI water) were not acquired due to the pronounced swelling and fracture of the SH-shell as displayed by the gray background. (b, top) ζ-potential measurements and (b, bottom) tan δ of the 2.0 wt % CCNF hydrogels shown as a function of NaCl concentration. The lines are drawn to guide the eye.
justified by the mismatch of the osmotic pressure in the SH (\(\Pi_{\text{SH}}\)) and \(\Pi_{\text{m}}\). However, for the case of 1000 mM NaCl, electrostatic interactions between the charged moieties of the SH (\(\text{NR}^+\) and \(\text{RCOO}^-\) for CCNF and PAA, respectively) and the electrolyte-rich media need to be accounted for. In this regard, colloidal stability has often been described as the balance between repulsive electrostatic forces and attractive forces, where ionic strength diminishes electrostatic repulsion between particles, leading to dominant attractive forces. Specifically for the case of rod-like particles, it has been shown that their assembly, induced by the increase of ionic strength, leads to the formation of a percolated network with pronounced elastic properties.37,38

As such, to evaluate the role of the osmotic pressure of the media on the SH stability, a systematic study of the SH swelling behavior was conducted in media containing different concentration ranges of NaCl or PEG at comparable values of \(\Pi_{\text{m}}\). The use of nonionic poly(ethylene glycol) (PEG) allowed the investigation of the SH swelling behavior due to changes in osmotic pressure, \(\Pi_{\text{m}}\). Contrarily, the use of NaCl coupled changes in \(\Pi_{\text{m}}\) and charge screening of both CCNF and PAA. The SH-shell enables the retention of CCNF, present in the SH-core, while allowing migration of both NaCl and PEG. It is expected that a mismatch in osmotic pressure as \(\Pi_{\text{SH}} > \Pi_{\text{m}}\) leads to water migration from the media toward the inside of the SH, resulting in a swelling ratio, \(SR > 1\), while a mismatch in osmotic pressure as \(\Pi_{\text{SH}} < \Pi_{\text{m}}\) would lead to an opposite trend and a \(SR < 1\). However, the SH did not exhibit substantial swelling or deswelling over a large span of NaCl concentrations, and consequently \(\Pi_{\text{m}}\) tested; the \(SR \approx 1\) (Figure 4a, top). Because different swelling behaviors are expected upon changes in \(\Pi_{\text{m}}\), we hypothesize, based on the swelling theory of ionic polymeric networks, that the increase of ionic strength gradually weakens CCNF–PAA binding but enhances the CCNF interactions due to charge screening and hydrophobic or hydrogen bonding interactions between the cellulose particle surfaces, augmenting the elasticity of the network and suppressing both swelling and deswelling.59 To test the hypothesis, a concentration series of a nonionic polymer, PEG, solutions were prepared to match the \(\Pi_{\text{m}}\) of the NaCl solutions. As expected, the use of PEG revealed a clear transition from a swelling (\(\Pi_{\text{m}}\) below \(\pm 5 \times 10^5 \text{Pa}\), \(\pm 6.26 \text{ wt } % \text{PEG}\)) to a deswelling regime upon increasing \(\Pi_{\text{m}}\) above \(5 \times 10^5 \text{Pa}\) (Figure 4a, bottom), a missing feature for the case of the NaCl containing media. The lack of further swelling at the lower PEG concentrations (\(\leq 1.74 \text{ wt } %\)) and the sedimented CCNF at the bottom of the vial indicated that swelling occurred up to the SH-shell fracture. As such, this experiment revealed that the swelling and deswelling of the SH could be either modulated via the osmotic pressure of the uncharged medium or even strongly suppressed through modulation of the ionic strength in the media, supporting our hypothesis. The remarkable survival of the SH in 100 mM NaCl solution at 80 °C also demonstrates the strong interactions present in the shell of these capsules in the presence of electrolyte.

The strength of the SH arises from both the shell and the core; hence, the two contributions must be considered separately. Because the SH-core is PAA-free, the effect of NaCl on the SH-core was probed through \(\zeta\)-potential and oscillatory rheology measurements of the 2.0 wt % CCNF hydrogels (not in the SH form) to gain information about inter fibrillar interactions within the SH-core (Figure 4b). The \(\zeta\)-potential of CCNF was found to decrease upon NaCl addition, indicating neutralization of the positively charged moieties via electrostatic screening as previously reported for similar systems.37,38 It is noted that the value of \(\zeta\)-potential = 30 mV sets an empirical threshold between repulsive (electrostatic dominated) and attractive (van der Waals and hydrogen bonds dominated) regimes and finds its root in the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory.40 Noticeably, addition of 10 mM NaCl was sufficient to overcome the repulsive electrostatic regime, leading to enhancement of attractive inter fibrillar attractions.40

The tan \(\delta\) of the CCNF hydrogels followed a similar trend as for \(\zeta\)-potential, indicating the formation of a more pronounced gel-like interconnected network upon addition of NaCl, as

Figure 5. (a) SAXS patterns of the CCNF–PAA films at different NaCl concentration fitted using a model of fractal cylinders. The scheme depicts the rod-like CCNF and parameters used in the model of fractal cylinders. (b) \(L\) (top) and \(D\) (center) values obtained by fitting of the fractal model to the SAXS data. The line is drawn to guide the eye. (b, bottom) Schematic representation of the possible mechanism of CCNF aggregation upon NaCl addition.
previously observed for similar systems. These results support the hypothesis of network strengthening in the SH-core due to charge screening and augmented CCNF–CCNF interactions. However, the properties of the solid precipitated SH-shell upon NaCl addition were not directly assessed.

Because insights into the SH-shell structure at the nanometer length scale are very complicated to obtain in situ, we mimicked the SH-shell structure by preparing CCNF–PAA films aged at different NaCl concentrations and probing their microstructure via SAXS (Figure 5a). All the SAXS patterns displayed similar high-q patterns, associated with the CCNF cross section, as for the pure CCNF (Figure S2), confirming the main contribution of CCNF to the scattering intensity in the CCNF–PAA films as well as unvaried CCNF cross section upon addition of NaCl.

A pronounced difference between samples was noticeable in the intermediate-q and low-q range. In these ranges, greater length scales than the CCNF cross section are probed (e.g., persistence length). The SAXS patterns did show the absence of a Guinier region (a plateau in the low-q region), indicating that the characteristic dimension of the object is not fully probed. Moreover, the low-q slopes substantially diverged from a $q^{-4}$ slope which is associated with scattering from dilute solutions of elongated objects, indicating a pronounced level of interactions between CCNF.

To better compare the SAXS patterns, the CCNF–PAA were modeled as fractal aggregates composed of cylinders, resembling CCNF, as building blocks, (as schematically represented in Figure 5a, inset). In this model, the contribution of the PAA is not included due to its negligible contribution to the scattering intensity. This is justified by the greater CCNF wt % in the CCNF–PAA film (composed by ca. 10.5 times the amount of CCNF compared to PAA, on a dry basis, as estimated for a 1:1 stoichiometric ratio ($f = 0.5$) between the oppositely charged moieties (see the Supporting Information for an estimation of $f$)). The $L$ yielded by the model, corresponding to the characteristic node-to-node distance between CCNF (Figure 5a, inset), did not show a pronounced variation in the 50–1000 mM NaCl range, indicating that the fractal network is constant. However, a smaller value of $L$ was obtained in the absence of NaCl (Figure 5b, top), suggesting that the presence of electrolyte weakens PAA–CCNF interactions, allowing some initial network expansion in the presence of NaCl. It is noted that the value of the correlation length of the aggregate, $\xi$, was found to be much greater (at least by 1 order of magnitude) than the values of radius of gyration, $R_g$, of the cylindrically shaped CCNF ($R_g^2 = (\text{radius}^2/2) + (\text{length}^2/12)$), indicating that both $L$ and $D$ are independent of the value of $\xi$ (values of $\xi$ in Figure S3).

The mass fractal dimension, $D$, scales with the number of primary CCNF particles, $i$, as $i \propto \left(\frac{\xi}{R_g}\right)^D$, assessing the density of CCNF within $\xi$. The values of $D$ obtained from the fractal model followed a gradual increase with increasing NaCl concentration from 50 to 1000 mM, indicating aggregation and densification of CCNF (Figure 5b, center, and schematic in Figure 5b, bottom). In the literature, the response of oppositely charged polyelectrolytes to subsequent addition of salt has been reported to (i) reduce aggregation up to dissolution, (ii) induce additional aggregation, or (iii) have a negligible effect depending on the characteristic of the polyelectrolytes.

Specific instances of (i) were provided by Hamad et al. and Spruijt et al., who found an increase in mesh size and a more pronounced liquid-like behavior in polyelectrolyte mixtures upon an increase of ionic strength due to diminishing polycation–polyanion attractive forces. The case of additional aggregation (ii) upon addition of NaCl has been reported by Dautzenberg and co-workers, termed secondary aggregation, and mainly attributed to the presence of strong ionic groups and a mismatch in charge density between the oppositely charged polyelectrolytes. Similarly, it is possible that, for the CCNF–PAA complexation the enhanced aggregation of the fibrillar particles upon NaCl addition arises due to the charge screening of neighboring, uncomplexed, charged moieties of CCNF, allowing these to aggregate further (schematic in Figure 5b, bottom). It is noted that the pattern of increasing $D$ upon NaCl addition does not apply to the case of 0 mM NaCl, where $D$ displayed a greater value than in the 50–500 mM NaCl range. This may be addressed by the substantially smaller building blocks of the network, as captured by the characteristic node-to-node distance between fibrils, $L$, in the absence of NaCl, which would allow better packing and consequently a denser network.

**SH as Microreactors for Biocatalysis.** Hydrogel beads have been broadly exploited for the encapsulation and release of active components. Nonetheless, the long-term entrapment of macromolecules has been stressed as an outstanding challenge, limiting their application and reusability. On this ground, we investigated the entrapment of a model enzyme, GOx, within SH by monitoring its catalytic activity. As expected, the GOx activity increased as a function of the concentration loaded into the SH. More interestingly, the activity of the GOx-loaded SH approached a plateau at higher concentrations, suggesting limited substrate diffusion into the SH matrix (Figure 6).

Figure 6. Catalytic activity of GOx-loaded SH as a function of the initial GOx concentration in the CCNF dispersion (employed for the production of the SH); the line is drawn to guide the eye. Inset displays the activity of GOx-loaded SH upon catalytic cycles and as a function of storage time ($4{°}C$) expressed in days. The 50 $\mu$g/mL GOx-loaded SH were employed to test their reusability over a time span of 8 days (Figure 6, inset). The GOx activity showed excellent retention of activity upon storage and catalytic cycles with only a minimal decay on the fifth day. The GOx activity measured on the eighth day was compared to a control sample of freshly prepared GOx-loaded SH, fabricated utilizing an 8 day vintage GOx-loaded CCNF dispersion. The GOx activity in the control sample showed a similar value to the GOx-loaded SH on the eighth day, indicating that no significant GOx leakage.
occurred upon storage or catalytic cycles. Moreover, the control sample showed a slightly lower value compared to the GOx-loaded SH on day 1, suggesting that the long-term GOx storage in aqueous media may be an influencing factor on its activity. The ability of the SH to retain enzymes for several days is a feature which differs from the previously reported alginate beads where substantial protein leakage occurred within a few hours.\textsuperscript{47,48} Moreover, the SH resilience in saline solutions, as demonstrated here by storage in phosphate buffer and NaCl differs from for instance, alginate beads which undergo dissolution under similar conditions.\textsuperscript{13,13,46} This highlights the potential of these SH in biotechnological applications.

**CONCLUSION**

This work aimed to characterize SH produced by a charge-driven interfacial complexation process. We found that via extruding, dropwise, a dispersion of positively charged cellulose nanofibrils, CCNF, into an aqueous bath containing negatively charged polymer, PAA, SH were formed. The SH were characterized by a liquid-like core, composed of uncomplexed CCNF and a solid-like shell formed by the CCNF-PAA complexation. The SH-shell did not redisperse in pure water and saline environments due to the strong CCNF-PAA complexation. The SH-shell did not redisperse in pure water and saline environments due to the strong CCNF-PAA complexation and possessed characteristic features of a semipermeable membrane, allowing the release of small macromolecules ($\leq$12.6 kDa) while retaining larger ones. Moreover, the semipermeable nature of the SH-shell allowed a tunable swelling behavior of the SH according to the osmotic pressure of the continuous media. We found that insensitivity to swelling was achieved in saline media, associated with the enhancement of interfibrillar interactions in both the SH-core and the SH-shell. Furthermore, we validated the applicability of SH as suitable matrices for physical enzyme entrapment, allowing the use and reuse of SH as microreactors from which substrate and product are freely able to migrate through the SH while avoiding enzyme leakage.

**ASSOCIATED CONTENT**

* Supporting Information
  The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsapm.9b01086.

  Conductometric titration of CCNF, estimation of the molar ratio ($f$), SAXS data of the 2.0 wt % CCNF dispersion and values of $\xi$, yielded by the model of fractal cylinders, as a function of NaCl concentration (PDF)

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