Catechol-Functionalized Chitosan/Iron Oxide Nanoparticle Composite Inspired by Mussel Thread Coating and Squid Beak Interfacial Chemistry

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ABSTRACT: Biological materials offer a wide range of multifunctional and structural properties that are currently not achieved in synthetic materials. Herein we report on the synthesis and preparation of bioinspired organic/inorganic composites that mimic the key physicochemical features associated with the mechanical strengthening of both squid beaks and mussel thread coatings using chitosan as an initial template. While chitosan is a well-known biocompatible material, it suffers from key drawbacks that have limited its usage in a wider range of structural biomedical applications. First, its load-bearing capability in hydrated conditions remains poor, and second it completely dissolves at pH < 6, preventing its use in mild acidic microenvironments. In order to overcome these intrinsic limitations, a chitosan-based organic/inorganic biocomposite is prepared that mimics the interfacial chemistry of squid beaks and mussel thread coating. Chitosan was functionalized with catechol moieties in a highly controlled fashion and combined with superparamagnetic iron oxide (γ-Fe₂O₃) nanoparticles to give composites that represent a significant improvement in functionality of chitosan-based biomaterials. The inorganic/organic (γ-Fe₂O₃/catechol) interfaces are stabilized and strengthened by coordination bonding, resulting in hybrid composites with improved stability at high temperatures, physiological pH conditions, and acid/base conditions. The inclusion of superparamagnetic particles also makes the composites stimuli-responsive.

Introduction

Synthetic materials that mimic the chemical and structural complexity of biological systems are the “holy grail” of bioinspired composite formulation. With increasing understanding of structure–property relationships of many natural materials at various length scales, from the molecular level up to the micro- and macro-scale, an increasing number of bioinspired synthetic strategies are emerging. Among the various length scales probed in biological materials, the molecular scale, which particularly includes the elucidation of primary sequence in protein-based materials, their post-translational modifications, or their interfacial chemistry, perhaps remains the most elusive. However, there are notable exceptions of biological materials whose molecular-scale designs have been elucidated, and those represent unique source of bioinspired synthesis strategies. Two such materials include squid beaks and mussel thread cuticle coatings, whose strengthening mechanisms at the molecular scale have recently been revealed. Squid beaks are highly sclerotized chitin-protein-water composites with graded structural properties that correlate with molecular gradients. The compliant region attached to the jaw muscular tissue is mostly composed of hydrated chitin (∼80 wt. pct water) with a small amount of hydrophilic proteins. Moving toward the hard rostrum, the water is gradually exchanged by Gly- and His-rich proteins. These proteins impregnate the chitinous nanofibrillar network, and are subsequently stabilized by covalent cross-linking between His and catecholic residues, the latter originating either from post-translational modification of Tyr into dihydroxyphenylalanine (DOPA), or from secretion of low-molecular weight catecholic compounds. This curing mechanism has a dual role in squid beak maturation. First, it results in a dense cross-linked network of proteins that enclose the chitin nanofibrillar network. Second, it drives water molecules out of the chitin network, stiffening the chitin fiber by localized dehydration. Hence, a key physicochemical characteristic is the controlled desolvation of molecular-bound water from the chitin polymer. In mussel thread coatings, a central and unusual...
characteristicthathardens theprotectivelayer (knownasthe cuticle) is the presence of DOPA-Fe³⁺ coordination bonds, which cross-link the cuticle proteins into dense micro- and nanoscale globular particles.⁴,⁵ Under mechanical stress, the load is transferred onto the dense DOPA-Fe³⁺ globular regions, circumventing cuticle failure through formation of microfractures, highlighting the role of the DOPA-Fe³⁺ coordination as a sacrificial bond.⁶

In the first stage of our composite strategy, we functionalized chitosan fibers (CS) with catecholic moieties (CAT) in a controlled manner, with the dual goals of (i) reducing the number of hydrophilic amine motifs along the backbone, and (ii) providing the polymer with side-chain functionality that triggers subsequent cross-linking (Scheme 1). Catecholic functional groups were chosen because of their versatile characteristics,⁷ including covalent cross-linking⁸ and coordination bonding.⁹ This is followed by induced coordination of functionalized chitosan with superparamagnetic iron oxide (γ-Fe₂O₃) nanoparticles, allowing catecholic side groups to form a robust coordinated-networked network with the surface of iron oxide nanoparticles, similar to the catechol-Fe³⁺ coordination cross-linked network observed in mussel thread coatings.⁶ The required functionalized chitosan polymer. 1-DOPA ; ¹H NMR (400 MHz; D₂O, δ = 7.26 ppm, 13C = 77.0 ppm) and water (1H, DHA, 1H, DHA, 7.50 (m, 3H, DOPA) 3.82 (s, 1H, chitosan), 3.18 (s, 3H, chitosan), 2.81 (s, 2H, DHA) 2.71–2.69 (m, 1H, DHA), 2.53–2.56 (m, 1H, DHA), 2.31–2.78 (m, 2H, DHA). DOPACS (3), ¹H NMR (400 MHz; D₂O, δ = 7.79–7.49 (m, 3H, DHA), 3.81 (s, 1H, chitosan), 3.63 (s, 2H, Chitosan), 3.07 (s, 3H, chitosan), 2.81–2.79 (m, 2H, DOPA). DHA CS (3), ¹H NMR (400 MHz; D₂O, δ = 7.79–7.49 (m, 3H, DHA), 3.81 (s, 1H, chitosan), 3.63 (s, 2H, Chitosan), 3.07 (s, 3H, chitosan), 2.81–2.78 (m, 2H, DHA), 2.71–2.69 (m, 1H, DHA), 2.53–2.56 (m, 1H, DHA).

Chitosan Polymer-Fe₂O₃ Nanoparticle Incorporation Preparation (mnp-CATCS). To a solution of requisite chitosan polymer (100 mg) in 15% acetic acid (20 mL) was added Fe₂O₃ nanoparticle (1 equiv or 0.1 equiv respective to CATCS monomer unit) in 1 mL in a single portion and allowed to stir for 72 h. The solution was dialyzed with water (3 × 4 ltr changes), and the solution decanted from unreacted nanoparticles into glass molds (~5 cm diameter) and cured under atmospheric conditions. The resulting cured film was washed with water to give the desired composites.

Surface Chemistry Analysis. 1H NMR spectra were obtained using a Bruker AVANCE I 400 MHz spectrometer in either deuterated dimethyl sulfoxide, chloroform, or water. Dimethyl sulfoxide (1H, D₂O, 1H = 2.50 ppm, 13C = 39.5 ppm), chloroform (1H = 7.26 ppm, 13C = 77.0 ppm) and water (1H = 4.79 ppm) were used as internal standards for NMR analysis. Arnows assay was performed as per literature for each polymer to identify the presence of catechol.¹³¹⁴ UV–vis spectra were obtained using a Thermo Scientific NanoDrop 2000c spectrophotometer for all modified chitosan polymers. Fourier transform infrared (FTIR) spectra were recorded for all polymers using a Bruker Vertex 70. For Raman spectroscopy, a continuous laser beam was focused on the sample through a confocal Raman microscope (CRM200, WITec) equipped with a piezo-scanner (P-500, Physik Instrumente). The diode-pumped 785 nm laser excitation (Toptica Photonics AG) was used in combination with a 20× objective lens (Nikon, NA = 0.4). The spectra were acquired using a CCD camera (DU401A-DR-DD, Andor) behind a grating (300 g mm⁻¹) spectrograph (Acton, Princeton Instruments Inc.) (for each spectrum a total of 30 s integration time was applied). The ScanCtrlSpectroscopyPlus software (version 2.08, Witec) was used for spectra processing.

Physical and Magnetic Properties Analysis. Transmission electron microscopy samples of the nanoparticles were prepared by drying a drop of the aqueous colloidal suspension on a copper grid. A JEOL 1010F transmission electron microscope operating at 200 kV was used for bright field imaging and particle size measurements were made using ImageJ software (see Figure S1a of the Supporting Information, SI). X-ray diffraction scans were performed on the iron oxide nanoparticle powder using a Shimadzu 6000 X-ray diffractometer with Cu Kα radiation of wavelength 1.54 Å at a scan rate of 0.5° min⁻¹ through the 2θ range of 10°–90°. The intensity vs 2θ plot was matched to reference JCPS files to indentify the phase and index the peaks (see Figure S1b of the SI). Magnetic measurements of the iron oxide nanoparticles as well as composite films were performed at room temperature up to an applied field strength of 20 kOe with a vibrating sample magnetometer attached to a Quantum Design PPMS-6500 physical property measurement system.

Mechanical Properties Analysis. Nanoindentation studies of composites were completed using a Hysitron TI950 tribolinder. Samples were mounted in epoxy resin before analysis. Dehydrated conditions: Using a 20 μm conical diamond probe with a load of 300 μN a series ~20–25 indentations across a 200 μm grid were
performed for each composite. Hydrated conditions: Samples were presoaked before analysis in ultrapure milli-Q water. With a 20 μm conical diamond probe a series of ∼20−25 indentations at a controlled load of 100 μN across a 200 μm grid were acquired for each composite. Thermal gravimetric analysis of the composite films was performed using a TA Instruments Q500 TGA system. Weight loss of bare iron oxide nanoparticles as well as pure chitosan was measured for reference. Samples (∼5 mg) were heated in the temperature range 30−700 °C at a ramp rate of 10 °C·min⁻¹ in air and N₂.

■ RESULTS AND DISCUSSION

Using water-soluble EDCI/HOBT carbodiimide chemistry, a series of CATCS polymers were prepared with a catecholic content in the range of 20−82%, representing the highest reported example of covalently bonded catechol into a polysaccharide backbone (see Table S1 of the SI). In tandem, recent developments in catechol functionalization of the chitosan backbone have emerged. A similar level of catecholic functionalization was recently reported on polysiloxane elastomers by Heo et al., with the goal of providing high affinity toward oxides. By limiting the addition of catechol, amidation could be achieved with highly controlled monomeric ratios. Typically, 1D HNMR (see Figures S2−S5 of the SI) spectrum showed resonances in the region of 6.64−6.81 ppm, attributed to the aryl motif of the catechol with reduction of the amine peak at 3.08 ppm, all of which confirmed the amidation of chitosan’s backbone. The “active” phenolic motifs were established through Arnow’s staining, an effective assay in identifying the presence of catecholic motifs. Iron oxide nanoparticles were incorporated into CATCS polymers, and the impregnation process was controlled such that molar ratios of 1:1 and 1:0.1 (CATCS: MNP) were obtained and cast into thin films. With ∼82% catechol functionalization (4a), the composites yielded a clustered microstructure, while with ∼47% functionalization, the composites (4b/d) were brittle.

During thin film deposition, appropriate films of uniform thickness (35−100 μm) were only obtained when the composites catecholic content was below 50% (4c/e), presumably due to the intrinsic hydrophobic interactions with the high catecholic content. These composites were subsequently characterized in more detail.

In order to probe the nature of the functionalized chitosan/Fe₂O₃ interfaces, the composites were investigated by FTIR and Raman spectroscopy at various stages of processing. FTIR spectroscopy (Figure 1a) of CATCS polymers showed the expected signals associated with the polysaccharide backbone and the presence of the catecholic motif including the aryl (1405 cm⁻¹) and phenolic alcohol bands (1267 cm⁻¹). Physical attachment of the catechol was confirmed with the presence of the amide I/II and carbonyl bands in the region 1532−1706 cm⁻¹. For unmodified chitosan, iron oxide addition resulted in a sharpening of the amine band with a shift of the CO alcohol band, suggesting close proximity of the iron oxide to these motifs. Solubility tests (see Table S2 of the SI) revealed that these observations are most likely a result of physical entrapment and not coordination for unmodified chiton-based composites. The FTIR spectral changes observed for mnp-CATCS composites were more pronounced, confirming the formation of the γ-Fe₂O₃-catechol coordination bond through loss of the phenolic band at 1260 cm⁻¹. Further indicative of the new coordination bonds, the aryl vibrational bands broadened and split into two distinct signals, which, commonly occurs when aryl groups are in close proximity to metals. The amide I/II bands associated with the amide bond grew in intensity, which is also attributed to the close proximity of these motifs to iron oxide. These changes in FTIR spectra signal highlighted the successful coordination of iron oxide with our catechol-chitosan composites.

Figure 1. FTIR analysis (A) of functional chitosan and composites, pH 3 and Raman spectra (B) of (1:1) mnp-DOPA_CS and mnp-DHA_CS composites.
Raman spectroscopy of mnp-CATCS (Figure 1b) showed the presence of key $[\text{M}-\text{O}]$ vibrational frequencies in the range 500 to 700 cm$^{-1}$, which also supports the presence of $\gamma$-Fe$_2$O$_3$-catechol coordination bonds. These vibrational frequencies have been previously outlined as key features for oxygen-metal chelation, with the 591 and 635 cm$^{-1}$ indicative of coordination of the metal center by the oxygens on C3 and C4 of the catechol ring, respectively. The 540 cm$^{-1}$ band arises upon charge transfer that proceeds only when both phenolic motifs are chelated to a transitional metal as is the case with $\gamma$-Fe$_2$O$_3$-catechol chelation. Upon wetting the samples, the signals associated with chelation remained unchanged even with applied strains 50% that of the strain to failure. These results suggest that the $\gamma$-Fe$_2$O$_3$-catechol coordinated network is stabilized by a relatively strong binding energy and that the coordinated bonds do not undergo reversible binding interactions, as has been documented for Fe$^{3+}$-DOPA rich mfp-1 protein but in contrast to functionalized PEG single-molecules. Further to this TGA measurement indicated that these prevailing coordination bonds are thermally stable up to 276 °C (see Figure S6 and Table S3 of the SI).

With higher catecholic (DOPA/DHA) content in the composites, water uptake decreased leading to a dehydration effect due to the increasing hydrophobic motifs expelling water. In tandem, this modification led to improved stability (Figure 2a), with all of the modified films shown to be resistant to a wider pH range from basic ($\sim$10) to acidic ($\sim$3) conditions. It has been well-documented that chitosan is soluble in mild acidic conditions with its exposed hydrophilic amine groups protonated at low pH. With our process, the newly formed amide bond decreases the charged state of chitosan at low pH, thus improving the stability of the CATCS composites at pH 3. Addition of iron oxide into neat chitosan composites gave materials that were still susceptible to mild acidic environments, with the composites completely degrading within 24hrs. This was not seen for the mnp-CATCS composites, highlighting the importance of $\gamma$-Fe$_2$O$_3$-catechol coordination in maintaining physical integrity and opening the pathway for use under physiological conditions, which is a key feature for biomedical applications. In summary, the vibrational spectroscopy data indicate that our composites successfully mimic key interfacial chemistry features from both squid beaks and mussel thread cuticles. First they mimic through catechol-assisted dehydration the controlled dehydration of chitin as in the squid beak; and second the functionalized chitin/inorganic interfaces are stabilized through the formation of $\gamma$-Fe$_2$O$_3$-catechol coordination bonds that mimic DOPA-Fe$^{3+}$ bonds detected in cuticle mussel threads.

The magnetic property measurements (Figure 2b; see Table S4 of the SI) showed the expected magnetization properties for 1:1 and 0.1:1 mnp-CATCS composites, with a 10- and 100-fold decrease in magnetization compared to bare $\gamma$-Fe$_2$O$_3$ (66.1 emu·g$^{-1}$). This demonstrates the first addition of superparamagnetic iron chitosan-based scaffolds. The appropriate decrease in magnetic saturation with mass fractions (CATCS:MNP; 10:1 and 100:1) indicates that: (i) iron oxide nanoparticles retain their magnetic properties once coordinate-bonded, and (ii) no unbound iron oxide is present within the composite.
The mechanical properties of the composites were probed by nanoindentation in both dry and hydrated states, using a spherico-conical tip (20 μm nominal radius) and Hertzian contact mechanics. Data summary (see Table S5 of the SI) and representative curves are presented in Figure 3. Nano-mechanical values of the elastic modulus of chitosan and other polysaccharides are usually obtained using the classical Oliver-Pharr analysis. However, we found that chitosan-based materials in this work systematically exhibited moduli approximately 3-fold higher when using the Oliver-Pharr method instead of the loading Hertzian portion of an indentation cycle, and attribute this phenomenon to poroelastic behavior. Chitosan film contain a certain degree of porosity, hence it is likely that the network is axially packed during indentation, leading to higher modulus values at the peak load. The Hertzian contact solution is thus deemed more accurate in order to obtain elastic moduli of our materials. The indentation response of functionalized chitosan in the dry state highlighted a 25% stiffer network with addition of the catechol motif. Along with the hydrogen bonding of chitosan, addition of the phenolic motif can induce additional aryl interactions, as well as increase dehydration and π−π organized packing. With MNP addition, the elastic modulus of mnp-DOPACs increased, going from 1.45 GPa (±0.06) for unreinforced DOPACs to 1.65 GPa (±0.17) with 0.1 M iron oxide, up to 1.88 GPa (±0.19) with 1 M iron oxide. The increase in modulus was more pronounced for mnp-DHACS composites, with elastic modulus values of 1.84 GPa (±0.21) and 2.37 GPa (±0.29) for 0.1 and 1 M iron oxide, respectively. We speculate that with 0.1 molar iron oxide, the moderate increase in elastic modulus is a result perhaps due to γ-Fe₂O₃-DOPA coordination bonds being masked by the inherently high stiffness of dry chitosan fibers. Increasing iron oxide content to 1 molar led to high increases in elastic modulus, with mnp-DHACS demonstrating nearly a 2-fold increase while in the case of mnp-DOPACs the elastic modulus only increased slightly. We speculate that this observation is a function of the amine side arm of DOPA imparting “sites” of irregular organizational packing in the composites, which decreases H-bonding and aryl π−π interactions.

In the hydrated state, differences between unmodified chitosan and the hybrid composites become more obvious, with the addition of MNPs mitigating the loss in stiffness upon hydration. Expressed in terms of the ratio E₆₆₆/E₆₀₀ (Figure 3b) the relative decrease was lower for functionalized chitosan, and even less pronounced for iron oxide reinforced composites. The E₆₆₆/E₆₀₀ ratio was 0.24 for raw chitosan, increasing to 0.37–0.48 for functionalized chitosan, and maximal values in the range 0.54–0.58 for MNPs-reinforced composites. Representative nanoindentation curves obtained under hydrated conditions (Figure 3c) demonstrate the clear effect of adding MNPs on the indentation response of mnp-CATcS composites. It should also be mentioned that in the wet state, no significant
differences were noticed for the two types of composites (mnp-DOPA_{CS} versus mnp-DHA_{CS}), with similar elastic moduli values obtained in both cases. Overall, the best-performing composite (mnp-DHA_{CS} 1:1 molar ratio) displayed a more than 3-fold increase in elastic modulus in comparison to raw chitosan in hydrated conditions with a value of 1.3 GPa, which is higher than currently available chitosan-based materials.\textsuperscript{36} In the hydrated state, our composites exhibit higher modulus (\textasciitilde50\%) and hardness (2-fold) compared to the native muscle thread cuticle (\textit{M. galloprovincialis}),\textsuperscript{37} while significantly less than the squid beak.\textsuperscript{2} $\gamma$-Fe$_2$O$_3$ coordination improves the mechanical properties of the functionalized chitosan, though we anticipate that further covalent cross-linking of the catechol motif would yield further improvements in modulus and hardness as observed in the squid beak.\textsuperscript{2}

Hybrid composites exhibit slight increases in mechanical properties in the dry state. Differences in mechanical response are much more dramatic in the hydrated state, where the elastic modulus of the hybrid composites is as much as 10-fold higher than the unmodified chitosan. Previous studies have underlined the key role of controlled water desolution and cross-linking in increasing the strength and stiffness of sclerotized/chitosan composites, including squid beaks.\textsuperscript{3} The present data can be explained on the basis of equivalent physicochemical mechanisms resulting from the chemical design inspired by our model systems. First, by mimicking the squid beak model (chitinous network dehydration), controlled functionalization of DOPA/DHA moieties imparts the chitosan network with hydrophobic aryl motifs, thereby preventing excessive water uptake, which has a direct role in plasticizing the chitosan network. Second, the $\gamma$-Fe$_2$O$_3$–catechol coordinated network acts as a mimic of the mussel thread coating chemistry (DOPA-Fe$^{3+}$ coordination), providing sites on the surface of the polysaccharide fibrils for efficient load transfer to the iron oxide particles. Finally, a dual role of these two mechanisms is also plausible. It is reasonable to assume that the dense network of $\gamma$-Fe$_2$O$_3$–catechol coordination bonds further prevents adsorption of water molecules on the surface of the chitosan chains, thereby mitigating the plasticization effect of water associated with interchain shearing during deformation. Overall, the combined mechanical and chemical degradation results highlight that the $\gamma$-Fe$_2$O$_3$–catechol coordination bond is an efficient means to prevent extreme losses in structural integrity (elastic modulus/hardness).

\section*{CONCLUSIONS}

One major challenge in bioinspired materials synthesis and processing resides in preparing materials that mimic multiple length scales of the biological system. Here we have established a route toward $\gamma$-Fe$_2$O$_3$-catechol clad composites that fulfills this goal, whereby chitosan was functionalized with catecholic motifs up to 82\% and coordinated bonded with $\gamma$-Fe$_2$O$_3$ nanoparticles. Raman spectroscopy confirmed bidentate $\gamma$-Fe$_2$O$_3$-catechol chelation, while nanoindentation measurements demonstrated that this form of cross-linking is an efficient means to prevent loss in structural integrity in the hydrated condition. This bioinspired strategy also allows us to overcome two key drawbacks of chitosan, namely its poor load-bearing capability in hydrated conditions and its solubility in mild acidic microenvironments, which chitosan or iron oxide doped chitosan cannot achieve. The composites exhibit multifunctional properties after coordination bonding, displaying super-paramagnetic behavior provided by the iron oxide nanoparticles. Biocompatible and magnetically responsive polymer-magnetic nanoparticle composites\textsuperscript{38,39} have been typically limited by their low stiffness in the physiological milieu, thus limiting the actuation forces that can be generated. The present coordinated-bonded hybrid composites, with an elastic modulus exceeding 1 GPa in hydrated conditions, overcomes this key limitation, opening the pathway for their use as stimuli-responsive materials in various regenerative applications.

\section*{ASSOCIATED CONTENT}

\section*{Supporting Information}

Experimental details; \textsuperscript{1}H NMR, TGA, TEM, XRD; nanoindentation and magnetization data for chitosan, catechol-chitosan and mnp-CAT\textsubscript{CS}. This material is available free of charge via the Internet at http://pubs.acs.org.

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\section*{Notes}

The authors declare no competing financial interest.

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REFERENCES

NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on August 7, 2013, with an error in Figure 4. The corrected version was reposted on August 8, 2013.