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Release-Modulated Antioxidant Activity of a Composite Curcumin-chitosan Polymer

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Curcumin is known to have immense therapeutic potential but is hindered by poor solubility and rapid degradation in solution. To overcome these shortcomings, curcumin has been conjugated to chitosan through a pendant glutaric anhydride linker using amide bond coupling chemistry. The hybrid polymer has been characterized by UV-visible, fluorescence and infrared spectroscopies as well as zeta potential measurements and SEM imaging. The conjugation reactivity was confirmed through gel permeation chromatography and quantification of un-conjugated curcumin. An analogous reaction of curcumin with glucosamine, a small molecule analog for chitosan was performed and the

purified product characterized by mass spectrometry, UV-visible, fluorescence, and infrared spectroscopies. Conjugation of curcumin to chitosan has greatly improved curcumin aqueous solubility and stability, with no significant curcumin degradation detected after one month in solution. The absorbance and fluorescence properties of curcumin are minimally perturbed (λ_{max} shifts of 2 nm and 5 nm, respectively) by the conjugation reaction. This conjugation strategy required use of one out of two curcumin phenols (one of the main antioxidant functional groups) for covalent linkage to chitosan, thus temporarily attenuating its antioxidant capacity. Hydrolysis-based release of curcumin from the polymer, however, is accompanied by full restoration of curcumin's antioxidant potential. Antioxidant assays show that curcumin radical scavenging potential is reduced by 40% after conjugation, but that full antioxidant potential is restored upon hydrolytic release from chitosan. Release studies show that curcumin is released over 19 days from the polymer and maintains a concentration of 0.23 ± 0.12 μM curcumin/mg polymer/mL solution based on 1% curcumin loading on the polymer. Release studies in the presence of carbonic anhydrase, an enzyme with known phenolic esterase activity, show no significant difference from non-enzymatic release studies, implying that simple ester hydrolysis is the dominant release mechanism. Conjugation of curcumin to chitosan through a phenol ester modification provides improved stability and solubility to curcumin, with ester hydrolysis restoring the full antioxidant potential of curcumin.

Introduction

Oxidative stress is characterized by an imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to readily remove the reactive intermediates or easily repair the subsequent damage.[1] The resulting indiscriminate damage caused by ROS can lead to severe metabolic dysfunctions, loss of cell integrity, hindrance of enzyme function, genomic instability, and other detrimental effects. Oxidative stress at the cellular level can ultimately lead to pathogenesis of many human disease states, such as inflammation, ischemia, atherosclerosis, arthritis, cancer, Parkinson's disease, aging and Alzheimer's disease.[2] To counteract oxidative stress, the body produces an armory of antioxidants to defend itself. These complex antioxidant systems, consisting of antioxidant enzymes and small antioxidant molecules, act in concert to decrease the ROS load. However, during times of excess ROS production, whether inspired by biological processes or external stimuli, such as acute radiation exposure, it is beneficial to supplement natural antioxidant capacities through therapeutic antioxidant agents. It is therefore desirable to seek new methods for bolstering the body's native antioxidant capacity.

Curcumin (diferuloylmethane) is a polyphenol extract from the rhizome of the Curcuma Longa herb from which the culinary spice, turmeric, is derived,

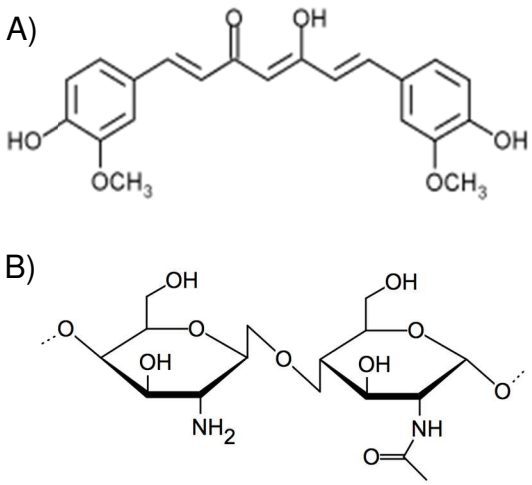


Figure 1. Chemical structures of curcumin (A) and chitosan (B).

(Figure 1A). It is a major component of turmeric that displays an intense yellow color and naturally fluoresces in the green spectrum.[2] The potent antioxidant activity of curcumin is provided by its diketone and phenol moieties that are known to scavenge free radicals.[3] Curcumin is known to interact with a variety of transcription factors, growth factors and their receptors, cytokines, enzymes, and genes. Specifically in the biological field of tumorigenesis, curcumin is a potent inhibitor of nuclear factor-κB (NF-κB), a transcription factor implicated in pathogeneses of several malignancies; inhibits the production of various angiogenic cytokines,[2, 4] and enhances the expression of the tumor suppressor protein p53 and its downstream target p21CIP1/WAF1.[5] Curcumin also has potent anti-inflammatory and wound healing properties. Curcumin quenches free radicals and reduces inflammation through NF-κB, COX-2, LOX, and iNOS inhibition.[3a, 6]

However, the effectiveness of curcumin *in vivo* is limited by low solubility in aqueous solution and rapid degradation.[7] Alkaline hydrolysis is the main process involved in the degradation of curcumin in buffer solutions, depleting 90% of curcumin in 30 minutes at physiological conditions.[8] Many analogues of curcumin have been created to increase its solubility, but they typically have reduced potency.[5a, 9] To improve the bioavailability of curcumin, it has been encapsulated in synthetic liposomes, polymeric nanoparticles, microspheres, and hydrogels, all of which demonstrated low residence time/ fast release kinetics.[10] In order to increase the release time for curcumin, we have been exploring covalent conjugation strategies that incorporate bio-degradable linkages. However, the three chemical functionalities on curcumin that most readily lend themselves to conjugation reactions, the two phenol moieties and the central diketone, are major components of curcumin's antioxidant potential.[3a] There is, therefore, a high probability

that covalent conjugation of curcumin to a delivery vehicle is concomitant with a partial loss of antioxidant potential. Therefore, a delivery system that enhances the solubility of curcumin, protects it from degradation, and retains its antioxidant properties could improve its bioavailability and effectiveness. As such, from our previous work encapsulating curcumin within polymer shells, it seemed feasible that a composite polymer of curcumin and chitosan could provide many of these enhanced properties.[11]

Chitosan (CS), a cationic polymer whose crystallinity is a function of the degree of deacetylation (range = 50–95%), is derived from chitin, an abundant polysaccharide found in crustacean shells (Figure 1B). CS has been shown to display wound-healing properties, is nontoxic, and has minimal foreign body response with accelerated angiogenesis.[12] To date, CS has been used in the medical field as wound dressings,[13] space filling implants[14] and drug delivery systems.[13a, 15] This work focuses on the development of a delivery platform for the stable presentation of curcumin that preserves the major pharmacologically relevant functional groups of curcumin for post-release efficacy.

Materials and Methods

Materials. Chitosan 90/200 was purchased from Heppe Medical Chitosan, GmbH (Halle, Germany). Curcumin (85% pure; demethoxycurcumin and bis-demethoxycurcumin as impurities), acetic acid, ethyl acetate (HPLC grade), 1-octanol, ascorbic acid, 2,2'-diphenylpicrylhydrazyl (DPPH), dichloromethane, glutaric anhydride, methanol (HPLC grade), 4-dimethylaminopyridine (DMAP), triethylamine, N-hydroxysulfosuccinimide (sulfo-NHS), 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), diethylether, polyethylene glycol sorbitan monolaurate (Tween 20), butylated hydroxyl

toluene (BHT), Folin Ciocalteu reagent and 10x phosphate-buffered saline were purchased from Sigma Aldrich (St. Louis, MO, USA) and used as received unless otherwise noted. Magnesium sulfate was purchased from Fisher Scientific (Pittsburgh, PA USA). Dextran molecular weight standards were purchased from PolySciences, Inc (Warrington, PA).

Methods. Fourier transform mass spectrometry, ESI-MS, was performed on a MS-FTICR-MSn, LTQ FT (Thermo Electron Corp., Waltham, MA). UV-visible spectroscopy was performed on a Cary 100 spectrometer (Agilent Technologies, Santa Clara, CA) and fluorescence spectroscopy on a Cary Eclipse Fluorimeter (Agilent Technologies, Santa Clara, CA). Gel Permeation Chromatography was performed on a Waters 1215 binary pump and 2420 evaporative light scattering detector (Waters Corporation, Milford MA). Zeta potential measurements were made on a Brookhaven Zeta PALS analyzer (Brookhaven Instruments Corp., Holtsville, NY). SEM imaging was conducted on a Zeiss Supra 35 (Carl Zeiss AG, Jena, Germany). SEM samples were sputter coated with a gold/palladium alloy prior to imaging.

Synthesis of 5-(4-((1E, 6E)-7-(4-hydroxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dienyl)-2-methoxyphenoxy)-5-oxopentanoic acid (acid-curcumin). Acid-curcumin was synthesized via a modification of published protocols.[16] The reaction was performed under air-free conditions using standard Schlenk reaction techniques. Tetrahydrofuran (THF) was dried in a Pure-solv solvent purification system (Innovative Technology, Inc., Amesbury, MA). Curcumin (2.7 mmol) and DMAP (0.5 mmol) were dissolved in 100.0 mL dry THF. After stirring for 10 min., triethylamine (4.0 mmol) was added to the reaction mixture. Glutaric anhydride (3.0 mmol) was dissolved in 5.0 mL THF and added

drop-wise to the reaction mixture via addition funnel. A reflux condenser was attached to the reaction vessel and the mixture refluxed overnight. Next, THF was removed using a rotatory evaporator producing a dark red oil. The oil was collected with 55.0 mL ethyl acetate and washed with 15.0 mL 1 M HCl. The organic layer was set aside and the aqueous layer was washed with 50.0 mL ethyl acetate, three times. The combined organic layers were dried over $MgSO_4$ and gravity filtered. Solvent was removed on a rotatory evaporator. 50 mg of the crude product was dissolved in 10.0 mL dichloromethane and loaded onto a silica gel column. Elution with dichloromethane:methanol (95:5) resulted in isolation of the title compound as an orange solid.

Synthesis of curcumin-modified glucosamine. Acid-curcumin (21 μmol) was dissolved in 500 μL methanol and added to 5.0 mL PBS buffer. Sulfo-NHS (23 μmol) was added to the acid-curcumin mixture and allowed to stir for 5 min at room temperature. The mixture was then added to D-glucosamine-HCl (21 μmol) dissolved in 15.0 mL 1% acetic acid. EDC (23 μmol) was added and the reaction stirred for 24 hrs in the dark at room temperature. The product was then extracted in 20.0 mL dichloromethane, three times, then dried over $MgSO_4$, filtered and dried on a rotatory evaporator. The product was purified with column chromatography (silica gel; 95:5 – 50:50 DCM: methanol gradient). ESI(+)-MS: calcd. for $C_{32}H_{33}O_{13}N$: 639.2. Found: 639.3 [M - H]+. Percent yield: 18.4%.

Synthesis of curcumin-chitosan polymer. Acid-curcumin (7.2 μmol) was dissolved in 5.0 mL PBS buffer with 200 μL of methanol. Sulfo-NHS (7.9 μmol) was added directly to the solution and allowed to stir at room temperature for 5 min. The solution was then added slowly to a solution of chitosan 90/200 (100 mg, 90 % deacetylated, 151-350 mPas

viscosity, Mw 200-300 kDa) dissolved in 15.0 mL 1% acetic acid. EDC (7.9 μmol) was added to the mixture and stirred for 24 hrs in the dark. The solution was dialyzed for 72 hrs (MWCO = 3400 Daltons) in the dark followed by lyophilization to obtain a yellow fibrous polymer. The product was then washed repeatedly with 70% ethanol until no trace of curcumin was detected in the rinse (monitored spectroscopically at 420 nm).

Determination of yield of curcumin-chitosan polymer. Curcumin-chitosan polymer was synthesized as above, with the exception that following dialysis the crude mixture was extracted with ethyl acetate until no absorbance at 415 nm was noted via UV-vis spectroscopy in the extracts. The total acid-curcumin in the extracts was then determined from a calibration curve of acid-curcumin in ethyl acetate.

Gel permeation chromatography of chitosan and curcumin-chitosan polymer. 0.2 % w/v Chitosan and curcumin-chitosan polymer samples were stirred overnight in 0.3 M acetic acid/0.1 M ammonium acetate, then filtered through a 0.22 um syringe filter. 100 uL samples were injected into an UltraHydrogel Linear column (7.8 mm I.D. x 30 cm, Waters) and eluted with 0.3 M acetic acid/0.1 M ammonium acetate at 0.5 mL/min flow rate. Average number molecular weights were calculated using a calibration of Dextran standards weighing 43,500, 66,700, 124,000, 196,000, 277,000, and 401,000 Da.

Determination of curcumin-chitosan polymer isoelectric point. The isoelectric point of the polymer was determined using a ZetaPALS Zeta Potential Analyzer with a BI-ZTU Titration Unit (Brookhaven Instruments, Holtsville, NY) auto-pH titrator. pH values were established via titration with nitric acid and potassium hydroxide.

Curcumin stability in solution as hybrid polymer. Curcumin-chitosan polymer (100 mg) was dissolved in 100.0 mL DI H_2O. The solution absorbance at 430 nm was measured once a week for 1 month as a 1:10 dilution.

Curcumin release studies. Carbonic anhydrase esterase activity was assayed against p-nitrophenyl acetate using the method of Polat, et al.[17] Carbonic anhydrase esterase activity, as measured against p-nitrophenyl acetate, was determined to be 1.7 ± 0.3 U/min/mL. One unit of enzyme activity was based on release of 1 μmol p-nitrophenol per minute at room temperature. Curcumin release with and without enzyme were conducted in a release solution consisting of 0.05 M trizma buffer with 0.025 M sulfuric acid (pH 7.0) prepared in nanopure water and 0.057 M L-ascorbic acid as preservative. Curcumin-chitosan polymer (5 mg) was added to 2.9 ml of release solution. At the same time, 0.125 mg/mL carbonic anhydrase buffer was prepared in the L-ascorbic acid buffer. To initiate the enzyme-mediated release study, 2.9 mL of the L-ascorbic acid buffer and 0.1 mL of the carbonic anhydrase buffer were placed into a centrifuge tube. As a control, the release study was performed in the above manner with the omission of carbonic anhydrase. Release study solutions were then covered in aluminum foil and placed in the dark at 37°C for release measurements.

For each time point measurement, the sample tubes were centrifuged at 2500 rpm for 5 minutes and supernatants were transferred to new centrifuge tubes. The curcumin-chitosan polymer pellets were re-suspended in 3.0 mL fresh release buffer. 3.0 ml of 0.1% (w/v) BHT (as preservative) solution in ethyl acetate was added to each supernatant. The supernatant solutions were mixed vigorously and the solvent layers were allowed to separate for five minutes. Curcumin released into solution was quantified by

measuring absorbance in the ethyl acetate layer at 420nm in a quartz cuvette. The process was repeated over a period of nineteen days.

Released curcumin sample preparation for DPPH and total phenolic content assays. 100.0 mg of curcumin-chitosan polymer was dissolved in 5.0 ml of 1% acetic acid and wrapped aluminum foil to protect against photo-degradation. After 5 minutes, 5.0 ml of ethyl acetate was added to the acetic acid solution, which was then vortexed. The yellowish layer of ethyl acetate was carefully separated using a micropipette and evaporated via rotavap. The resulting yellowish solid was re-dissolved in 4.0 ml methanol and UV-Vis absorbance was measured at 420 nm. The average concentration of three trials was calculated using curcumin's standard curve. For total phenolic content determination, the extracted yellow solid obtained via rotavap was re-dissolved in 2.0 ml methanol and UV-Vis absorbance was measured at 420 nm. The average concentration of three trials was calculated using the former equation for standard curve of curcumin in methanol.

Antioxidant studies. Determination of antioxidant capacity using DPPH was derived from published protocols.[18] Due to solubility differences between acid-curcumin and curcumin-chitosan polymer, the DPPH studies were performed under two sets of solvent conditions.

Curcumin/acid-curcumin/curcumin-glucosamine conjugate DPPH studies: 50 μL of various concentrations (0.05–0.2 mg/mL) of curcumin, acid-curcumin, or curcumin-glucosamine conjugate were added to 5.0 mL 0.004% (DPPH) in methanol. The solution was incubated in the dark for 30 min then the absorbance was read at 517 nm.

Curcumin/curcumin-chitosan polymer/chitosan DPPH studies: Various concentrations (0.05–0.2 mg/mL) of curcumin, curcumin-chitosan polymer, chitosan, and released curcumin were prepared in 5.0 mL 1% acetic acid containing 0.025% Tween 20. Curcumin equivalents to the curcumin-chitosan polymer were determined from a calibration curve of curcumin in 1% acetic acid containing 0.025% Tween 20. To these solutions with curcumin, chitosan, or curcumin-chitosan polymer, 1.0 mL of 0.004% DPPH in methanol was added. The solution was incubated in the dark at 37°C for 30 min. then the absorbance was read at 515 nm. For both DPPH studies, radical scavenging percentages were calculated from:

(Control OD – Sample OD)/Control OD) x 100

where OD = optical density.

Total phenolic content. The total phenolic content in released curcumin was determined using Folin Ciocalteu (FC) reagent via a method developed by Singleton and Rossi expressing total phenolic content as Gallic acid equivalents.[19] A 0.5 ml solution of curcumin in methanol (3.8 to 18.9) µg/ml was mixed with 2.5 ml of 10% (v/v) FC reagent and 2.5 ml of 7.5% (w/v) Na_2CO_3 prepared in the deionized water. A blank solution was simultaneously prepared with 0.5 ml methanol, 2.5 ml 10 % (v/v) FC reagent and 2.5 ml of 7.5 % (w/v) Na_2CO_3 prepared in the deionized water. The samples were incubated in a thermostat at 37° C for 120 minutes followed by measuring the absorbance at 765 nm. This procedure is repeated for standard curcumin (20 to 100) ug/ml and Gallic acid (20 to 100) µg/ml respectively. The phenolic contents of the extracted curcumin and standard curcumin are expressed as the Gallic acid equivalent (mg of GA/g of extract) using a standard curve of phenolic content of Gallic acid from the assay.

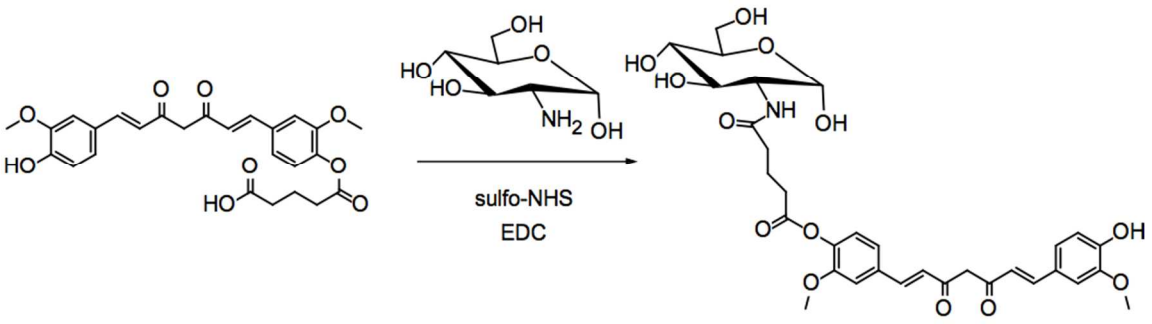
Results and Discussion

Curcumin modification. Curcumin has been modified with a pendant carboxylic acid via standard coupling chemistry with glutaric anhydride utilizing DMAP and triethylamine, resulting in "acid-curcumin", Scheme S1.[16] Isolation of the mono-carboxylate derivative was achieved through column chromatography. This modification allows facile conjugation of curcumin to amine bearing molecules such as chitosan and D-glucosamine. High resolution Fourier transform mass spectrometry conclusively identifies the identity of the product with a parent ion peak found at 483.16 amu [M+H]+. Characterization of acid-curcumin via UV-visible spectroscopy and fluorescence measurements was undertaken to verify that the spectral properties of curcumin were minimally affected by the modification procedure (Figure S1). The ability to retain the optical properties of curcumin throughout the conjugation process is deemed critical to retaining the therapeutic properties of curcumin, as breakdown of the curcumin backbone to less therapeutically relevant compounds is accompanied by a loss in the signature absorbance/fluorescence bands for curcumin.[20] The UV-visible spectra of curcumin and acid-curcumin in DMSO are nearly identical, with only a 2 nm blue shift from 418 to 416 nm upon modification of curcumin, suggesting that the chromophore backbone of curcumin is unperturbed by the conjugation reaction, Figure S1(A). Likewise, fluorescence emission spectra of curcumin and acid-modified curcumin reveal only a 5 nm blue shift for the product from 518 to 513 nm, Figure S1(B). Comparison of the infrared spectra of curcumin vs. modified curcumin reveals that the compounds have nearly identical IR spectra with two important exceptions, Figure S2. The IR spectrum

for acid-modified curcumin displays -C=O stretching bands at 1705 and 1755 cm^{-1}, implying the addition of a carboxylic acid to the curcumin structure, and the stretching band related to the phenolic –OH of curcumin at 3504 cm^{-1} has been attenuated in the product. The reduction in intensity for the phenolic -OH band implies that the modification of one phenol moiety through esterification was successful.

Conjugation of acid-curcumin to D-glucosamine. Addition of a pendant carboxylic moiety to curcumin allows for facile conjugation to the amine moiety of chitosan. However, as a feasibility test for the curcumin-chitosan conjugation, acid-modified curcumin was conjugated to D-glucosamine using standard amide bond forming chemistry, Scheme 1. Reacting a 1 to 1 molar ratio of acid-modified curcumin with D-glucosamine in the presence of a small excess of sulfo-NHS and EDC affords the curcumin glucosamine conjugate. The conjugation reaction proceeds with a yield of 18.4%. Curcumin-glucosamine conjugation has been confirmed by mass spectrometry and FTIR. The main evidence of the successful conjugation is found in FT-MS analysis, which reveals the expected peak for the conjugate at 639.3 [M-H]+, Figure 2. The infrared spectrum of the curcumin-glucosamine conjugate contains strong stretching bands for both glucosamine and curcumin as well as a strong carbonyl stretching band at 1763 cm^{-1}, Figure S3.

Scheme 1.



Conjugation of acid-modified curcumin to glucosamine minimally affects the spectroscopic properties of the curcumin chromophore, as evidenced in the optical spectra shown in Figure S4. The UV-visible absorption maximum and fluorescence emission maximum at 420 and 539 nm, respectively, are within 5 nm of those reported for curcumin.

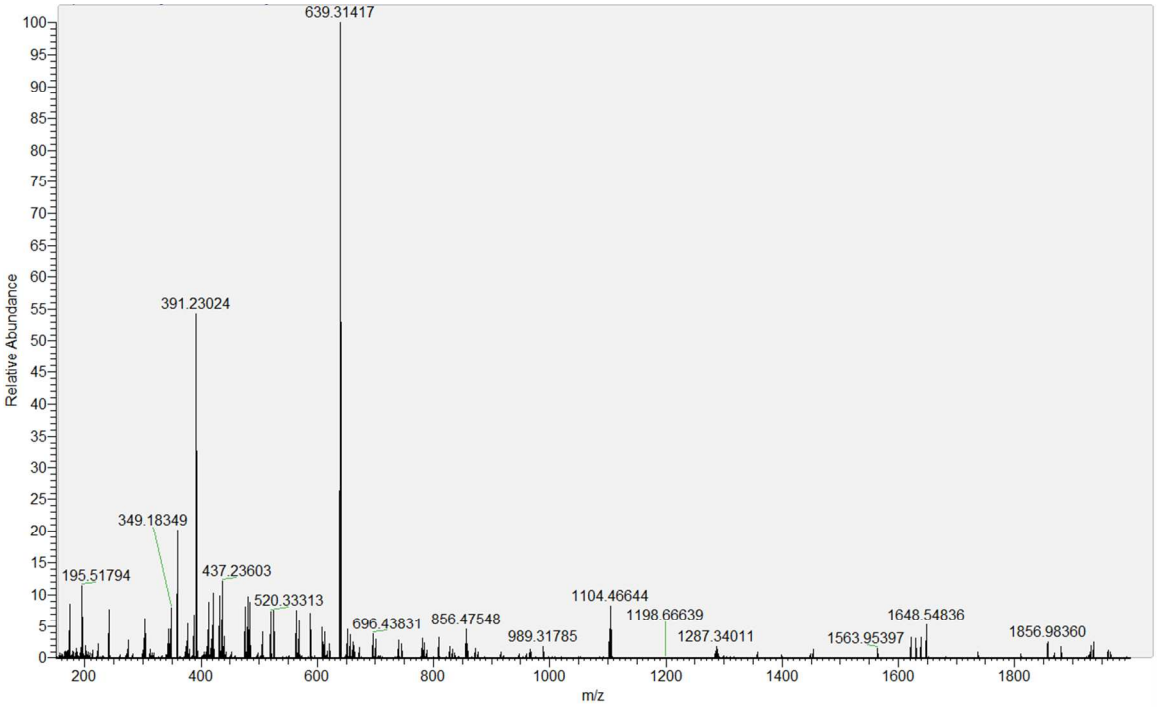
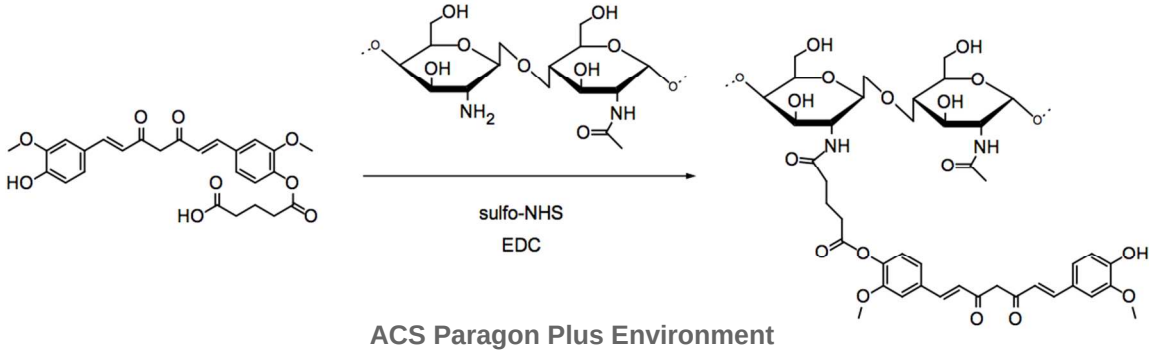


Figure 2. FT-MS (positive ion mode) of the curcumin-glucosamine conjugate.

Conjugation of acid-curcumin to chitosan. The successful conjugation reaction between curcumin and glucosamine was used in the analogous reaction between acid-curcumin and chitosan, Scheme 2. Reacting acid-curcumin with chitosan in the presence

Scheme 2.



of sulfo-NHS and EDC followed by dialysis and lyophilization yielded a spongy polymeric substance that bears the morphological characteristics of chitosan and the optical properties of curcumin, Figure 3. Due to the low solubility of curcumin in 1% acetic acid (even with the assistance of methanol) and the limitations of dissolving

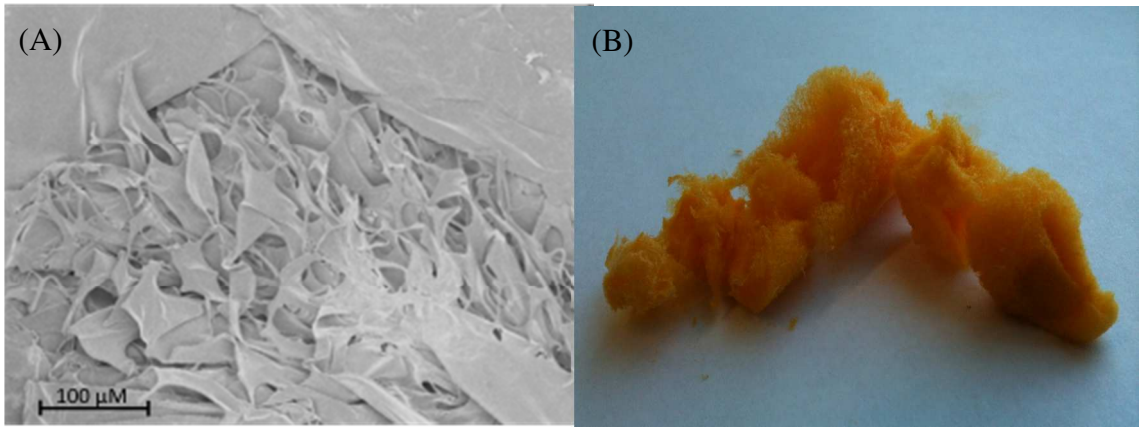


Figure 3. A) SEM image of the curcumin-chitosan hybrid polymer displaying a fibrous morphology. EHT = 2kV (B) Photograph of the curcumin-chitosan hybrid polymer reveals a bright orange-yellow color reminiscent of curcumin and a spongy consistency characteristic of chitosan.

chitosan without the solution becoming overly viscous, it was practical to use an acid-curcumin-chitosan ratio equivalent to labeling chitosan on 1% of the available amine groups. Extraction of the crude polymer with ethyl acetate leads to recovery of 32.2% of the polymer, suggesting to a yield of 67.7% for the conjugation reaction. For a polymer molecular weight of 200-300 kDa and 90% deacetylation, this is equivalent to the addition of 600-900 molecules of curcumin per polymer strand. SEM images of the polymer reveal a fibrous polymeric structure that is typical for chitosan, Figure 3(A).[21] The curcumin-chitosan polymer has a deep orange-yellow color that is typical of curcumin, Figure 3(B). The polymer, as an acetate salt is soluble in 18 MΩ water in

which it acts as a weak acid. As much as 5 mg/mL of the polymer can be dissolved in plain water. The curcumin-chitsosan polymer can also be dissolved in dilute HCl and then successfully dispersed in PBS buffer or cell culture media up to 4 mg/mL concentration. Gel permeation chromatography (GPC), Figure S5, reveals that the polymer has an increased apparent number average molecular weight (M_n) after conjugation. Calibration with dextran standards yields M_n for chitosan as 299517 Da and the curcumin-chitosan polymer as 322,822 Da. This gain in mass overestimates the degree of curcumin addition at conjugation to 4.9% of available amines on chitosan. Theoretically, a 100% yield would result in only 1% labelling. This suggests that conjugation of curcumin to chitosan either alters the shape of the polymer in solution or the rigidity of the polymer, thus artificially skewing the apparent M_n measurement with GPC.[22]

The FTIR spectrum of the curcumin-chitosan polymer contains all of the peaks that are present in the curcumin-glucosamine conjugate, Figure S3. Both samples contain an amide carbonyl stretching band at 1763 cm^{-1} indicating a successful coupling. In the polymer, however, the mass ratio of curcumin to chitosan is low (on the order of 1%) and so the IR bands associated with curcumin are weaker than in the curcumin-glucosamine spectrum.

The conjugation of acid-curcumin to CS was also monitored by measuring the zeta potential of the polymer before and after modification. As the amine groups on CS are pH labile, the conjugation of curcumin to those amine groups and subsequent conversion to uncharged amide groups will affect the overall charge on the polymer at a given pH. A plot of zeta potential vs. pH for both chitosan and the curcumin-chitosan polymer is

shown in Figure S6. It is evident from the shift in isoelectric point to higher pH (7.4 to 8.1) for the polymer that the conjugation reaction has reduced the number of free charge-bearing amines on chitosan.

Analogous to the curcumin-glucosamine conjugation, the strong optical absorption profile of curcumin remains intact after conjugation to chitosan. However, the absorbance maximum for curcumin has blue-shifted to 380 nm (vs. 420 nm for curcumin), Figure 4. This may reflect the fact that the curcumin-chitosan spectrum was collected in acidic aqueous solution, whereas aqueous spectra for unconjugated curcumin are collected in basic aqueous or organic media.[23] The fluorescence emission of curcumin remains after conjugation to chitosan, with an emission maximum of 538 nm in 1% acetic acid, Figure 4.

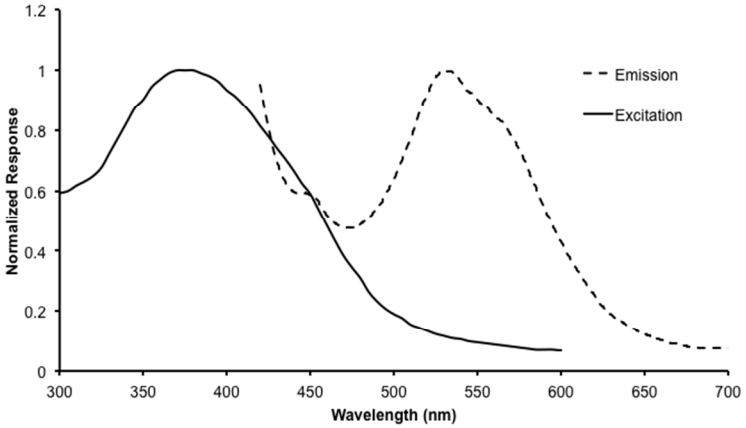


Figure 4. UV/visible absorption spectrum (solid line) and fluorescence emission spectrum (dashed line) of curcumin-chitosan hybrid polymer in 1% acetic acid.

Previous reports have demonstrated that curcumin is 90% decomposed within 30 minutes in aqueous environments.[20] However, the curcumin-chitosan polymer was stable in solution in DI H_2O for up to one month with minimal change in solubility and optical (absorbance and fluorescence) properties, confirming reports of the protective effect imparted on curcumin by chitosan.[24] The ability of polymer conjugation to prolong curcumin solution lifetime and increase aqueous solubility has also been reported when

using poly(ethylene glycol) (PEG) conjugates, where curcumin was detected in solution up to 24 hours after exposure to cells.[25]

Antioxidant capacity of curcumin and curcumin-derivatives. In order to test the antioxidant capacity of curcumin after modification, a DPPH radical scavenging assay was performed on curcumin, acid-curcumin, curcumin-glucosamine conjugate and the curcumin-chitosan polymer.[18a] DPPH assays for the curcumin-glucosamine conjugate were performed in methanol, however the poor solubility of the curcumin-chitosan polymer was prohibitive to performing the DPPH assay on the polymeric form using the same conditions as the other compounds, and required a modified form of the assay using 1% acetic acid with 0.025% Tween 20. For each solvent condition, the radical scavenging abilities for the various reagents were compared to that of unmodified curcumin, Figure 5. Before modification, curcumin displayed 93% radical scavenging activity. Modification of one of the phenol groups of curcumin lowers the antioxidant capacity to 85%. The reduced antioxidant capacity of curcumin-glucosamine is most likely due to usage of one of the phenol moieties on curcumin for tethering the molecule. Conjugation of curcumin to

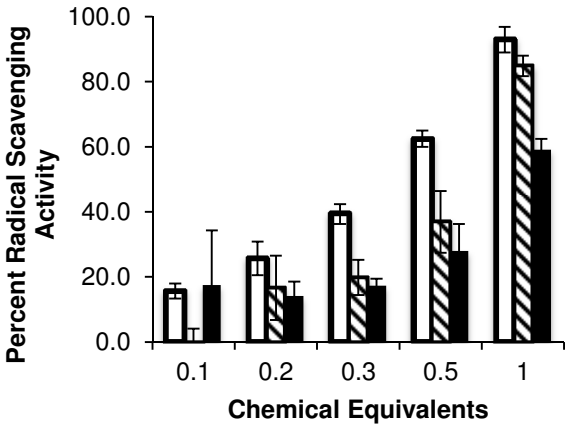


Figure 5. Radical scavenging assay results for curcumin (white bar), acid-modified curcumin (Striped bar), and curcumin-glucosamine conjugate (black bar) in methanol.

glucosamine reduces radical scavenging capabilities to 60%, Figure 5. The further reduction in antioxidant capacity may reflect the presence of hydrogen bonding interactions between glucosamine and curcumin that stabilize groups that would typically participate in radical quenching reactions. However, the tethered curcumin molecules display 60% activity, rather than 50%, of the radical quenching ability of curcumin which may reflect antioxidant activity of the β-diketone moiety contributing to radical quenching reactions.[3c]

The antioxidant capacity of curcumin-chitosan polymer, chitosan and post-release curcumin (*vide infra*) were also assessed using a DPPH assay, but was performed in 1% acetic acid with 0.025% Tween 20 due to the limited solubility of the polymer in methanol. Tween 20 was included to effectively solubilize curcumin in the aqueous solution for use as a control for the DPPH assay. Similar to the DPPH assay in methanol, curcumin was found to demonstrate 92.2% radical quenching at a 1:1 ratio with DPPH. Curcumin-chitosan polymer performed in a manner analogous to the curcumin-glucosamine conjugate with radical quenching ability reduced to 53.2% after modification and conjugation of curcumin, Figure 6. The reduction in radical quenching ability is again contributed to usage of one of the two phenol groups of curcumin for the conjugation reaction. As a control, the radical scavenging ability of unmodified

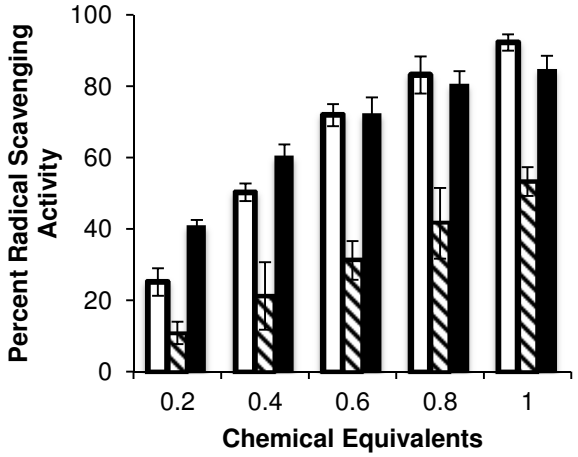


Figure 6. Radical scavenging assay results for curcumin (white bar), curcumin-chitosan polymer (striped bar), and released-curcumin (black bar) in 1% acetic acid.

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chitosan was also measured and found to be 12% (data not shown). This may explain why curcumin-chitosan polymer displays radical scavenging above 50% even though half of the available phenol groups have been modified.

Notably, the curcumin released from the curcumin-chitosan polymer (*vide infra*) has regained all of its radical scavenging capabilities. This implies that covalently bound curcumin is released through ester hydrolysis, which would result in re-formation of the phenol that was modified to attach curcumin to the polymer. The reconstitution of curcumin further confirmed in two ways. First, ESI-MS confirmed the presence of curcumin as the dominant peak (M+H 369.13). In order to confirm that this peak was not the result of fragmentation during ionization in the mass spectrometer, phenolic content assays were performed on the extracted curcumin following release from the polymer, Table 1. The assay, which determines the total amount of phenol groups in a sample relative to a Gallic acid standard, confirms that the post-released curcumin has a phenolic assay count very similar to the unmodified curcumin used in this study.[19]

Table 1. Phenolic content assay results for unmodified and post-release curcumin.

Sample	Total phenolic content (mg GAE/g)
Extracted curcumin	422.9 ± 0.7
Standard curcumin	372.5 ± 0.9
Standard curcumin (values taken from ref. [19, 26])	448.4 ± 8.3

Curcumin release studies. Release studies were performed on the curcumin-chitosan polymer in its fibrous polymer morphology both with and without the presence of carbonic anhydrase. Carbonic anhydrase was chosen for its well-characterized ability to cleave phenolic esters such as the one used to tether curcumin to the chitosan backbone. For the curcumin-chitosan polymer, curcumin is released steadily over the course of 19 days from the polymer and follows first-order kinetics, Figure 7.[27] The effective dose of curcumin released over this time is 230 ± 120 nM curcumin/mg polymer/mL solution based on 1% curcumin loading on the polymer. The release rates measured here are dramatically longer than previous reports involving PEG-conjugated curcumin derivatives, suggesting that the curcumin-chitosan polymer may be more useful in applications where a longer release time is needed.[25] Curcumin is released from the polymer at the same rate regardless of the presence of functionally competent carbonic anhydrase; suggesting that the length of the connecting tether between chitosan and curcumin may not be sufficient for allowing carbonic anhydrase to cleave the ester bond,

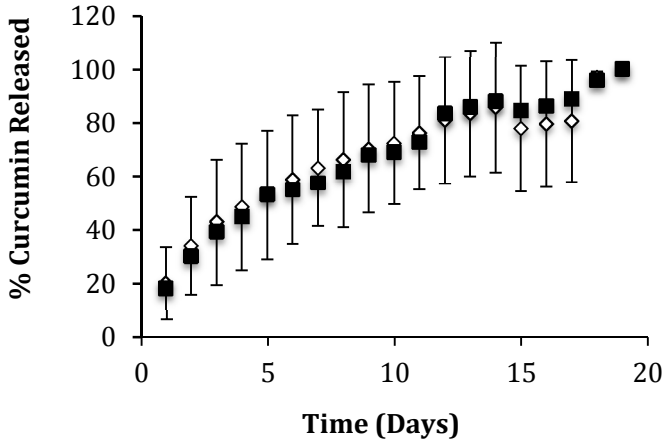

Figure 7. Curcumin release from curcumin-chitosan polymer with (black squares) and without (white diamonds) carbonic anhydrase.

or that simple ester hydrolysis is likely to be the main release pathway for the curcumin.

Conclusions

Curcumin is a potent antioxidant and is a promising agent in treatments and therapies against diseases and injuries, such as cancer and inflammation; however, its low solubility limits its bioavailability and clinical efficacy. To overcome these limitations, curcumin has been conjugated to chitosan polymer. Additionally, curcumin was conjugated to the small molecule analog of chitosan, glucosamine, to further characterize the conjugation product. Covalent conjugation of curcumin to chitosan not only protects curcumin from rapid degradation, but also provides a means for controlled delivery to various tissues for specific applications. The attachment modality of curcumin to chitosan reported here contains biodegradable linkages to facilitate both hydrolytic and enzyme catalyzed release and was designed to release curcumin in its pre-modification form. Non-enzyme catalyzed hydrolytic release was determined to be the predominant release mechanism in this system. The antioxidant capacity of curcumin throughout the conjugation and release processes has been monitored. Release studies have been performed to compare the rates of enzymatic vs. non-enzymatic release. Though antioxidant capacity is temporarily reduced, due to modification of one of the phenol groups, it is restored once curcumin is released from the polymer. The conjugation to chitosan therefor provides a viable strategy to stabilize curcumin for applications involving long-term release delivery. The hydrolysis-triggered release of curcumin from the polymer provides a steady concentration of curcumin over the period of the release study. This indicates that curcumin-chitosan polymer is a promising candidate carrier to

deliver therapeutic doses of curcumin over extended periods of time while maintaining potency for antioxidant applications.

ASSOCIATED CONTENT

Supporting Information. Additional spectra for modified-curcumin and Zeta potential measurements on the curcumin-chitosan polymer are located in the supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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