Communication

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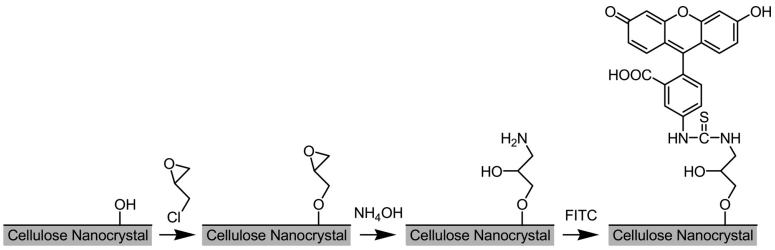

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Fluorescently Labeled Cellulose Nanocrystals for Bioimaging Applications

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The delivery and targeting of therapeutic and diagnostic agents with nanoparticles represents the forefront of nanomedicine.[1] Several nanoparticle-based technologies are already commercially available and in clinical use, such as liposomal formulations for cancer therapy, colloidal gold for in vitro diagnostics, and magnetic nanoparticles for in vivo imaging.[2] Suitable nanoparticulate delivery systems need to offer the desired biodistribution, an effective delivery of the cargo to the targeted tissue, and adequate rate of clearance from the host.[3]

The rate by which potential carriers are removed from the blood circulation of a host depends on several factors. According to a recent literature review, for a prolonged blood circulation time, carriers must be small, composed of natural compounds, and present a neutral and hydrophilic surface.[4] Polysaccharides, a class of hydrophilic natural polymers, have recently attracted attention as surface coatings for drug carriers.[5] In addition to prolonging the circulation time of the carrier, polysaccharide coatings offer reactive surface groups for chemical coupling of functional agents.[5] The many attributes of polysaccharides make polysaccharide nanoparticles, such as cellulose nanocrystals,[6−8] promising candidates for targeted delivery of therapeutics.

The cellular uptake and biodistribution of nanoparticulate delivery systems is often studied using fluorescence techniques.[9] Fluorophores, either covalently attached or noncovalently associated with the nanoparticles, allow quantification and localization of the nanoparticles inside the cells or host system. One of the most widely used fluorophores in fluorescence methods is fluorescein-5′-isothiocyanate (FITC).[10] The present paper describes a method to label cellulose nanocrystals with FITC for fluorescence bioassay and bioimaging applications.

Cellulose nanocrystals were prepared from milled (60-mesh) dissolving grade softwood sulfite pulp (Temalfa 93A-A from Tembec, Inc.) as described by Beck-Candanedo et al.,[11] using 64 wt % sulfuric acid (10 mL/g cellulose), a temperature of 45 °C, and a hydrolysis time of 60 min. An atomic force microscopy image of the obtained crystals, deposited from a 0.001 wt % aqueous suspension onto a mica substrate, is shown in Figure 1.

To covalently attach FITC moieties to the surface of the nanocrystals, we followed the reaction pathway illustrated in Scheme 1. First, the surface of the nanocrystals was decorated with epoxy functional groups via reaction with epichlorohydrin (6 mmol/g cellulose) in 1 M sodium hydroxide (132 mL/g cellulose) according to the method by Porath and Fornstedt.[12] After 2 h at 60 °C, the reaction mixture was dialyzed (Spectra/Por 4 dialysis tubing) against deionized water (Millipore Direct-Q 5, 18.2 MΩ· cm) until the pH was below 12. Next, the epoxy ring was opened with ammonium hydroxide to introduce primary amino groups. After adjusting the pH to 12 with 50% (w/v) sodium hydroxide, ammonium hydroxide (29.4%, 5 mL/g cellulose) was added and the reaction mixture heated to 60 °C for 2 h. The reaction mixture was dialyzed until the pH was 7.

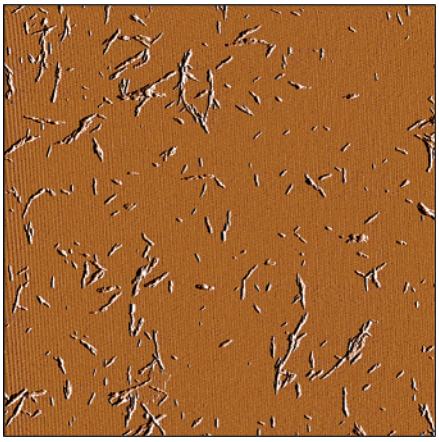
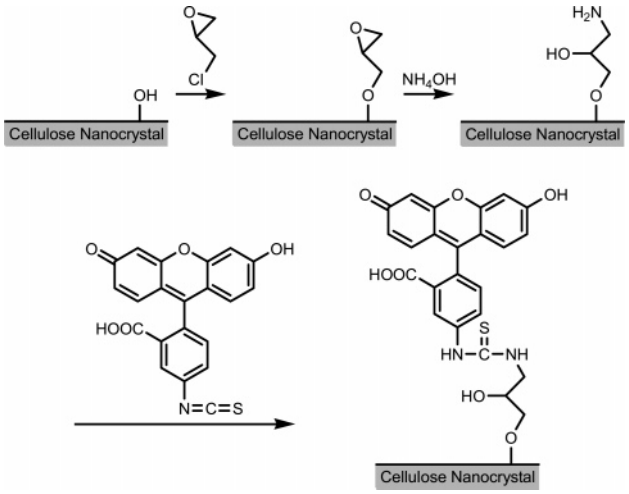


Figure 1. AFM amplitude image (intermittent contact mode) of cellulose nanocrystals (scan size: 5 μm).

Scheme 1



Finally, the primary amino group was reacted with the isothiocyanate group of FITC to form a thiourea. Following the method of Swoboda and Hasselbach,[13] FITC (0.32 mmol/g cellulose) was added to the aminated nanocrystals in 50 mM sodium borate buffer solution (50 mL/g cellulose), containing ethylene glycol tetraacetic acid (5 mM), sodium chloride (0.15 M), and sucrose (0.3 M). The reaction mixture was stirred overnight in the dark and then dialyzed for 5 days. The suspension was sonicated (10 min, 200 W, ice bath cooling), centrifuged (10 min, 4550 G, 25 °C), and filtered through a syringe filter (0.45 μm) to remove any aggregates. The final suspension (0.5 wt %) had a pH of 6.

Figure 2 shows aqueous suspensions of unlabeled and FITC-labeled cellulose nanocrystals. The unlabeled suspension was colorless and slightly opaque, whereas the FITC-labeled suspension appeared clear and yellow.

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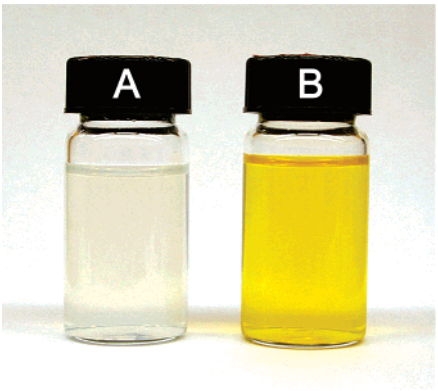

Figure 2. Aqueous suspensions of (A) cellulose nanocrystals (0.8 wt %) and (B) FITC-labeled cellulose nanocrystals (0.5 wt %).

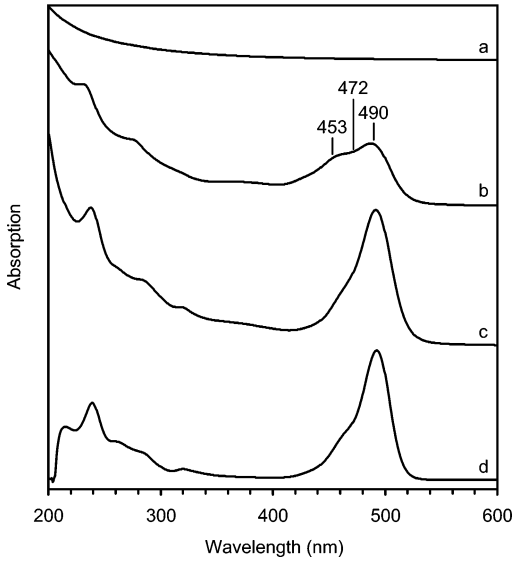

Figure 3. UV/vis absorption spectra of (a) an aqueous suspension of unlabeled cellulose nanocrystals (0.05 wt %), (b) an aqueous suspension of FITC-labeled cellulose nanocrystals (0.05 wt %), (c) the same suspension as in (b) with pH adjusted to that of the FITC solution, (d) a solution of FITC in ammonium hydroxide (0.0006 wt %, pH 11).

The FITC content of the labeled cellulose nanocrystals was determined by UV/vis spectroscopy. Figure 3 compares the UV/vis absorption spectrum of FITC-labeled cellulose nanocrystals with the spectra of unlabeled crystals and free FITC in solution. The UV/vis absorption spectrum of FITC depends strongly on pH.[14] In aqueous solution, FITC exists in cationic, neutral, anionic, or dianionic form. The dianionic form, having both the carboxyl and hydroxyl group deprotonated, predominates at pH values above 6.6.[15] At pH values between 4.3 and 6.6, anionic FITC moieties occur, in which only one of the two groups is deprotonated at a time.

Unlabeled cellulose nanocrystals (spectrum a in Figure 3) did not show any absorption peaks in the wavelength range of 200 to 600 nm. The spectrum of the FITC-labeled nanocrystals (spectrum b) showed absorption maxima of both the dianionic (490 nm) and the anionic (453 and 472 nm) form of FITC, in accordance with a suspension pH of 6.[14] To obtain a direct correlation of FITC content and absorption intensity, the pH of the suspension was adjusted to 11, resulting in a UV/vis spectrum (spectrum c) typical of the dianionic form and similar to that of free FITC in ammonium hydroxide (spectrum d), with absorption maxima at 490, 322, 283, and 239 nm.[14] According to previous studies,[9] the fluorescence properties of FITC in a fluorescing conjugate are not essentially different from those of free FITC. Using the absorption intensities at 490 nm of free FITC in ammonium hydroxide (spectrum d) and FITC-labeled cellulose nanocrystals at pH 11 (spectrum c), we calculated a FITC content of 0.03 mmol/g of cellulose, equivalent to 5 FITC moieties per 1000 anhydroglucose units. Assuming a nanocrystal diameter of 4.5 nm,[11] a density of 1.6 g/cm^3,[16] and neglecting the end surfaces of the particles, the surface concentration is 0.037 FITC moieties per nm^2 or 1 FITC moiety per 27 nm^2.

In conclusion, we have developed a simple method, involving a three-step reaction pathway, to covalently attach fluorescent FITC molecules to the surface of cellulose nanocrystals. Fluorescently labeled cellulose nanocrystals enable the use of fluorescence techniques, such as spectrofluorometry, fluorescence microscopy, and flow cytometry, to study, for example, the interaction of cellulose nanocrystals with cells and the biodistribution of cellulose nanocrystals in vivo. In a collaborative effort, we are currently investigating the uptake of FITC-labeled cellulose nanocrystals by different types of mammalian cells.

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