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Radiolabeled Molecular Imaging Probes for the *in Vivo* Evaluation of Cellulose Nanocrystals for Biomedical Applications

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KEYWORDS

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ABSTRACT

Cellulose nanocrystals (CNCs) have remarkable potential to improve the delivery of diagnostic and therapeutic agents to tumors, however, the *in vivo* studies on CNC biodistribution are still limited. We developed CNC-based imaging probes for the *in vitro* and *in vivo* evaluation using two labeling strategies: site-specific hydrazone linkage to the terminal aldehyde of the CNC and non-site-specific activation using 1,1'-carbonyldiimidazole (CDI). The *in vivo* behavior of unmodified CNC, DOTA-CNC (ald.), and DOTA-CNC (OH) was investigated in healthy and 4T1 breast cancer mouse models. They displayed good biocompatibility in cell models. Moreover, the biodistribution profile and SPECT/CT imaging confirmed that the accumulation of ^{111}In -labeled DOTA-CNC (ald.) and ^{111}In -DOTA-CNC (OH) was primarily in hepatic, splenic, and pulmonary ducts in accordance with the clearance of nontargeted nanoparticles. The developed CNC imaging probes can be used to obtain information with non-invasive imaging on the behavior *in vivo* to guide structural optimization for targeted delivery.

INTRODUCTION

The development of biocompatible nanomaterials has had a tremendous impact on the targeted delivery of therapeutic and diagnostic agents in cancer.¹⁻⁵ Nanomaterials have been extensively used as nanocarriers in order to achieve successful delivery of therapeutic payloads, contrast agents and diagnostic/therapeutic radionuclides to the tumor while reducing systemic side effects after administration *in vivo*.⁶⁻⁸ Molecular imaging techniques are already widely employed in the early detection of malignant lesions, tumor biochemical characterization, disease staging and monitoring of treatment response.⁹⁻¹⁰ Single-photon emission computed tomography (SPECT), based on the detection of gamma (γ) radiation emitted by radioactive isotopes, such as Tc-99m, In-111, I-123, and Lu-177, is an important non-invasive imaging method in oncology clinical practice. SPECT imaging is a widely used modality in the clinic due to its relatively low cost compared to positron emission tomography (PET), often facile radiopharmaceutical synthesis, and the availability of a number of isotopes with relatively long (>10 hours) and multi-day half-lives allowing for transport of the prepared radiotracers.¹¹⁻¹² Additionally, the energy resolution in SPECT instrumentation allows for the simultaneous imaging with multiple isotopes.¹³ With the appropriate choice of radiotracer, SPECT imaging can render valuable information on tissue biochemistry, function, and metabolism. The development of nanoparticle-based imaging probes for nuclear imaging has created the capability to determine the disease location, monitor pathological and molecular changes related to payload delivery, as well as the comprehensive view of the material behavior *in vivo* in order to use as a guideline for therapeutic and diagnostic interventions with nanoscale drug delivery systems.¹⁴⁻¹⁶

Cellulose is an inexhaustible, renewably-sourced biopolymer present in plants, select micro-organisms, and even in some marine invertebrates.¹⁷⁻²⁰ The natural cellulose consists of amorphous (disordered) and crystalline regions. When mechanical (e.g. high-pressure homogenization, ultrasound) and chemical (e.g. strong acid hydrolysis) treatments are applied to bulk cellulose, the highly crystalline regions can be simply separated from the amorphous fractions to yield cellulose nanocrystals (CNCs).²¹⁻²² CNCs are

needle-like nanoparticles comprising of glucose repeating units linked together by glycosidic oxygen bridges forming a linear homopolymer. The dimensions of CNCs vary depending on the source of the bulk cellulose and the treatment conditions. Acid hydrolysis of cotton (used in this study) typically yields CNCs of 5–10 nm in width and 100–300 nm in length, demonstrating a high aspect (L/D) ratio.²³⁻²⁴ The as-hydrolyzed CNC surface is covered with numerous hydroxyl groups from the glucose units and has an aldehyde group at the reducing end of the polymer chain that allow several types of chemical modifications, for example using: esterification, amination, oxidation, and polymer engraftment.²⁵⁻²⁷ The abovementioned physicochemical properties of CNCs offer several advantages for biomedical and drug delivery applications, including the use as an excipient for the controlled release of hydrophobic drugs and enhancing drug bioavailability, loading capacity in aerogels, and in viral delivery applications.^{26, 28-31} The biosafety of novel nanomaterials has always been a critical issue because the changes in size, morphology, surface chemistry, charge and the degree of crystallinity can affect the behavior of the nanomaterials *in vitro* and *in vivo*.³²⁻³³ CNC-based biomaterials have demonstrated good biocompatibility, biodegradability, and low cytotoxicity in cell models.³⁴⁻³⁵ However, the reports on CNC-based drug delivery system behavior after systemic administration are still limited.³⁶

Here, we developed CNC-based nuclear imaging probes for evaluating their behavior in biological systems *in vitro* and *in vivo*. The macrocyclic radiometal chelator, DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), was covalently installed on the surface of CNCs by two different conjugation methods. Firstly, DOTA was conjugated to the aldehyde group (–CHO) at the reducing end of CNC as a hydrazone. Secondly, the hydroxyl groups (–OH) on the CNCs surface were activated with 1,1'-carbonyldiimidazole (CDI) and further conjugated with a DOTA-amine. The *in vitro* cytotoxicity of the two types of DOTA-installed CNCs was investigated in murine RAW 264.7 macrophages and murine 4T1 mammary adenocarcinoma cell line. Then, the DOTA-installed CNC were

radiolabeled with ^{111}In ($t_{1/2} = 2.80$ d, $E_{\gamma} = 171.280$ keV (I_{γ} 90%) and $E_{\gamma} = 245.395$ keV (I_{γ} 94%)), a diagnostic radionuclide for SPECT imaging (**Figure 1a–1b**). The *in vivo* fate of DOTA-CNC prepared using the two synthetic strategies was determined in healthy and 4T1-tumor-bearing mice after intravenous administration by *in vivo* small-animal SPECT/CT imaging and *ex vivo* gamma counting of the excised tissues.

Figure 1. The schematic representations of (a) ^{111}In -DOTA-CNC (ald.) and (b) ^{111}In -DOTA-CNC (OH).

EXPERIMENTAL SECTION

Materials and chemicals

CNCs were prepared from cotton fibers (Whatman[®] filter paper) through acid hydrolysis as described in the literature.³⁷⁻³⁸ Prior to the synthetic modifications, CNCs were washed with 5× DMSO and 2× ultrapure water in order to remove the acid and sulfate residues, then collected back in powder form by lyophilizer. All chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA), VWR

(Radnor, PA, USA), Macrocyclics (Plano, TX, USA), and Thermo Fisher Scientific (Waltham, MA, USA), and were used without further purification. The dialysis tubing was purchased from Spectrum Labs (Rancho Dominguez, CA, USA). Ultrapure water (18.2 M Ω) was prepared on a Milli-Q Integral 10 water purification system. The cell viability assay, culturing media, and other procedures for cell experiments are described in the Supporting Information (SI).

DOTA-CNCs preparation

In this study, there were two strategies of installing the DOTA chelator to the CNCs. First is the conjugation of DOTA hydrazide with aldehyde group at the reducing end of CNCs. The DOTA functionalized hydrazide (1,4,7,10-tetraazacyclododecane-1,4,7-tris (acetic acid)-10-acethydrazide or DOTA-NH-NH₂) was synthesized in-house from 1,4,7,10-tetraazacyclododecane-1,4,7-tris-*tert*-butyl acetate-10-acetic acid precursor (DOTA-tris (*t*-Bu ester), Macrocyclics, Plano, TX, USA) through amide coupling reaction and Boc-deprotection as described in the SI. The CNCs were dispersed in anhydrous DMSO at 5 mg/ml in a conical centrifuge tube and sonicated with tip sonicator (tip ϕ = 3 mm., SONIC Inc., CT, USA) at 20% amplitude for 5 min. Then, the CNCs solution was transferred to a round bottom flask and flushed with argon flow for 10 min with constant magnetic agitation. The DOTA-NH-NH₂ (1 eq. w/w) was added to the CNCs dispersion under argon atmosphere. The reaction was left for 4 days in temperature-controlled oil bath at 50–60 °C. The reaction was terminated by centrifugation at 4000 rpm, 5 min to collect the pellet of DOTA-CNC (ald.). The DOTA-NH-NH₂ residues in the product pellet were washed out with 4 $\times$ fresh DMSO and dialysed against ultrapure water in a dialysis tube (MWCO 3.5–5.0 kDa) for at least 4 days while changing the medium at least twice a day. The purified DOTA-CNC (ald.) in aqueous solution was dried in a lyophilizer.

The DOTA-amine (1,4,7,10-tetraazacyclododecane-1,4,7-tris (acetic acid)-10-(4-aminobutyl) acetamide or DOTA-NH₂) was conjugated to the hydroxyl groups on the CNC surface through CDI-mediated amide coupling reaction. The CNCs were dissolved in anhydrous DMSO at a concentration of 5 mg/ml and subjected to sonication as described above. CDI (2 eq. w/w) in anhydrous DMSO was slowly added to the CNCs solution under argon flow and constant agitation. The CDI activation reaction was run overnight in an oil bath at 50–60 °C under an argon atmosphere. The reaction was terminated by centrifugation and the excess CDI was washed out with 3× fresh DMSO. The CDI-activated CNC pellet was redispersed in anhydrous DMSO under argon. The DOTA-NH₂ in DMSO solution (1 eq. w/w, Macrocylics, Plano, TX, USA) was added dropwise to the CDI-activated CNCs solution. The reaction was left stirring under argon atmosphere at 50–60 °C for 4 days. The termination and purification procedures of DOTA-CNC (OH) were the same as described in DOTA-CNC (ald.).

Material characterization

The elemental compositions of both DOTA-CNC (ald.) and DOTA-CNC (OH) were analyzed with a vario MICRO cube CHNS analyser (Elementar Analysensysteme, GmbH, Germany). The results were reported as a percent composition (C, H, and N). The Fourier Transform Infrared Spectroscopy (FTIR, Vertex 70, Bruker, MA, USA) with attenuated total reflectance (ATR, MIRacle, PIKE Technologies, WI, USA) was used to confirm the conjugation of the DOTA chelator to the CNCs surface in both DOTA-CNC (ald.) and DOTA-CNC (OH). The surface charge (ζ -potential) of unmodified CNC, DOTA-CNC (ald.), and DOTA-CNC (OH) was determined from the NP electrophoretic mobility by Zetasizer Nano ZS instrument (Malvern, Worcestershire, UK) at 500 μ g/ml in aqueous solution. Moreover, the morphology and dimensions of unmodified and modified CNCs were characterized by 120 kV transmission electron microscopy (Tecnai 12 Bio-Twin FEI, Hillsboro, OR, USA). The unmodified and modified CNCs were dissolved in ultrapure water at 1 mg/ml and sonicated for 5 min.

Then, 5 µl of each CNC solution were applied on glow discharge treated carbon support film hexagonal 300 mesh copper grids (Electron Microscopy Sciences, PA, USA) and further negatively stained with 1% uranyl acetate for 2 min before leaving for 2 h of air-drying prior to the measurements.

In vitro cytotoxicity studies

The *in vitro* cytotoxicity was performed using a commercial CellTiter-Glo[®] cell viability assay (Promega Corp., Madison, WI, USA). Murine RAW 264.7 macrophages and murine 4T1 mammary adenocarcinoma cell lines were selected as immune and tumor cell models. The cells were seeded overnight on a 96-well plate at density of 15,000 cells per well in 100 µl DMEM and RPMI-1640 media for RAW 264.7 and 4T1 cells, respectively. The medium was discarded and replaced with 100 µl of unmodified CNC, DOTA-CNC (ald.), and DOTA-CNC (OH) solutions at 5, 25, and 100 µg/ml, prepared in complete growth media for each cell line. Triton X-100 solution (1% v/v) and fresh medium were used as negative and positive controls of viability, respectively. Cells were incubated for 6, 24, 48, and 72 h at an incubator at 37 °C (5% CO₂ concentration and 95% relative humidity). The assay was carried out according to the instructions from the manufacturer, reading the luminescence from the ATP activity produced in each well with Varioskan LUX multimode microplate reader (Thermo Fisher Scientific Inc., NY, USA). All measurements were done in triplicate.

¹¹¹In-radiolabeling of DOTA-modified CNCs

The DOTA-CNC (ald.) and DOTA-CNC (OH) were radiolabeled with ¹¹¹InCl₃ (Mallinckrodt Medical B.V., Petten, the Netherlands). All radiolabeling solutions were prepared in ultrapure water rendered metal-free by treatment with Chelex 100 ion exchange resin (Bio-Rad, Hercules, CA, USA). Both DOTA-modified CNCs (1 mg) were dispersed in 0.2 M ammonium acetate buffer (pH 4.0) at a concentration of 1 mg/ml in a Protein LoBind microtube (Eppendorf, Hamburg, Germany). The solutions were sonicated with tip sonicator at 20% amplitude for 3 min before radiolabeling. The ¹¹¹InCl₃ solution

(30–150 MBq) was added to the DOTA-CNCs solution and the reaction mixtures were incubated at 100 °C for 60 min on a shaker. The radiolabeling reaction was quenched with 100 µl of 50 mM EDTA solution and allowed to equilibrate to room temperature before purification. The ^{111}In -DOTA-CNCs pellet was collected by centrifugation at 10 000g, 5 min. The supernatant was discarded and the product pellets were washed with 3×50 mM of EDTA solution, 2 times with $1 \times \text{PBS}$ (pH 7.4), and $1 \times$ ultrapure water in order to release the non-specifically absorbed ^{111}In on the CNC surface. The radioactivity in the pellet and supernatant was measured by VDC-405 dose calibrator (Comer Group, Joure, the Netherlands) after every step. The purified product pellets were resuspended in sterilized $1 \times \text{PBS}$ (pH 7.4) and sonicated for 2 min before supplementing the buffer with the Solutol HS 15 solubilizer (BASF ChemTrade GmbH, Burgbernheim, Germany) to a final concentration of 5% (v/v). The quality control of the products was checked by radio-TLC. The products were spotted on the glass microfiber chromatography sheet impregnated with a salicylic acid (iTLC-SA, Agilent Technologies, Folsom, CA, USA) and chromatogram was run in 0.2 M of sodium acetate buffer (pH 4.0) where the ^{111}In -DOTA-CNC products retains at the origin and the free $^{111}\text{In}^{3+}$ moves along with the solvent ($R_f = 0.74$).

Radiolabel stability

The radiolabel stability of ^{111}In -DOTA-CNC (ald.) and ^{111}In -DOTA-CNC (OH) were conducted in simulated physiological conditions: $1 \times \text{PBS}$ (pH 7.4) and 50% human plasma. The ^{111}In -DOTA-CNCs were dissolved in those conditions at 100 µg/ml and incubated at 37 °C with constant shaking at 300 rpm (Eppendorf Thermomixer® C, Eppendorf, Hamburg, Germany). Samples were drawn at specified time points (6, 24, 48, and 72 h) for radio-TLC in order to determine the percent of intact ^{111}In on the CNC. All assays were carried out in triplicate.

EDTA and Fe³⁺ challenges

The ¹¹¹In-DOTA-CNC (ald.) and ¹¹¹In-DOTA-CNC (OH) were incubated in EDTA (2 mM) and FeCl₃ (0.2 mM) at nanocrystal concentration of 100 µg/ml at 37 °C, 300 rpm. Samples were spotted on the radio-TLC at 3, 24, 48, 72, 96, and 120 h. The radio-TLC chromatogram was run in 0.2 M sodium acetate buffer (pH 4.0). The percent of ¹¹¹In intact in the CNC was quantified by autoradiography (Fujifilm Fluorescent Image Analyzer (FLA-5100), Fuji Film Photo, Tokyo, Japan). The experiments were run in triplicate.

Ex vivo biodistribution

The *ex vivo* biodistribution study was carried out in healthy female mice (CD-1, Charles River, weighing 26–29 g, aged 8–12 weeks) and 4T1 tumor-bearing mice (Balb/c, Charles River, weighing 15–20 g, aged 8–12 weeks) models. All animal experiments were conducted under a project license approved by the National Board of Animal Experimentation in Finland (ELLA, license number ESAVI/12132/04.10.07/2017). For 4T1 tumor-bearing mice, 1×10⁶ cells of 4T1 in 50 µl of additive-free RPMI-1640 medium were inoculated to the surgically exposed right inguinal mammary fat pad under 2.5% isoflurane anesthesia in medical air:oxygen carrier (0.6 and 0.4 L/min, respectively) and 100 µl of local anesthesia infiltrated at the incision site (1:1 5 mg/ml bupivacaine : 10 mg/ml lidocaine). The tumor was allowed to develop for 9 days before the administration of radiolabeled CNC. The 4T1-inoculated mice received carprofen at 5 mg/kg dose (Norocarp, 50 mg/ml, Norbrook Laboratories, Newry, Northern Ireland) at 24 and 48 h after the surgery. The ¹¹¹In-DOTA-CNCs formulations (0.1–0.9 MBq) were intravenously injected at a dose of 50 µg in 150 µl of sterilized 1× PBS (pH 7.4)–5% Solutol HS 15 *via* the tail vein. At predetermined time points (6, 24, and 48 h), the mice were sacrificed by CO₂ asphyxiation followed by cervical dislocation. The collected tissue samples were blood, pancreas, spleen, stomach,

small intestine, large intestine, kidney, liver, lung, heart, skeletal muscle, bone with marrow (tibia), occipital bone, brain, and tumor. All tissue samples were individually weighted in 5-ml scintillation tubes and the radioactivity was measured on an automatic gamma counter 1480 Wizard[®] 3” (PerkinElmer[™] Life Sciences, Waltham, MA, USA). The results were calculated and reported as percent of injected dose per weight of tissue (%ID/g). The number of animals was set to 3–5 mice per time point and nanocrystal type for statistical analysis.

In vivo SPECT/CT imaging

The *in vivo* SPECT/CT imaging was performed with a small animal SPECT/CT system (Bioscan NanoSPECT/CT, Mediso, Hungary) at the Helsinki *In Vivo* Animal Imaging Platform (HAIP), University of Helsinki. The mice were divided into two groups: healthy mice (CD-1) and 4T1-tumor bearing mice (Balb/c). The 4T1 xenograft to the Balb/c mice was done with the same procedures and conditions as previously described in *ex vivo* biodistribution. The ¹¹¹In-DOTA-CNCs formulations (2.0 MBq) were intravenously administered at a dose of 100 µg in 150 µl of sterilized 1× PBS (pH 7.4)–5% Solutol HS 15 *via* the tail vein. The number of animals was set at two mice per time point and formulation. The whole-body SPECT/CT scans were performed under 2.0% isoflurane anesthesia in oxygen carrier (0.6 L/min) at 1, 24, and 48 h after the administration. The scanning protocol was SCOUT CT SPECT ¹¹¹In and the parameters were set at 360°, 20 projections × 100s per projection, and 60 min. After each SPECT scan, the 45 kVp X-ray source was used to acquire the CT image (Helical, 240 projections, 6 min). All images were reconstructed by Nucline[™] acquisition software (Mediso, Hungary) and analyzed by VivoQuant[™] software (InviCRO LLC, USA).

RESULTS AND DISCUSSION

DOTA-modified CNCs characterization

The CNC nanoprobe, DOTA-CNC (ald.) and DOTA-CNC (OH), were successfully prepared through hydrazide-aldehyde and amide coupling reactions, respectively. The DOTA-CNC (ald.) was prepared by the conjugation of DOTA-hydrazide (1,4,7,10-tetraazacyclododecane-1,4,7-tris (acetic acid)-10-acethydrazide) to the aldehyde group at the reducing end of CNC. The DOTA-hydrazide chelator was successfully synthesized and characterized by ^1H and ^{13}C NMR, and ESI-TOF MS analysis before the conjugation to the CNC (see SI). The DOTA-CNC (OH) was prepared from the amide coupling reaction between DOTA-amine (1,4,7,10-tetraazacyclododecane-1,4,7-tris (acetic acid)-10-(4-aminobutyl) acetamide) and CDI-activated hydroxyl group on the surface of CNC. The DOTA-CNC (ald.) and DOTA-CNC (OH) were further characterized with ATR-FTIR spectroscopy. The indicated amine II band (N-H bending and C-H stretching) appeared at the wavelength of 1590 cm^{-1} , as well as the corresponding amide I band (C=O stretching) at 1665 cm^{-1} , demonstrating successful conjugations of DOTA to the CNC surface with both strategies (**Figure S3**). Moreover, the elemental compositions (C, H, and N) of the unmodified and modified CNC were also quantified (**Table 1**). There was no significant difference in C and H compositions, because C and H are major constituents of the CNC homopolymer, however, there was an increase in N content from the nitrogen bonding after the conjugations. The N content of DOTA-CNC (ald.) was 0.10 % while in DOTA-CNC (OH) the value was 9-fold higher due to a higher number of CDI-activated hydroxyl conjugating sites for the DOTA-amine. Furthermore, the surface charge of unmodified and modified CNC is also an important parameter determining material dispersity, cell interactions and plasma protein adsorption in vivo. The DOTA-modified CNCs showed an increasing trend in surface charge in which the ζ -potential of DOTA-CNC (OH) was about 2-fold higher than unmodified CNC while DOTA-CNC (ald.) was slightly increased (**Table 1**).

Table 1. Elemental compositions analysis and ζ -potential values of unmodified and modified CNCs

Samples	Elemental compositions			ζ -Potential (mV)
	%C	%H	%N	
Unmodified CNC	43.15	6.07	-	-57.23 ± 2.40
DOTA-CNC (ald.)	43.22	6.13	0.10	-41.93 ± 0.75
DOTA-CNC (OH)	43.06	6.07	0.90	-29.60 ± 0.89

*Results are presented as average values (n=2) for elemental analysis and mean $\pm$ s.d. (n=3) for ζ -potential.

However, the DOTA-modified CNCs still maintained an optimal negative charge for stabilizing the CNC nanoparticles in suspension and reducing the nonspecific adsorption of plasma proteins in vivo.³⁹

TEM images were acquired to determine the morphology of the CNCs after the modifications.

Figure 2. TEM images of CNC NPs (a) unmodified CNC, (b) DOTA-CNC (ald.), and (c) DOTA-CNC (OH), scale bar = 500 nm.

The unmodified and modified CNCs demonstrated a uniform needle-like shape with average dimensions of 13-15 nm in width and 168-196 nm in length (**Table 2**, **Figure 2** and **Figure S4**). As expected, the conjugation of DOTA to the CNC surface did not much alter the dimensions of CNC in both strategies. Therefore, with matching physicochemical properties, they can be used as a tracer for the unmodified CNC in vivo. Additionally, the morphology suggests that use as a therapeutic and diagnostic

delivery carrier for passive extravasation (< 200 nm) through the leaky tumor vasculature might be possible.⁴⁰

Table 2. Average dimensions of unmodified and modified CNCs.

Samples	Width (nm)	Length (nm)
Unmodified CNC	12.71 ± 3.99	167.65 ± 30.28
DOTA-CNC (ald.)	13.63 ± 3.43	168.59 ± 25.76
DOTA-CNC (OH)	14.82 ± 2.72	195.71 ± 26.17

*Results are presented as mean ± s.d. (n=50)

In vitro cytotoxicity studies

The in vitro cytotoxicity of unmodified and modified CNCs was investigated to evaluate the initial biosafety of the nanoparticles. The cytocompatibility assay with unmodified and DOTA-modified CNCs was carried out in a macrophage cell line (RAW 264.7), which is a good model of nanoparticle uptake and clearance in vivo. In addition, a tumor cell line (4T1 breast cancer) for the preliminary assessment of suitability for drug delivery applications was also studied. The unmodified and modified CNC were incubated with the cells at concentrations of 5, 25, and 100 µg/ml for 6, 24, 48, and 72 h. The cell viability was determined by the measurement of metabolic activity-generated luminescence from the ATP production in viable cells when reacting with CellTiter-Glo® cell viability assay reagent. The results demonstrated that the three types of CNCs displayed good biocompatibility at all tested concentrations with the percent of viable cells exceeding 80% after 48 h incubation for both of the cell lines (**Figure 3**).

Figure 3. Cell cytotoxicity studies in (a) RAW 264.7 macrophage and (b) 4T1 breast cancer cell lines after incubation with unmodified CNC, DOTA-CNC (ald.), and DOTA-CNC (OH) NPs at 5, 25, and 100 $\mu\text{g/ml}$ for 6, 24, 48, and 72 h. Error bars represent the mean $\pm$ s.d. ($n = 3$) in comparison with the control. The statistical hypothesis was evaluated by Student's t -test where the significant probabilities were set at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

However, the cell viability was slightly decreased below 80% when the cells had been incubated for 72 h with DOTA-CNC (ald.) and DOTA-CNC (OH) at the highest concentration (100 $\mu\text{g/ml}$). The cell viability for the unmodified CNC sample was maintained high even after 72 h. The high concentration of the modified CNCs and the incubation times exceeding the doubling time of the cells in culture can induce cell apoptosis and antiproliferative effect.⁴¹ Moreover, the surface modifications can affect the cell–nanoparticle interactions and cytotoxicity.⁴² In general, all CNCs showed no significant cytotoxicity and did not induce the changes in morphology indicating an inflammatory response in vitro in both non-

tumor and tumor cell lines at the concentration up to 100 µg/ml, demonstrating the potential to study CNC NPs behavior in vivo.

¹¹¹In-DOTA-CNC radiolabeling

Both DOTA-CNC (ald.) and DOTA-CNC (OH) were successfully labeled with ¹¹¹In using standard radiolabeling methods. The decay-corrected radiolabeling yield for DOTA-CNC (ald.) was only modest 7 to 18 % due to the lower number of conjugated DOTA chelator dependent on the availability of the terminal aldehydes, whereas DOTA-CNC (OH) was labeled at a considerably higher yield of 54 to 65 %. The radiolabeling results for the ¹¹¹In-DOTA-CNC NP batches used in the biodistribution and SPECT/CT studies are compiled in **Table 3**. The initial activities for the radiolabeling synthesis for the *ex vivo* biodistribution study were 30 and 35 MBq for DOTA-CNC (ald.) and DOTA-CNC (OH), respectively. For the SPECT/CT imaging study we increased the starting activities to 150 MBq and 50 MBq for DOTA-CNC (ald.) and DOTA-CNC (OH), respectively, in order to be able to inject 2 MBq of the radiolabeled nanocrystals without exceeding a nanoparticle dose of 100 µg. The increase in starting radioactivity to 150 MBq for DOTA-CNC (ald.) resulted in a radiolabeled product with comparable specific radioactivity to the DOTA-CNC (OH) albeit at much lower radiochemical yield which is impractical given the relatively high cost of the radioisotope. Additionally, the observed low radiolabeling yield of the ¹¹¹In-DOTA-CNC (ald.) might result from the degradation of the hydrazone bond between the DOTA chelator and the CNC in the acidic conditions needed for the radiolabeling with ¹¹¹In. Therefore, reactions proceeding in neutral or basic conditions might be more successful for the radiolabeling of CNC through the terminal aldehyde. Also, the hydrazone linkage step could be further optimized in order to incorporate more of the DOTA chelator. After diligent washing to remove any non-specifically adsorbed ¹¹¹In³⁺, all of the constructs exhibited high radiochemical purity and dispersion stability in the 1× PBS (pH 7.4)–5% Solutol HS 15 formulation.

Table 3. Radiolabeled ^{111}In -DOTA-CNC NP products used in the biodistribution and SPECT/CT imaging studies.

Radiolabeled product	Study	RCY [%]	Radiochemical purity [%]	Specific radioactivity [MBq/mg]
^{111}In -DOTA-CNC (ald.)	Biodistribution	7a	99.3	2
	SPECT Imaging	18b	99.5	22
^{111}In -DOTA-CNC (OH)	Biodistribution	65	99.3	18
	SPECT Imaging	54	98.9	23

^aStarting radioactivity 30 MBq; ^bStarting radioactivity 150 MBq; RCY = Radiochemical yield

^{111}In -DOTA-CNCs in vitro stability and EDTA/ Fe^{3+} challenges

Complex biological responses such as plasma protein corona formation, immune recognition, material degradation and clearance occur when NPs are administered *in vivo*. The study of the radiolabel stability under stimulated biological conditions and the EDTA transchelation and Fe^{3+} radiometal displacement challenges can provide preliminary information about the physiological stability of ^{111}In -DOTA-CNC radiolabeling over time. This information is crucial for the interpretation of the results of non-invasive imaging, as the chemical identity of the radioactive signal cannot be determined from the image alone. The DOTA-CNC (ald.) and DOTA-CNC (OH) NPs were labelled with $^{111}\text{InCl}_3$ prior to the *ex vivo* biodistribution and *in vivo* SPECT imaging experiments. Then, small aliquots of those ^{111}In -DOTA-CNC NPs formulations were drawn out for the stability assays. The ^{111}In -DOTA-CNC (ald.) and ^{111}In -DOTA-CNC (OH) NPs were incubated under physiological conditions, 1× PBS (pH 7.4) and 50% human plasma at 37 °C. Both ^{111}In -DOTA-CNC (ald.) and ^{111}In -DOTA-CNC (OH) NPs were found to be stable over 48 h incubation period in both conditions, maintaining over 97% of the ^{111}In -DOTA complex intact before starting to release the radiolabel after 72 h (**Figure 4a**). Moreover, the ^{111}In -DOTA-CNC (ald.) and ^{111}In -DOTA-CNC (OH) NPs were also incubated in 2 mM of EDTA (excess) at 37 °C for 120 h

period in order to evaluate the potential for transchelation of ^{111}In with another ligand outcompeting the DOTA chelator. The radio-TLC was acquired to monitor the transchelation. In the radio-TLC setup, the ^{111}In -EDTA complex moves along with the solvent front while the ^{111}In -DOTA-CNCs are retained at the origin. The results revealed that there was approximately 9% of ^{111}In transchelation into EDTA over 120 h incubation for both formulations (**Figure 4b**), which is in good agreement with the reported stability of the ^{111}In -DOTA complex.⁴³⁻⁴⁴ Additionally, challenge with trivalent iron was conducted to simulate the possibility of a physiological concentration of Fe^{3+} to outcompete the ^{111}In in order to occupy the binding cavity of the DOTA chelator. Both ^{111}In -DOTA-CNC NPs formulations were incubated at 37 °C with 0.2 mM of FeCl_3 , 15-fold excess to the average Fe^{3+} concentration in human blood (~13 μM). The samples were monitored over 120 h and the percent of intact ^{111}In radiolabel was determined by radio-TLC. The ^{111}In -DOTA-CNC (OH) NPs proved out to be relatively stable (94% intact) over the 120 h incubation. The ^{111}In -DOTA-CNC (ald.) could maintain over 99% stability until 72 h when free ^{111}In started to release from the ^{111}In -DOTA complex (**Figure 4b**). This suggests that the two labeling methods used do not yield radiolabeled products of drastically different stability prompting the use of either *in vivo*.

Figure 4. (a) ^{111}In -DOTA-CNC NPs *in vitro* stability in 1× PBS (pH 7.4) and 50% human plasma, (b) EDTA and Fe^{3+} challenges of ^{111}In -DOTA-CNC NPs (n=3).

Ex vivo biodistribution

The *ex vivo* biodistribution evaluation of the non-targeted nanostructured carrier in animal models is a preliminary step toward the assessment of their potential for theranostic (*therapeutic* and *diagnostic*) applications. The biodistribution of radioactivity after injection of DOTA-CNC (ald.) and DOTA-CNC (OH) NPs was studied in healthy female CD-1 mice and in 4T1-tumor bearing Balb/c female mice in order to compare the distribution profiles of both types of CNC NPs in both animal models, the latter bearing an aggressive mammary fat pad tumor with the potential of nanomaterial accumulation by the enhanced permeation and retention (EPR) effect. The DOTA-CNC (ald.) and DOTA-CNC (OH) NPs were successfully labeled with ^{111}In , showing 99.3% and 99.5% radiochemical purity, respectively (**Figure S5a–S5b**). The final concentration of ^{111}In -DOTA-CNC NPs before injection was fixed at 2 mg/kg in 150 μl formulation and the injected activities were 0.1 and 0.9 MBq for ^{111}In -DOTA-CNC (ald.) and ^{111}In -DOTA-CNC (OH) NPs, respectively. The greater number of DOTA chelator groups conjugated to the CNC using the non-site-specific CDI activation explains the difference in the injected

activity, as more ^{111}In was chelated by DOTA-CNC (OH) than by ^{111}In -DOTA-CNC (ald.). However, given the inherent sensitivity of radiometric detection methods, the hydrazone linkage is also a viable labeling strategy for *ex vivo* studies, as it renders a large portion of the surface -OH groups in the CNC available for the subsequent conjugation of therapeutic payloads and fluorescent labels. Both formulations of ^{111}In -DOTA-CNC NPs were intravenously injected to the mice. The animals were sacrificed and select tissues were dissected for gamma counting at 6, 24, and 48 h post injection (p.i.). The uptake of radioactivity in an individual tissue was measured by automatic gamma counter and reported as a mean %ID/g of tissue ($n=3-5$) (**Figure 5**). Overall, both formulations of ^{111}In -DOTA-CNC NPs showed hepatic (20–30 %ID/g), splenic (15–30 %ID/g), and pulmonary (10–40 %ID/g) accumulations, while only a small uptake was seen in the kidney (1–5 %ID/g) after 6, 24, and 48 h p.i. This demonstrates the high uptake of CNC NPs in mononuclear phagocyte system (MPS), which is in agreement with the behavior and clearance of nontargeted NPs *in vivo*.⁴⁵ Moreover, there were no major uptake changes observed in heart, muscle, tibia and occipital bones, brain, and the 4T1 tumor over 48 h. Interestingly, there was a high accumulation of radioactivity after injection of ^{111}In -DOTA-CNC (OH) NPs in lung (125–150 %ID/g) at 6 h p.i in both animal models, but this drastically decreased within the first 24 h p.i. (**Figure 5c and 5d**). This might be due to the observed increase of the ζ -potential of DOTA-CNC (OH) NPs comparing to the unmodified CNC NPs (**Table 1**), which might affect the dispersion stability and circulation of the DOTA-CNC NPs *in vivo*. Further optimization of CNC surface functionalization and conjugation of targeting ligands needs to be carried out in order to improve the biodistribution profile and reduce the MPS uptake of the CNC. Only a slight radioactivity uptake in tumor (0.30–0.75 %ID/g) was seen, although the percentage of administered NPs reaching the tumor through EPR effect can be exploited to 5% maximum regardless of the nanoparticle type⁴⁶ (**Figure S6**). However, the NP surface chemistry and cancer-specific factors (*e.g.*, tumor type, growth rate, location and interstitial pressure) have to be taken into consideration in

order to elucidate the behavior of a specific type of NPs in the intrinsically heterogeneous tumor microenvironment.⁴⁷⁻⁴⁸ Therefore, the introduction of specific active targeting moiety and surface-stabilizing coating, such as PEGylation, to the CNC surface might enhance the tumor-targeting capability, prolong systemic circulation and reduce the uptake in MPS.

In vivo SPECT/CT imaging

The SPECT/CT imaging was carried out in order to monitor a in real-time the pharmacokinetic behavior of ¹¹¹In-DOTA-CNC (ald.) and ¹¹¹In-DOTA-CNC (OH) NPs *in vivo*. The experiment was conducted in both healthy mice and in 4T1-tumor bearing mice. The ¹¹¹In-labeled DOTA-CNC (ald.) and DOTA-CNC (OH) NPs were prepared separately from the particles used for the *ex vivo* biodistribution, and the radiochemical purities were 99.3% and 98.9% before injection, respectively (**Figure S5c–S5d**). Owing to the limitations of the sensitivity of SPECT, the injected activity in this experiment was higher than in the *ex vivo* biodistribution in order to obtain representative SPECT images. Therefore, the final activity of both formulations was injected at 2 MBq in 150 µl of formulated solution while the concentration of CNC was maintained below 4 mg/kg per body weight per injection in order to prevent aggregation and possible adverse effects related to the nanomaterial administration. Notably, the systemic toxicity or guidelines for safe dosage of CNC in mice has not been reported, and such studies are warranted in the future. However, some previous reports include observations of complications related to the pulmonary accumulation of intravenously administered CNCs at the dose of ~20 mg/kg⁴⁹, which is related to the stability of the injected dispersion, and we have aimed to obviate these problems by using a lower injected dose as well as improving the dispersion stability of the DOTA-CNC.

Figure 5. The *ex vivo* biodistribution of radioactivity after the injection of ^{111}In -DOTA-CNC (ald.) and ^{111}In -DOTA-CNC (OH) NPs in healthy CD-1 mice (**a and c**) and in 4T1-tumor bearing Balb/c mice (**b and d**), respectively, at 6, 24, and 48 h p.i. The values represent mean $\pm$ s.d. (n=3–5). %ID/g = percentage injected dose per gram of tissue, S.I. denotes small intestine and L.I. large intestine.

In our experiment, no acute adverse effects related to the nanomaterial administration were observed. Whole-body SPECT/CT scans were acquired at 1, 24, and 48 p.i. for 60 min followed by a 6 min CT scan. Overall, the coronal SPECT/CT distribution profile of both ^{111}In -DOTA-CNC NPs formulations corresponded well with what was observed in *ex vivo* biodistribution in which the radioactivity

accumulated substantially in the principal MPS organs: the liver, lung, and spleen. Similarly, in the experiment with ^{111}In -DOTA-CNC (OH) NPs pronounced pulmonary radioactivity accumulation was observed in the early time point in both animal models before accumulation to the liver and spleen at the later time points (**Figure 6**).

Figure 6. Representative coronal SPECT/CT fusion images after *i.v.* administration of ^{111}In -DOTA-CNC (ald.) (**a and b**) and ^{111}In -DOTA-CNC (OH) (**c and d**) NPs in healthy and 4T1 tumor-bearing animal models at 1, 24, and 48 h p.i., respectively. Lu denotes for lung, Li for liver, Sp for spleen, and Bl for bladder.

CONCLUSIONS

In summary, we have developed new molecular imaging probes based on nanocrystalline cellulose radiolabeled with the diagnostic radionuclide ^{111}In for SPECT imaging and evaluated their biodistribution profiles in healthy and tumor-bearing mice. The DOTA-modified CNCs were successfully prepared using two strategies in which the DOTA-hydrazide and DOTA-amine were conjugated through the aldehyde group (DOTA-CNC (ald.)) at reducing end of CNC polymer and to the hydroxyl groups (DOTA-CNC (OH)) on the surface of the CNC, respectively. Both strategies yielded stably labeled ^{111}In -DOTA-CNC NPs, suggesting the possibility to track these nanoprobe behavior over time with low dissolution of the ^{111}In -DOTA complex in vivo. Moreover, the DOTA-CNC (OH) NPs demonstrated a higher specific activity than DOTA-CNC (ald.) NPs when labeled with ^{111}In and started with less initial activity of $^{111}\text{InCl}_3$. This reveals a better radiolabeling efficacy and allows to minimize the injected amount of CNC NPs whilst maintaining sufficient radioactive dose for SPECT/CT imaging. Both of the DOTA-modified CNC nanoprobe showed good biocompatibility in both non-tumor and tumor cell models in which the cell viability was maintained high after 72 h incubation with the CNCs in vitro. Both the ex vivo biodistribution and in vivo SPECT/CT imaging in healthy and tumor-bearing mice showed a similar pharmacokinetic biodistribution profile for ^{111}In -DOTA-CNC (ald.) and ^{111}In -DOTA-CNC (OH) NPs, where the nanoprobe were mainly uptaken by the MPS organs. However, the ^{111}In -DOTA-CNC (OH) NPs showed a high but transient accumulation in the lung in vivo at the early time points after systemic administration likely due to the loss of surface charge or dispersion-stabilizing properties during the chemical modification. Therefore, the current efforts are oriented towards the optimization and improvement of conjugation chemistry in order to maintain a proper surface charge property of the CNC while maintaining sufficient radioactivity for theranostic applications, to reduce the MPS uptake, and to include tumor-targeting vectors. To this end, the terminal aldehyde modification might be a more appealing strategy as it renders the OH-groups on the CNC surface free for subsequent

functionalization and payload incorporation. The developed imaging probes provide a convenient avenue towards the investigation of CNC drug delivery system behavior in vivo to guide structural optimization for biomedical applications and clinical translation.

SUPPORTING INFORMATION

Cell culturing materials and methods, DOTA-hydrazide synthesis and related data, ATR-FTIR characterization, CNCs size distribution, radiochemical purity of ^{111}In -DOTA-CNCs, and results for tumor uptake (PDF). The Supporting Information is available free of charge on the ACS Publications website at <https://pubs.acs.org/>.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

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Supporting Information

Radiolabeled Molecular Imaging Probes for the *in Vivo* Evaluation of Cellulose Nanocrystals for Biomedical Applications

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Cell culturing materials and methods. Murine RAW 264.7 macrophages and 4T1 mammary adenocarcinoma cell lines were acquired from American Type Culture Collection (ATCC), VA, USA. T75 cell culturing flasks and 96 well-plate were purchased from Corning Inc., NY, USA. Dulbecco's phosphate buffer saline (10×DPBS), Dulbecco's Modified Eagle medium (DMEM) for RAW 264.7 macrophages, Roswell Park Memorial Institute 1640 medium (RPMI-1640) for 4T1, GlutaMAX (100×), fetal bovine serum (FBS), Penicillin-Streptomycin (10,000 U/ml), and TrypLE™ (1×) were purchased from Life Technologies Gibco, Carlsbad, CA, USA. Hyclone™ Non-essential amino acids (100×) was acquired from GE Healthcare Life Sciences, Chicago, IL, USA. All culturing media were supplemented with 1× non-essential amino acids, 10% FBS, 1×GlutaMAX and 1% Penicillin-Streptomycin and aseptically filtered through sterilized 0.22 µm filter unit before use. The cell incubator was set at 37 °C with 5% CO₂ and 95% relative humidity.

DOTA-hydrazide synthesis. The 1,4,7,10-tetraazacyclododecane-1,4,7-tris (acetic acid)-10-acethydrazide or DOTA-hydrazide was prepared by the hydrazinolysis reaction (**Figure S1**). The 1,4,7,10-tetraazacyclododecane-1,4,7-tris-tert-butyl acetate-10-acetic acid (Boc-DOTA-COOH) precursor (1 eq., 100 mg, 30 mM, Macrocyclics™ Inc., TX, USA) was dissolved in dried DMF under argon atmosphere. The HATU (1.2 eq., 79.7 mg, 36 mM) was separately dissolved in dried DMF and added to the Boc-DOTA-COOH solution. The reaction was left stirring at room temperature under argon atmosphere for 10 min. The *N,N*-Diisopropylethylamine or DIPEA (2 eq., 61 µL, 60 mM) was added to the reaction mixture and left for 30 min while stirring. Then, the hydrazine solution in 1.0 M THF (1.1 eq., 7 µL, 33 mM) was subsequently added to the reaction mixture. The reaction was left stirring overnight under argon. The completed reaction was monitored by TLC Silica gel 60 RP-18 F₂₅₄s coated on aluminium sheet (EMD Millipore Corporation, Billerica, MA, USA) with the DCM : MeOH (9:1) eluent system (*R_f* = 0.67). The

liquid-liquid extraction was performed in order to remove water soluble by products and DMF with 2×20 mL ethyl acetate, 10 mL water, and 10 mL saturated brine solution. The combined organic layer was dried over sodium sulfate (Na₂SO₄) before filtered through WhatmanTM filter paper (ϕ = 55 mm, GE Healthcare Life Sciences, Buckinghamshire, UK) and evaporated to dryness.

The product was re-dissolved in 1 mL of MeOH, and then 3 mL of ACS reagent grade 37% HCl solution was added. The Boc-deprotection reaction was left agitating in an open system under the fume hood for 4 h. All solvents were removed by vacuum rotary evaporator. The product was repeatedly washed and evaporated with 7× 6 mL of DCM in order to remove the concentrated HCl. The viscous yellowish oil product was again re-dissolved in 1 mL of MeOH and the solution was passed through a reverse-phase Sep-Pak[®] Plus C18 SPE cartridge (Waters Corporation, Milford, MA, USA), which was preconditioned with 2 mL of acetonitrile and 10 mL of water. The DOTA-hydrazide was eluted out from the C18 cartridge by 1.5 mL of acetonitrile:water (70:30) mixture. The eluted solution was evaporated to dryness and characterized by ¹H-NMR, ¹³C-NMR, and ESI-TOF MS.

The DOTA-hydrazide (72 mg, yield 72%) was successfully synthesized and characterized with ¹H and ¹³C NMR (Varian Mercury 300 spectrometer model). Chemical shifts are reported as a ppm (δ) calibrated with the solvent residual peak position. The assignments of H and C positions are according to the scheme in *Figures S2*.

¹H-NMR (300 MHz, *d*₆-DMSO), (a) δ = 2.49 ppm (s, 16H), (b) δ = 3.01 – 3.55 ppm, br, 6H, (c) δ = 3.68 ppm, m, 2H, (d) δ = 3.83 – 4.12 ppm, t, 2H, (e) δ = 10.24 ppm, s, 1H, and *d*₆-DMSO solvent residual peak δ = 2.50 ppm.

^{13}C -NMR (300 MHz, d_6 -DMSO), (a) $\delta = 49.58$ ppm, (b) $\delta = 52.94$ ppm, and (c) $\delta = 170.28$ ppm, and d_6 -DMSO solvent residual peak $\delta = 39.51$ ppm.

Additionally, the ESI-TOF MS (microTOF, Bruker Daltonics[®], Billerica, MA, USA) $[\text{M}+\text{H}]^+$ showed a m/z ratio corresponding to 419.2134 (m/z calc. 419.2210).

Figure S1. The synthesis scheme of DOTA-hydrazide through hydrazinolysis reaction.

Figure S2. NMR assignments of DOTA-hydrazide

Figure S3. ATR-FTIR characterization of CNC unmodified CNC, DOTA-CNC (ald.), and DOTA-CNC (OH).

Figure S4. The width and length distribution of CNC NPs (a) unmodified CNC, (b) DOTA-CNC (ald.), and (c) DOTA-CNC (OH), n=50.

Figure S5. The autoradiography of injected formulations of **(a and c)** ^{111}In -DOTA-CNC (ald.) and **(b and d)** ^{111}In -DOTA-CNC (OH) for *ex vivo* biodistribution and SPECT/CT imaging, respectively. %RCP denotes for percent of radiochemical purity of final formulation.

Figure S6. Radioactivity uptake in tumor after *i.v.* administration of ^{111}In -DOTA-CNC (ald.) and ^{111}In -DOTA-DND (OH) in 4T1-tumor bearing mice after 6, 24, and 48 h p.i. (n=3).