Folic Acid-Conjugated Cellulose Nanocrystals Show High Folate-Receptor Binding Affinity and Uptake by KB and Breast Cancer Cells

Katelyn Rose Bittleman,† Shuping Dong,‡ Maren Roman,*‡§ and Yong Woo Lee†∥

†Virginia Tech-Wake Forest University School of Biomedical Engineering and Sciences (MC0298), 325 Stanger Street, Blacksburg, Virginia 24061, United States
‡Macromolecules Innovation Institute (MC0201), Virginia Tech, 1075 Life Science Circle, Blacksburg, Virginia 24061, United States
§Department of Sustainable Biomaterials (MC0323), Virginia Tech, 310 West Campus Drive, Blacksburg, Virginia 24061, United States
∥Department of Biomedical Engineering and Mechanics (MC0298), Virginia Tech, 325 Stanger Street, Blacksburg, Virginia 24061, United States

Supporting Information

ABSTRACT: The study evaluates cellulose nanocrystals (CNCs) as nanocarriers for targeted, intracellular delivery of molecular agents. CNCs were labeled with fluorescein-5′-isothiocyanate as an imaging agent and conjugated to folic acid (FA) as a targeting ligand. The CNC conjugates were characterized by UV−vis spectroscopy, ζ-potential analysis, dynamic light scattering, and atomic force microscopy. Cellular binding/uptake of the FA-conjugated CNCs by KB and MDA-MB-468 cells was quantified with cellular uptake assays. Internalization of the particles was confirmed by confocal microscopy. Uptake mechanisms were determined by inhibition studies with chlorpromazine and genistein. Binding affinity was qualitatively assessed with a free folate inhibition assay. Both KB and MDA-MB-468 cells exhibited significant and folate-receptor specific binding/uptake of FA-conjugated CNCs. Clathrin-mediated endocytosis was a significant uptake mechanism in both cell types, whereas caveolae-mediated endocytosis only played a significant role in MDA-MB-468 cells. Uptake inhibition of FA-conjugated CNCs by KB cells required high concentrations (>1 mM) of free FA. The observed FR-specific internalization of FA-conjugated CNCs by FR-positive cancer cells and tumors and their remarkable high affinity for the FR demonstrate the great potential of CNCs as novel nanocarriers for imaging agents and chemotherapeutics in the early detection and treatment of cancer.

INTRODUCTION

One of the most promising applications of nanotechnology is the selective delivery of molecules to specific cells of the body. This can be accomplished with novel nanoscale delivery systems in which selective targeting agents, therapeutic drugs, and/or imaging probes are conjugated to the surface or encapsulated within the particle.1−4 As one of several targeting strategies, receptor-mediated cell targeting has become an attractive approach for the early detection and treatment of cancer in recent years, and many suitable cancer-specific receptors have been identified.5 Many factors need to be considered in the design of nanoscale carrier systems for imaging or therapeutic applications. One must optimize parameters, such as particle shape, size, surface chemistry, cytotoxicity, and circulation time.6 Decuzzi et al. investigated optimal size and shape of nanoparticles for increased circulation time and accumulation at tumor sites. Spherical particles were shown to be inefficient because they tend to flow toward the center of blood vessels with laminar flow, whereas irregularly shaped or high-aspect-ratio nanoparticles tend to be pushed to the walls of blood vessels, similar to platelets. This increases the likelihood of nanoparticles entering tumor tissues through fenestrations in the vasculature.6,7 Cellulose nanocrystals (CNCs) are elongated nanoparticles ranging in average length from 100 to 200 nm and average height from 3 to 5 nm if derived from wood pulp.8 This size range is expected to be too large for rapid renal clearance yet small enough for evasion of the mononuclear phagocytic system.9 However, because of their elongated shape, CNCs can be expected to orient themselves in the direction of blood flow and eventually pass through glomerular fenestrations in the kidney, enabling ultimate excretion in the urine.10 Further-
more, their high aspect ratio likely causes increased fenestration penetration in tumor vasculature compared to spherical particles, which many carriers are by design, e.g., liposomes, metal nanoparticles, and dendrimers. The starting material, cellulose, is extremely abundant in nature, inexpensive, and has excellent strength properties. Additionally, the surface of CNCs contains multiple hydroxyl groups, which are amenable to chemical modification for ligand targeting, labeling with imaging probes, and drug conjugating. Finally, toxicity studies have indicated a lack of or low toxicity for CNCs.

Folate receptors (FRs), mediating cellular uptake of folic acid (FA), also known as vitamin B9, are overexpressed on the plasma membrane of many cancer cell types, including breast, ovarian, lung, kidney, brain, and endometrial cancer, whereas normal tissues seldom express the FRs. FA is a key vitamin for cell division and is needed by all cells to proliferate. However, healthy cells can also take up other forms of folate via the reduced folate carrier and the proton-coupled folate transporter, which does not actively bind FA. Overexpression of the FR is essential for rapidly dividing cells, such as cancer cells.

Development of a noninvasive, inert screening strategy would greatly reduce late-stage diagnoses of cancer, minimize unnecessary risk to patients, and increase chances of survival. According to the American Cancer Society, the 5 year survival rate for lung cancer increases from 4 to 54% when diagnosed in early versus late stage. Likewise, kidney cancer has a 5 year survival rate of 92 versus 12% with early- and late-stage diagnoses, respectively. Currently, cancer screening methods are limited to breast, ovarian, colorectal, and prostate cancers. Since the FR is overexpressed in many cancer types, targeting strategies for the FR might enable us to screen for additional cancers, such as lung, kidney, and brain cancers, whose early detection would be extremely useful.

In a previous study, we showed that fluorescently labeled, FA-conjugated CNCs enable the detection of FR-positive human (DBTRG-05MG, H4) and rat (C6) brain tumor cells. In the study reported here, we investigated uptake of these particles by KB and human breast cancer cells (MDA-MB-468). We present confocal microscopy images that unequivocally and for the first time demonstrate internalization of FA-conjugated CNCs by FR-positive cancer cells. Furthermore, with a competition assay we show that FA-conjugated CNCs have a remarkably high affinity for the FR.

### RESULTS AND DISCUSSION

CNCs were synthesized from dissolving-grade, bleached wood pulp and functionalized with fluorescein-5′-isothiocyanate (FITC) for detection and quantification. The fluorescently labeled CNCs (FITC-CNC) were subsequently functionalized with FA for FR targeting. Measurements of the FITC and FA contents by UV−vis spectroscopy yielded FITC contents of 7.4 and 3.7 wt % for FITC-CNC and the FA-conjugate (FITC-CNC-FA), respectively, and an FA content of 22.9 wt % for FITC-CNC-FA (Table 1). ζ-Potential analysis showed that both FITC-CNC and FITC-CNC-FA had a negative particle surface charge that was similar to that of unmodified CNCs. Particle analysis by dynamic light scattering (DLS) revealed that the hydrodynamic diameters of FITC-CNC and FITC-CNC-FA were larger than that of unmodified CNCs. However, atomic force microscopy (AFM) images confirmed that both conjugates still had the initial elongated morphology (Figure 1).

FR-α expression by KB and MDA-MB-468 cells was quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunofluorescent staining. KB and MDA-MB-468 cells had significantly higher levels of FR-α mRNA and protein expression than human aortic endothelial cells (HAEC), the negative control (Figure S1). FR-α mRNA expression of KB cells was over 8 million fold higher and that of MDA-MB-468 cells over 7000-fold higher than FR-α mRNA expression of HAEC (Figure S1a). Little to no expression of FR-α protein was detected in HAEC, however, KB and MDA-MB-468 cells had detectable protein expression levels for FR-α (Figure S1b).

Incubation of KB and MDA-MB-468 cells in the presence of free FITC or nontargeted FITC-CNC resulted in little to no binding/uptake of these entities (Figure 2); furthermore, binding/uptake of FITC-CNC was lower than that of free

### Table 1. Properties of CNCs and CNC Conjugates

| sample     | cellulose content (wt %) | FITC content (wt %) | FA content (wt %) | hydrodynamic diameter (nm) | ζ-potential (mV) |
|------------|--------------------------|---------------------|-------------------|-----------------------------|-----------------|
| CNCs       | 100.0                    | 0                   | 0                 | 81 ± 2                      | −47 ± 2         |
| FITC-CNC   | 92.6                     | 7.4                 | 3.7               | 109 ± 23                    | 0.36            |
| FITC-CNC-FA| 73.4                     | 22.9                | 10.1              | 204 ± 50                    | 0.24            |

On the basis of the molar mass of the anhydroglucose unit. Data shown are means ± standard deviations. PDI: polydispersity index.
FITC. FITC molecules ($pK_a = 4.4$) are known to enter cells in their neutral form by passive diffusion across the plasma membrane, which may explain the greater uptake of FITC molecules relative to that of the larger, more hydrophilic, and negatively charged FITC-CNC particles. Incubation in the presence of FR-targeted FITC-CNC-FA resulted in significant binding/uptake of the nanoparticles (Figure 2), many times greater (>1000 times that of FITC-CNC) than could be expected from the slightly higher CNC concentration (99.2 μg/mL for FITC-CNC-FA versus 62.6 μg/mL for FITC-CNC, particle concentrations were normalized by FITC content). The fact that FITC-CNC-FA is bound/internalized despite its negative charge indicates that charge–charge repulsion is sufficiently suppressed at the ionic strength of the cell culture medium or weaker than the receptor–ligand affinity.

To confirm targeting specificity, we conducted a competition assay in which KB cells were incubated in the presence of FITC-CNC-FA and increasing amounts of free FA. As the free FA concentration increased to 25 mM, decreasing amounts of FITC-CNC-FA were bound/taken up by KB cells (Figure 3). The decrease in binding/uptake of FITC-CNC-FA in the presence of free FA suggests that FITC-CNC-FA uptake by FR-negative cells will be minimal, i.e., that FITC-CNC-FA is selective toward FR-positive cells. The high concentration of free FA that was needed to significantly reduce FITC-CNC-FA binding/uptake by KB cells is remarkable. Previous studies showed a significant decrease in binding/uptake of FA conjugates at free FA concentrations of 1 mM or less. Here, although the concentration of bound FA in the culture medium was only 0.07 mM, a free FA concentration of 5 mM caused a smaller than 40% reduction in FITC-CNC-FA binding/uptake. Nukolova et al. reported a similar observation for folate-decorated nanogels. It has been suggested that the carboxyl group ($\alpha$ or $\gamma$) used to conjugate folate to other ligands or particles has a direct effect on the affinity of FA for the FR. Leamon et al. have further suggested that FR-mediated endocytosis is strongly influenced by the steric environment around the folate’s 4-aminobenzoic acid moiety. The apparently high binding affinity of FITC-CNC-FA suggests that low concentrations are sufficient to achieve significant cellular uptake in vivo at human serum folate concentrations (9–14 nM). Preliminary targeting experiments in a mouse model (Figure S2) indicated in vivo targeting efficacy.

Next, we investigated the mechanism for endocytosis of the FR-targeted CNC conjugates. The endocytic pathway via FR is known to be mediated by two proteins, clathrin and caveolin. KB and MDA-MB-468 cells pretreated with a clathrin inhibitor showed significantly reduced binding/uptake of FITC-CNC-FA (Figure 4). In MDA-MB-468 cells, binding/uptake of FITC-CNC-FA was also significantly reduced by a caveolae inhibitor. These results suggest that the mechanism for FITC-CNC-FA uptake via the FR is cell-dependent and nonexclusive. Both cell types exhibited primarily clathrin-mediated endocytosis. However, in MDA-MB-468 cells, caveolae-mediated endocytosis was also significant. Many studies have reported cell type-specific dependence on either clathrin or caveolin for FR-mediated endocytosis. In our previous study, human and rat astrocytoma cells (DBTRG-05MG and C6, respectively), which are non-neuronal brain cells, internalized FITC-CNC-FA primarily via caveolae-mediated endocytosis, whereas human ganglioma cells (H4), which are neuronal brain cells, internalized FITC-CNC-FA

![Figure 2. Cellular binding/uptake of free FITC (i–iii), FITC-CNC (iv–vi), and FITC-CNC-FA (vii–ix). KB (A) and MDA-MB-468 (B) cells were exposed to either free FITC or FITC-CNC conjugates for 2 h, stained with Alexa Fluor 594, and imaged by fluorescence microscopy. Images shown in (A) and (B) are representative of cell membrane (i, iv, vii), bound/uptaken FITC (ii, v, viii), and overlay images (iii, vi, ix) for each group ($n = 4$) (bar: 100 μm). Quantitative analysis of bound/uptaken particles (C). Data shown are means ± SDs for each group ($n = 4$) of relative fluorescence intensity normalized against cell membrane area (*$p < 0.05$ vs free FITC, **$p < 0.05$ vs FITC-CNC).](https://doi.org/10.1021/acsomega.8b01619)
primarily via clathrin-mediated endocytosis. Suen et al. have suggested that particle size also plays an important role in the mechanism of FR internalization triggered by nanoparticle binding. Their data show that nanoparticles with hydrodynamic diameters of about 50 and 120 nm are internalized by both caveolae- and clathrin-mediated endocytosis pathways, whereas larger nanoparticles with a hydrodynamic diameter of around 250 nm are only internalized by caveolae-mediated endocytosis. The fact that clathrin-mediated endocytosis is observed with FITC-CNC-FA, having a hydrodynamic diameter of about 200 nm, indicates either that the size limit for this uptake mechanism is above 200 nm or that the size limit depends on cell type.

Because of its large depth of field, relative to cell height, fluorescence microscopy cannot distinguish between mem-

Figure 3. Effects of free FA on cellular binding/uptake of FITC-CNC-FA by KB cells. Cells were exposed to FITC-CNC-FA and increasing concentrations of FA for 2 h, stained with Alexa Fluor 594, and imaged by fluorescence microscopy. Data shown are representative images of cell membrane and bound/uptaken FITC for each group (n = 4) (bar: 100 μm) (A) and means ± SDs for each group (n = 4) of fluorescence intensity normalized against cell membrane area (B) (*p < 0.05 vs control).

Figure 4. Effects of endocytosis inhibitors on cellular binding/uptake of FITC-CNC-FA. KB (A) and MDA-MB-648 (B) cells were pretreated with phosphate-buffered saline (PBS), 5 μg/mL chlorpromazine (clathrin inhibitor) or 200 μM genistein (caveolae inhibitor) 30 min, exposed to FITC-CNC-FA in the presence of the inhibitors for 2 h, stained with Alexa Fluor 594, and imaged by fluorescence microscopy. (A) and (B) show representative images of cell membrane and bound/uptaken FITC for each group (n = 4) (bar: 100 μm). Data shown in (C) are means ± SDs for each group (n = 4) of fluorescence intensity normalized against cell membrane area (*p < 0.05 vs PBS).
brane binding and cellular uptake of nanoparticles. To assess whether FITC-CNC-FA particles are being internalized by KB and MDA-MB-468 cells, we recorded z-stacks of KB and MDA-MB-468 cells incubated in the presence of FITC-CNC-FA with a confocal microscope. Cross-sectional confocal microscopy images confirmed that in addition to binding externally to the cell membrane, FITC-CNC-FA are being internalized by FR-positive cancer cells (Figure 5).

**CONCLUSIONS**

Advances in the biomedical field have led to the discovery of numerous nanoscale delivery vehicles. CNCs have characteristics that make them an excellent choice as a nanocarrier. We have demonstrated that once conjugated with FA, CNCs are internalized by cells via both clathrin- and caveolae-dependent, FR-mediated endocytosis pathways. We have also shown that FA-conjugated CNCs have a remarkably high affinity for the FR and thus offer highly efficient targeting of FR-positive cancer cells. The ability of FITC-CNC-FA particles to bind specifically to the membranes of FR-positive cancer cells, demonstrated in this and our previous study, makes them promising diagnostic imaging agents for the early detection of cancer. Moreover, the internalization of FITC-CNC-FA particles by FR-positive cancer cells, proven here for the first time, might enable the selective intracellular delivery of chemotherapeutic agents to such cells.

**EXPERIMENTAL PROCEDURES**

**Materials.** Dissolving-grade softwood sulfite pulp (Temalfa 93A-A) was kindly provided by Tembec, Inc. (Montréal, QC, CA). FITC, FA, genistein, doxorubicin, and dimethylsulfoxide were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Chlorpromazine was purchased from MP Biomedicals (Santa Ana, CA). Folate-free RPMI 1640 medium, medium 200, low serum growth supplement, Concanavalin A Alexa Fluor 594 and 633 conjugates, and donkey anti-goat Alexa Fluor 488 were purchased from Invitrogen Corp. (Carlsbad, CA). Goat poly Immunoglobulin G (IgG) FR-α and goat non-IgG primary antibodies and bovine serum albumin (BSA) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibiotics (penicillin and streptomycin) and fetal bovine serum (FBS) were purchased from Mediatech, Inc. (Manassas, VA). RNeasy Mini Kit was purchased from Qiagen (Valencia, CA). Reverse Transcription System and CellTiter-Blue Cell Viability Assay Kit were purchased from Promega (Madison, WI). TaqMan Universal PCR Master Mix, gene-specific TaqMan PCR probes and primers were purchased from Applied Biosystems (Carlsbad, CA). VECTASHIELD HardSet Mounting Media with and without 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Vector Labs (Burlingame, CA). Attachment factor solution (AFS) was purchased from Cell Applications (San Diego, CA). The human cancer cell lines KB and MDA-MB-468 were purchased from American Type Culture Collection (Manassas, VA), whereas HAEC were purchased from Invitrogen Corp. (Carlsbad, CA). All other reagents and supplies were purchased from Fisher Scientific (Pittsburgh, PA).

**METHODS**

**CNC Preparation and CNC Conjugate Synthesis.** CNCs and CNC conjugates were prepared, as described previously. Briefly, a CNC suspension in deionized water was prepared by hydrolysis of softwood sulfite pulp with 64 wt % sulfuric acid at 45 °C for 1 h with an acid-to-pulp ratio of 10 mL/g. FITC was conjugated to the CNCs after amination of the surface hydroxyl groups with epichlorohydrin and ammonium hydroxide. FA was conjugated to the FITC-labeled CNCs (FITC-CNC) with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and N-hydroxysulfosuccinimide to yield FA-conjugated, FITC-labeled CNCs (FITC-CNC-FA).

**UV–Vis Spectroscopy.** UV–vis absorption spectra were recorded using dilute suspensions of known concentration between 0.001 and 0.01 wt % in standard cuvettes, having an optical path length of 1 cm, with a Thermo Scientific Evolution 300 UV–vis spectrometer.
\(\zeta\)-Potentials. \(\zeta\)-potentials were measured in triplicate at 25 ± 0.1 °C in the absence of added electrolyte with a Malvern Zetazizer NanoZS particle analyzer (Malvern Instruments Ltd., Malvern, U.K.). Samples had a concentration of 0.01 wt % and were analyzed in Malvern DTS1060-folded capillary cells after sonication for 10 min in a 70 W Cole-Parmer 8890 ultrasonic cleaner. Reported values are mean \(\zeta\)-potentials obtained with the Zetasizer Nano 4.2 software using the Hückel model.

Dynamic Light Scattering (DLS). DLS measurements were conducted in triplicate at 25 ± 0.1 °C with a Malvern Zetazizer NanoZS particle analyzer (Malvern Instruments Ltd., Malvern, U.K.). Samples had a concentration of 0.01 wt % and were analyzed in 12 mm square glass cuvettes (Malvern PCS 1115) after filtration through 0.45 μm poly(vinylidene fluoride) syringe filters. Reported values are mean Peak 1 diameters obtained with the General Purpose algorithm of the Zetasizer Nano 4.2 software.

Atomic Force Microscopy (AFM). AFM images were recorded with an Asylum Research MFP-3D Bio atomic force microscope (Asylum Research, Santa Barbara, CA). Aqueous suspensions were diluted to 0.001 wt % and sonicated at 150 W for 3 min. Single drops of the suspensions were placed on freshly cleaved mica surfaces and allowed to dry under ambient conditions. Samples were scanned in intermittent contact mode under ambient conditions with Olympus AC160TS tips (nominal tip radius <10 nm, spring constant 42 N/m).

Cell Culture. HAEC were grown in Medium 200 supplemented with low serum growth supplement. KB and MDA-MB-468 cells were grown in folate-free RPMI 1640 medium supplemented with 10% FBS. Cells were grown at 37 °C and 5% CO2. All experiments were conducted in basal medium.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA from HAEC, KB, and MDA-MB-468 cells was isolated and purified using RNeasy Mini Kit according to the protocol of the manufacturer. Quantitative real-time RT-PCR using TaqMan probes and primers were used for gene expression analyses, as described previously. Amplification of individual genes was performed with Applied Biosystems 7300 real-time PCR system using TaqMan Universal PCR Master Mix and a standard thermal cycler protocol. TaqMan Gene Expression Assay Reagents for human FR-α and glyceraldehyde 3-phosphate dehydrogenase were used for specific probes and primers of PCR amplifications. The threshold cycle (C_T) was determined, and relative quantification was calculated by the comparative C_T method as described previously. Results were observed and analyzed using a 7300 Real-time PCR System and 7300 Real-time PCR System Sequence Detection Software v1.2.3 (Applied Biosystems, Carlsbad, CA).

Fluorescence Microscopy. HAEC, KB, and MDA-MB-468 cells were seeded onto Lab-Tek Chamber Slide systems coated with AFS and grown to confluence. Before imaging, cells were washed and mounted with VECTASHIELD HardSet Mounting Medium with or without DAPI. Images were obtained with a Leica AF6000 fluorescent microscope (Leica Microsystems Inc., Buffalo Grove, IL). A quantitative comparison of experimental groups was conducted by measuring the total fluorescence intensity normalized by cell area using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescent Staining. Cells were washed and fixed with 4% paraformaldehyde for 15 min. Cells were again washed and blocked with 3% BSA for 1 h. Cells were then treated with 4 μg/mL goat polyclonal IgG FR-α primary antibody or goat non IgG antibody in 1% BSA solution overnight at 4 °C. Cells were thoroughly washed and treated with 2 μg/mL donkey anti-goat Alexa Fluor 488 in 1% BSA solution for 2 h at room temperature.

Binding/Uptake Assay. Cells were incubated with FITC, FITC-CNC, or FITC-CNC-FA at a FITC concentration of 5 μg/mL for 2 h, corresponding to FITC-CNC and FITC-CNC-FA concentrations of 67.6 and 135.1 μg/mL, respectively. Cells were washed twice with phosphate-buffered saline (PBS), and the plasma membranes were briefly stained with 50 ng/μL of Concanavalin A Alexa Fluor 594 for 2 min. Cells were then fixed with cold ethanol for 1 h.

Free Folate Inhibition Assay. Cells were incubated with FITC-CNC-FA at a FITC concentration of 5 μg/mL in the presence of 0, 5, 10, or 25 mM FA for 2 h. Cells were washed twice with PBS, and the plasma membranes were briefly stained with 50 ng/μL of Concanavalin A Alexa Fluor 594 for 2 min. Cells were then fixed with cold ethanol for 1 h.

Binding/Uptake Mechanism Assay. Cells were pre-treated with PBS, 5 μg/mL chlorpromazine, or 200 μM genistein for 30 min. Growth medium was replaced with basal medium containing FITC-CNC-FA at a FITC concentration of 5 μg/mL and either PBS, 5 μg/mL chlorpromazine (clathrin inhibitor), or 200 μM genistein (caveolae inhibitor). After 2 h of exposure, cells were washed twice with PBS, and the plasma membranes were briefly stained with 50 ng/μL of Concanavalin A Alexa Fluor 594 for 2 min. Cells were then fixed with cold ethanol for 1 h.

Confocal Microscopy. KB and MDA-MB-468 cells were seeded onto round coverslips coated with AFS and allowed to grow for 24 h at 37 °C. Cells were incubated with FITC, FITC-CNC, or FITC-CNC-FA at a FITC concentration of 5 μg/mL for 2 h. Cells were washed twice with PBS, and the plasma membranes were briefly stained with 50 ng/μL of Concanavalin A Alexa Fluor 633 for 2 min. Cells were then fixed with cold ethanol for 1 h.

Statistical Analysis. All statistical analysis of data was performed using SigmaPlot 11 (SPSS Inc., Chicago, IL). One-way analysis of variance was used to compare mean responses among the treatments. For each endpoint, the treatment means were compared using the Holm–Sidak method. Statistical probability of \(p < 0.05\) was considered significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01619.

FR-α expression in HAEC KB, and MDA-MB-468 cells; preliminary in vivo targeting data in a mouse model; tumor sizes over the course of in vivo study; mouse weights over the course of the in vivo study (PDF)
Corresponding Author
*E-mail: maren.roman@vt.edu. Phone: (540) 231-1421. Fax: (540) 231-8176.

ORCID
Maren Roman: 0000-0001-6622-8591

Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
AFM, atomic force microscopy; AFS, attachment factor; BSA, bovine serum albumin; CNCs, cellulose nanocrystals; DAPI, 4',6-diamidino-2-phenylindole; DLS, dynamic light scattering; FA, folic acid; FBS, fetal bovine serum; FITC, fluorescein-5-isothiocyanate; FR, folate receptor; IgG, Immunoglobulin G; HAEC, human aortic endothelial cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction

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