JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Synthetic Polymer Nanoparticles Conjugated with $FimH_A$ from *E. coli* Pili to Emulate the Bacterial Mode of Epithelial Internalization

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Supporting Information

ABSTRACT: Amphiphilic block copolymer nanoparticles are conjugated with uropathogenic *Escherichia coli* type 1 pilus adhesin $FimH_A$ through amidation chemistry to enable bladder epithelial cell binding and internalization of the nanoparticles in vitro.

Urinary tract infections (UTI) account annually for approximately 7 million office visits and 100 000 hospitalizations and are among the most common bacterial infections in the United States.[1] Almost half of all women will experience an episode of UTI during their lifetime, and 1 in 3 women will require antimicrobial treatment by the age of 24.[2] Although females are more susceptible to UTI than are males, many subpopulations that are also at increased risk include infants and the elderly as well as patients with diabetes, spinal cord injuries, and urologic abnormalities, resulting in economic costs associated with UTI of $2 billion annually, even more than a decade ago.[3,4]

Uropathogenic strains of *Escherichia coli* (UPEC) are the predominant cause of community-onset and healthcare-associated UTI.[5,6] Consequently, understanding the compositional and pathogenetic details of UPEC is important for the development of therapeutic systems. UPEC's ability to attach to superficial epithelial cells of the bladder is mediated by heteropolymeric surface fibers called type 1 pili. Type 1 pili consist of a thick pilus rod composed of a helical polymer of the major subunit FimA plus a thin, flexible tip fibrillum comprised of the adapter FimF, fibrillar subunit FimG and tip adhesin FimH.[7] The terminal, adhesive domain of FimH (denoted $FimH_A$) allows the pathogen to bind mannose moieties decorating the centers of the 16 nm uroplakin complexes found on the luminal surface of bladder epithelial cells.[8−10] Earlier work has shown that type 1 pili are necessary and sufficient to mediate internalization of UPEC into bladder epithelial cells,[11] and this activity is conferred by the mannose binding pocket within the FimH adhesin domain.[12,13] Internalized bacteria can replicate within the epithelial cell cytoplasm to form biofilm-like communities[14] and later establish a quiescent reservoir within bladder epithelium that avoids immune detection and might later re-emerge to cause recurrent UTI.[13,14]

Our primary interest is to develop nanoparticle carriers that bind and enter bladder epithelial cells to deliver antimicrobial agents directly to the niche occupied by these harbored bacteria. In this study, therefore, we have designed multifunctional shell cross-linked knedel-like nanoparticles (SCKs) that can be conjugated with $FimH_A$ for bladder epithelial cell binding and internalization, for the purpose of targeted delivery of various antimicrobial agents for the treatment and prevention of UTIs. The SCKs were constructed from aqueous assembly of poly(acrylic acid)-*block*-polystyrene block copolymers (PAA-*b*-PS) into micelles, followed by covalent cross-linking throughout the shell region to afford discrete, robust shell cross-linked nanostructures. The amphiphilic core−shell morphology provides opportunities to partition the various functions of therapeutic packaging,[15,16] cell targeting, and imaging within different regions of the nanoparticle framework.[17] In this study, the residual reactive acrylic acid residues on the surfaces and throughout the shell layers of the SCKs were utilized for amidation with native lysines of $FimH_A$ to conjugate the adhesin and promote epithelial cell targeting and internalization and with the amine terminus of the fluorescein-based dye, 5-((5-aminopentyl)thioureidyl)fluorescein (FITC cadaverine) to permit fluorescence microscopic imaging (Figure 1).

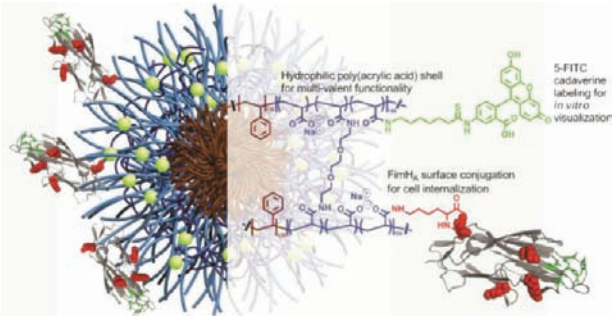

Figure 1. Schematic and chemical representation of $FimH_A$-functionalized and FITC cadaverine-labeled SCK nanoparticles.

Received: October 2, 2011
Published: February 23, 2012

dx.doi.org/10.1021/ja2091917 | *J. Am. Chem. Soc.* 2012, 134, 3938−3941

The multifunctional SCK nanoparticles were prepared from previously reported PAA_{120}-*b*-PS_{100} amphiphilic block copolymers,[16] through aqueous micellization and further covalent cross-linking and chemical modification reactions. Water (a selective solvent for the PAA block segment) was introduced to a THF (a good solvent for both PAA and PS) solution of the block copolymer dropwise via a syringe pump to form discrete spherical micelles. The PAA shell regions of these micelles were then cross-linked via amidation chemistry with 2,2′-(ethylenedioxy)bis-(ethylamine) (EDDA), as a diamine cross-linker, in the presence of 1-[3′-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (EDCI) to afford SCK nanoparticles. 5-FITC cadaverine was attached through additional amidation chemistry using the coupling agent EDCI, rendering the SCKs fluorescent, to facilitate in vitro tracking of SCKs during functional studies (Figure 2A; see Supporting Information for detailed experimental

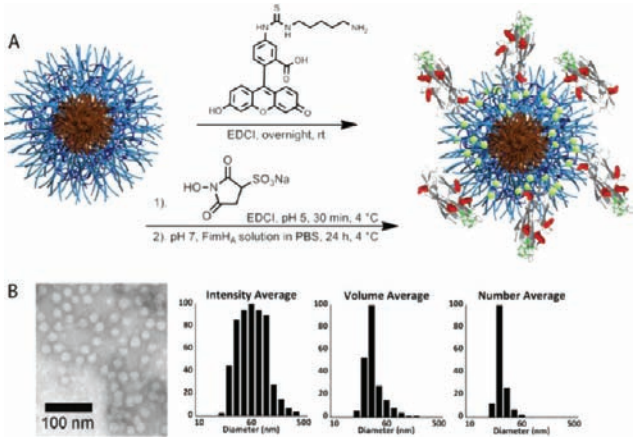

Figure 2. (A) Schematic representation of 5-FITC cadaverine labeling and $FimH_A$ conjugation to the shell of SCK nanoparticles via amidation chemistry. (B) TEM and DLS characterization of 5-FITC cadaverine-tagged, $FimH_A$-conjugated SCK nanoparticles. Diameter (TEM) = 20 ± 3 nm, $D_{h(intensity)}$ = 80 ± 30 nm, $D_{h(volume)}$ = 50 ± 20 nm, $D_{h(number)}$ = 40 ± 10 nm.

procedures). The conjugation of the targeting ligand $FimH_A$ was then accomplished by nonselective reaction between any of the four lysine residues on $FimH_A$ and residual carboxylic acids on the SCK surface, following a previously established method.[18] The reaction was carried out by first lowering the pH of the SCK solution in nanopure water to 5 with acetic acid and incubating at 4 °C for 30 min. Addition of sulfonated *N*-hydroxysuccinimide and coupling agent EDCI followed, and the reaction mixture was then allowed to stir at 4 °C for another 30 min before the pH of the solution was adjusted to 7−8 by addition of sodium phosphate. $FimH_A$ (0.65 mg/mL in PBS, pH 7.4) was added to the reaction solution slowly, and the conjugation process was allowed to proceed for 24 h at 4 °C. Purification by extensive washing using a centrifugal filtration device (MWCO 100 kDa) with PBS solution was performed to remove $FimH_A$ that was not associated with the nanoparticles. The final products were characterized physicochemically to determine their sizes, compositions, and structures, and the effects of $FimH_A$ conjugation on their biological behaviors were evaluated.

The dimensions of the 5-FITC cadaverine-labeled and $FimH_A$-functionalized SCK nanoparticles were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS) (Figure 2B). The circularly shaped images observed by TEM suggested that these nanoparticles were spherical with a narrow size distribution of 20 ± 3 nm. The number average hydrodynamic diameter of SCK nanoparticles was determined to be 40 ± 10 nm by DLS.

$FimH_A$ functionalization efficiency in a biologically active form was determined by immunogold electron microscopy (EM), using primary rabbit anti-$FimH_A$ and secondary antirabbit IgG conjugated to 6 nm gold particles. The SCK nanoparticles were readily visualized by EM, and gold particle labeling of $FimH_A$-SCKs (33%) was significantly higher than that of naked SCKs (5.6%) or of $FimH_A$-SCKs omitting primary antibody (8.8%; $p < 0.005$ for both comparisons; Figure 3).

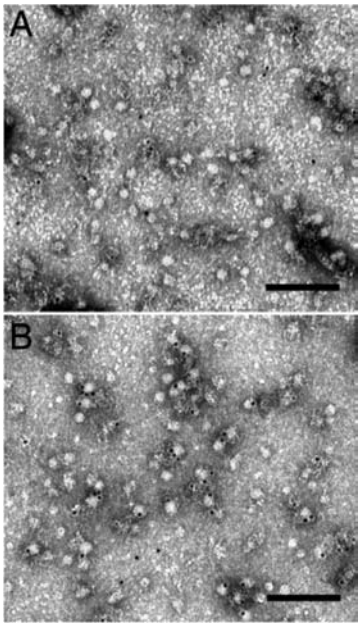

Figure 3. Functionalization of $FimH_A$ onto SCK surfaces. Naked SCKs (A) and $FimH_A$-functionalized SCKs (B) viewed by immunogold TEM after incubation with rabbit anti-FimH antibody and gold-conjugated antirabbit IgG. Labeling of $FimH_A$-SCKs was significantly greater than that of naked SCKs; representative images are shown. Scale bars, 100 nm.

Since the efficiency of the immunolabeling process was not precisely known, we concluded that the conjugation process accomplished $FimH_A$ attachment to a minimum of one third of SCKs.

We next demonstrated $FimH_A$-SCK-specific binding to cultured bladder epithelial cells. We then assessed epithelial cell binding and internalization by fluorescent confocal microscopy. Cells were grown to subconfluence on sterile glass coverslips, overlaid with 5-FITC cadaverine-labeled, naked or $FimH_A$-SCKs, washed, and stained with AlexaFluor 594-conjugated wheat germ to visualize the cell surfaces. By fluorescence microscopy the association of SCKs with the cell surfaces was shown to be dose dependent, $FimH_A$ specific, and inhibited by addition of mannose to the medium (Figure 4). Confocal analysis demonstrated several examples of internalized SCKs (Figure 5), though the majority of particles were localized to the cell surfaces, and internalized SCKs were only occasionally observed.

We then prepared samples by incubating $FimH_A$ protein and nanoparticles in the absence vs presence of the amidation coupling agents to evaluate the relative amount of $FimH_A$ that

dx.doi.org/10.1021/ja2091917 | *J. Am. Chem. Soc.* 2012, 134, 3938−3941

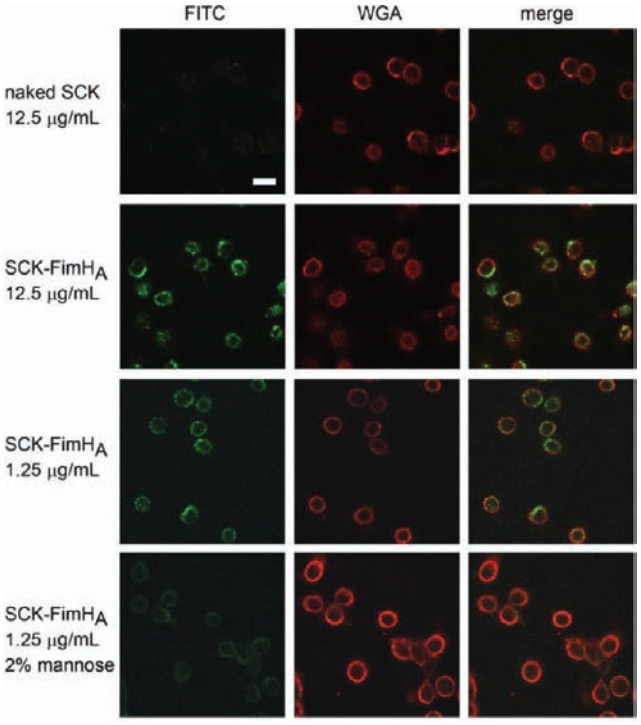

Figure 4. Binding of FimH$_A$-SCK nanoparticles to cultured bladder epithelial cells. Monolayers of 5637 cells were treated with the indicated doses of 5-FITC cadaverine-tagged (green) SCKs, naked or conjugated with FimH$_A$, and with or without 2% mannose. Cells were liberated from the surface and stained circumferentially with AlexaFluor 594-conjugated wheat germ agglutinin (WGA; red). Scale bar (upper left panel), 20 μm.

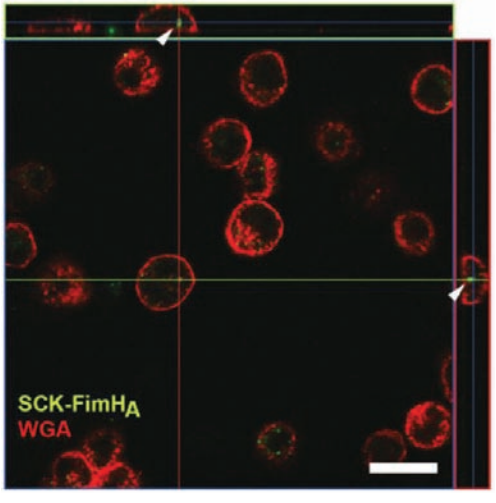

Figure 5. Epithelial cell internalization of FimH$_A$-SCKs. In this example of an internalized particle, the SCK of interest is viewed at the cross-hairs in the *XY* plane; orthogonal views (top and right of the panel) show the particle encircled by the red cell-surface stain (arrowheads). Scale bar, 20 μm.

was physically associated vs covalently conjugated with the SCKs, respectively. More free protein (noncovalently attached) was observed in samples that lacked the coupling agents; however, even the samples that had undergone the complete conjugation conditions contained a minor amount of free protein or protein that was strongly but noncovalently (perhaps electrostatically) associated with the SCKs. On sodium dodecyl sulfate (SDS) gels, the nanoparticles remained mostly in the sample wells, but a portion entered the stacking gel (5% acrylamide) and stopped at the interface of the resolving gel (12% acrylamide). By Western blot, FimH$_A$ was colocalized with the FITC signal arising from SCKs in sample wells and at the gel interface. This colocalized signal indicates covalent, detergent-resistant SCK-FimH$_A$ association, although the final functionalized SCKs, even with centrifugal filtration, existed as a mixture of physically and covalently associated protein and nanoparticle.

We have demonstrated the feasibility of leveraging a known bacterial–host interaction to direct nanostructures to an epithelial cell surface for potential therapeutic use. We recognize a number of opportunities for our ongoing work to optimize facets of our synthetic scheme: increasing FimH$_A$ density on the SCK surface (with full covalent attachment), improving the orientation of conjugated FimH$_A$ (i.e., altering lysine content to make the mannose binding pocket consistently available upon conjugation to SCKs), and promoting increased internalization of SCKs into epithelial cells (e.g., by varying the size and shape of the functionalized SCKs).[19,20] FimH$_A$ is readily amenable to genetic engineering approaches to optimize these parameters and to study the efficiency of alternative conjugation chemistries, and the conservation of mannose-binding capacity in engineered FimH$_A$ variants can be confirmed by readily available methods. Aside from the primary therapeutic aim, an antimicrobial-bearing SCK that would coat the luminal surface of the bladder would also be of interest for prophylaxis against UTI in special populations (those with indwelling catheters, who intermittently catheterize, or who are undergoing cystoscopy or other procedures). Upon identification of a lead FimH$_A$ construct, we will employ a murine model of cystitis[21] to begin studying in vivo activities.

An array of antimicrobials might be considered for loading into the functionalized SCKs described here, but silver is an attractive potential cargo,[22−26] as it may be loaded in cationic form or incorporated into hydrophobic organometallic compounds[27] and might be effective against reservoir bacteria that appear metabolically quiescent. In fact, our previous work demonstrated loading and release of cationic and organometallic silver with SCKs as well as antimicrobial activity of these silver-loaded systems against both *E. coli* and *Pseudomonas aeruginosa* at physiologically relevant concentrations.[15]

Finally, functionalized SCKs targeting bladder epithelial cells might be employed in antigen delivery for epithelial vaccines. FimH itself has been studied as a component of candidate vaccines against UTI,[28−30] but results so far have not translated into human efficacy. However, if alternative antigens and adjuvants that induce protective antibody and/or T-cell responses are identified, these could be adapted for presentation alongside FimH$_A$ on the SCK shell, where epithelial adhesion would promote prolonged antigen exposure in the urinary tract.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, including chemical syntheses, particle characterization and in vitro imaging. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank W. Beatty for technical imaging expertise. This project has been funded by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (R01-DK082546), by a March of Dimes Basil O'Connor Award (5-FY09-105), and by the Welch Foundation through the W. T. Doherty-Welch Chair in Chemistry, grant no. A-0001.

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