Cite as: G. L. Weiss *et al.*, *Science* 10.1126/science.aaz9866 (2020).

Architecture and function of human uromodulin filaments in urinary tract infections

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Uromodulin is the most abundant protein in human urine and forms filaments that antagonize the adhesion of uropathogens; however, filament structure and mechanism of protection remain poorly understood. We used cryo-electron tomography to show that the uromodulin filament consists of a zigzag-shaped backbone with laterally protruding arms. N-glycosylation mapping and biophysical assays revealed that uromodulin acts as a multivalent ligand for the bacterial type 1 pilus adhesin, presenting specific epitopes on the regularly spaced arms. Imaging of uromodulin-uropathogen interactions in vitro and in patient urine showed that uromodulin filaments associate with uropathogens and mediate bacterial aggregation, which likely prevents adhesion and allows clearance by micturition. These results provide a framework for understanding uromodulin in urinary tract infections and in its more enigmatic roles in physiology and disease.

The glycoprotein Uromodulin (Umod) is secreted in the kidney and is the most abundant urinary protein (1). A *UMOD* promoter variant in ~80% of the human population drives a two-fold increase of urinary Umod levels (2), resulting in reduced susceptibility to bacterial urinary tract infections (UTIs) (3). Uropathogenic *Escherichia coli* (UPEC) utilize adhesive type 1 pili to attach to high-mannose type N-glycans displayed on the uroepithelial surface (4). Umod has been suggested to act as a soluble adhesion antagonist for UPECs (5–11).

Mature Umod consists of three epidermal growth factor-like domains (EGF I–III), a cysteine-rich domain (D8C), a fourth EGF domain (EGF IV) and the bipartite zona pellucida module (subdomains ZP-N and ZP-C) (Fig. 1A) (1). Produced as a glycosylphosphatidylinositol-anchored precursor, Umod is then cleaved by the protease hepsin and assembles into homopolymeric filaments with an average length of ~2.5 μ m (12, 13). Despite its multiple roles in human health and disease (1), the molecular architecture and interactions of Umod in urine are poorly understood.

We first developed a protocol to purify stable Umod filaments from urine (table S1 and fig. S1A). Mass spectrometry (MS) of dissociated Umod monomers from different donors revealed highly similar, broad mass distribution profiles (fig. S1, B to D), showing that Umod glycosylation was gender- and genotype-independent. Next, we established a site-specific N-

glycosylation map of Umod by LC-MS/MS analysis of tryptic glycopeptides (Fig. 1A, figs. S2 to S6, and tables S2 and S3). We identified individual N-glycans attached to asparagine (Asn) residues 38, 76, 80, 232, 275, 322, 396 and 513 (figs. S2 to S6 and tables S2 and S3). High-mannose type N-glycans were found at Asn275 and Asn513, while other N-glycans were confirmed to be di-, tri-, or tetra-antennary complex types. Because UPEC adhere to uroepithelial cells via type 1 pili that specifically recognize high-mannose type N-glycans (4, 14), both glycans at Asn275 and Asn513 were candidates for mediating Umod's antiadhesive activity.

To contextualize the glycan arrangement within Umod polymers, we imaged Umod filaments by cryo-electron tomography (cryoET) (15) (Fig. 1B and movie S1). One orientation (Fig. 1C) was consistent with the previously observed zigzag shape (12, 13, 16, 17), though filaments showed different degrees of curvature and irregular rotations around the long axis. A second, prominent orientation had a fishbone-like appearance, with a central core filament and regularly protruding "arms" (Fig. 1D). We calculated a structure by subtomogram averaging, which resolved repeating filament-subunits arranged in a helix with 180° twist and 6.5 nm rise. The filament core consisted of 8.5 nm long modules zigzagging at 95° angles. The 12.5 nm long arm segments were flexible and protruded at 45° angles (Fig. 1E, fig. S7, and movie S1).

To assign Umod protein domains to densities in the

subtomogram average, we studied an elastase-digested form of Umod (eUmod), wherein Umod is cleaved between the D8C and EGF IV domains (18). eUmod retained the zigzag core structure, while densities for the arms were absent (Fig. 1F; fig. S7; fig. S8, B to D; fig. S9; and movie S2). Based on the fitting of the previously published crystal structure of truncated Umod (EGF IV/ZP-module) (16) into our subtomogram averages, we propose a model in which the ZP module polymerizes into the filament backbone, while the EGF I-III and D8C domains constitute the protruding arms (Fig. 1G and fig. S9, C and D). Our “alternating ZP-stacking” model differs from previously suggested architectures (16, 19) of the Umod filament (fig. S10).

Next, we investigated the interaction between Umod filaments and the adhesin, FimH, of type 1-piliated UPEC strains. FimH at the pilus tip recognizes terminal mannosides in high-mannose type N-glycans of the uroepithelial receptor uroplakin 1a (4, 20). Binding of type 1-piliated cells to Umod filaments had been demonstrated, but the exact FimH binding site in Umod remained unknown (6). We recorded the affinity of the isolated FimH lectin domain (FimH_L) to Umod filaments and obtained a single apparent dissociation constant of 2.2×10^{-8} M (Fig. 2A). Consistent with the high affinity of FimH_L for Umod, spontaneous dissociation of FimH_L from Umod filaments was very slow, with a half-life of 2.1 hours (Fig. 2B). As FimH_L binds mannosides with a 2000-fold higher affinity than full-length FimH as a consequence of the ability of FimH to form catch bonds under tensile mechanical force (21, 22), we calculated a K_d of $\sim 4 \times 10^{-5}$ M for the Umod/FimH complex in the absence of shear stress. Using analytical gel filtration and gel band densitometry, we determined the stoichiometry of the Umod/FimH_L complex to be 1:2 (Fig. 2, C and D, and fig. S11).

To test which of the two high-mannose type N-glycans of Umod was recognized by FimH, we analyzed the Umod·(FimH_L)₂ complex by cryoET. Differences between Umod·(FimH_L)₂ and native Umod were already visible in individual tomograms (fig. S12, A and B, and movie S3). The Umod·(FimH_L)₂ subtomogram average revealed a prominent additional density on the Umod arms, which may be sufficient to accommodate two FimH_L (Fig. 2, E and F; fig. S12, C to F; and movie S3). No significant additional density was seen at the core of Umod·(FimH_L)₂ filaments or on eUmod (contains only the high mannose-glycan at Asn513) incubated with FimH_L (fig. S12, G to L). Together, our data demonstrate that the Man5–9 glycan at Asn275 of the Umod arm is the only accessible FimH_L recognition site.

We next analyzed binding of Umod filaments to a mixture of type 1-piliated and non-piliated *E. coli* cells by cryoET imaging. Each type 1-piliated cell exhibited a significant increase in the local Umod filament concentration around the cells ($n = 6$ tomograms) (Fig. 3, A and B; fig. S13, A and B;

and movie S4). Contact sites between pili and Umod were mainly at the FimH-containing pilus tips (Fig. 3A). In contrast, no Umod filaments accumulated around non-piliated cells ($n = 7$ tomograms; fig. S13, C and D) or piliated cells incubated with eUmod ($n = 3$ tomograms; fig. S14). *E. coli*-Umod association therefore requires both the presence of type 1 pili and the glycosylated Umod arm.

Using light microscopy, we investigated bacteria-Umod interactions on a larger scale. Incubation of type 1-piliated *E. coli* with Umod filaments resulted in the aggregation of bacteria and the formation of clumps consisting of tens to hundreds of bacteria (Fig. 3C). Cell clumping occurred over a wide range of Umod concentrations and was inhibited by an excess of D-mannose (Fig. 3C), indicating that clumping was caused by FimH binding to Umod glycans. Pre-formed clumps, however, proved to be resistant against dissociation by D-mannose (fig. S15). TIRF microscopy showed that cell aggregates co-localized with Umod (Fig. 3D). Umod-dependent cell aggregation also occurred with a UPEC strain (fig. S16), but was not detected with non-piliated *E. coli* or with piliated *E. coli* incubated with eUmod (figs. S17 and S18). To visualize the Umod-induced cell aggregates by cryoET, we thinned the sample using cryo-focused ion beam (FIB) milling (23). The cryotomograms consistently revealed bacteria that were tightly surrounded by a dense mesh of Umod filaments ($n = 17$ tomograms; Fig. 3E, fig. S19, and movie S5).

In addition to type 1 pili, many UPEC strains express multiple pili with diverse glycan specificities. The UPEC strain CFT073 encodes type 1 pili (mannoside specific lectin), F9/Fml pili [lectin specific for galactosides], and S-pili [lectin specific for sialic acid] (24–26). We therefore tested the inhibition of Umod-mediated cell clumping of CFT073 by the addition of D-mannose, D-galactose and sialic acid. While D-mannose alone only slightly decreased cell aggregation (fig. S20, A and B), the addition of a cocktail of all three monosaccharides proved to be most effective in reducing Umod-mediated cell clumping (fig. S20B). This indicates that the complex-type Umod glycans might interact with different types of pilus adhesins presented by different uropathogens.

We verified these findings by analyzing unprocessed urine from patients with clinically diagnosed UTIs by light microscopy and cryoET. Urine from a patient with an *E. coli* UTI revealed bacterial clumps that were embedded into fibrous structures (Fig. 4A). Individual bacteria of the same sample were heavily piliated and always surrounded by numerous Umod filaments (Fig. 4B and movie S6; $n = 27$ tomograms); several contact sites between pilus tips and Umod were resolved (Fig. 4C and movie S6). Furthermore, we analyzed urine from UTI patients with other pathogens, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Streptococcus mitis*. In all cases, we observed cell aggregates associated with fibrous structures in light microscopy, and 67–

71% of cells imaged by cryoET confirmed the presence of Umod filaments (fig. S21 and movie S7).

In conclusion, our data provide a 3D structure of native Umod filaments and support the hypothesis that the polymerization of Umod into a multivalent filament is required for its function as a FimH antagonist to effectively compete with the high concentration of uroplakin Ia on the urinary epithelium (4, 14, 27). The intrinsic flexibility of Umod filaments allows their adaptation to the bacterial surface, multivalent binding to pili from the same bacterium and eventually encapsulation of entire cells. Analogous to the mechanism by which antibody-crosslinked clumps of enchain *Salmonella* cells facilitates the removal of pathogens from the gut (28), Umod-mediated cell aggregation may have a role in efficient clearance of bacteria via micturition.

Our study also sheds light on interactions between Umod and pathogens other than type 1-piliated UPECs. UPEC genomes, for instance, encode up to 16 fimbrial gene clusters and UPEC switch expression between pilus types with distinct receptor specificities (24, 29–31). The seven different complex type N-glycans on Umod (Fig. 1A) may therefore competitively inhibit adhesion of other pathogens to other epithelial receptors. Finally, the resolved site-specific N-glycosylation pattern and architecture of Umod filaments will serve as a framework for studying the mechanisms underlying Umod's roles in the regulation of salt transport, kidney diseases and innate immunity (1, 19).

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ACKNOWLEDGMENTS

We thank the Functional Genomics Center Zürich, specifically S. Chesnov and P. Hunziker, for performing the MALDI-MS analysis and Edman sequencing, H. Debaix for the genotyping, E. Olinger and A. Yoshifuji for support in urinary Umod analyses, P. Tittmann and M. Peterek for technical support during EM data collection and ScopeM for instrument access at ETH Zürich. F. Eisenstein is acknowledged for help with subtomogram average processing and J. Xu for help with analyzing cryoET data. We thank G. Navarra and B. Ernst (University of Basel) for providing the fluorescent mannoside GN-FP. C. Giese is acknowledged for providing *E. coli* AAEC189 [pSH2] cells. **Funding:** GLW was supported by a Boehringer Ingelheim Fonds PhD fellowship. MP was supported by the Swiss National Science Foundation (#31003A_179255), the European Research Council (#679209) and the NOMIS foundation. RG was supported by the Swiss National Science Foundation (310030B_176403/1 and 31003A_156304). OD was supported by the Swiss National Centre of Competence in Research Kidney Control of Homeostasis (NCCR Kidney.CH) program, the Swiss National Science Foundation (310030_189044) and the Rare Disease Initiative Zürich (Radiz). JT was supported by the Swiss National Science Foundation (PZ00P3_161147; PZ00P3_183777). MP and RG were also supported by basic funding from ETH Zürich. **Author contributions:** G.L.W., J.J.S. and M.M.S. contributed equally. G.L.W., J.J.S., M.M.S., O.D., M.P. and R.G. designed experiments. J.J.S., M.M.S. and J.E. purified and biophysically analyzed Umod. C.-W.L. and M.A. performed and analyzed MS experiments. G.L.W. collected and processed cryoET data. D.S.Z. constructed the expression plasmid and performed TIRF microscopy. J.J.S. performed and analyzed light microscopy experiments. G.L.W. and J.T. collected patient urine. G.L.W., J.J.S., M.M.S., M.P. and R.G. wrote the manuscript with comments from all authors. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** All subtomogram averages shown in this study were uploaded to the Electron Microscopy Databank (EMD) together with their respective half maps, masks for FSC calculations and an example tomogram. Accession numbers: EMD-11128/EMD-11129 (native Umod), EMD-11130/EMD11131 (eUmod), EMD-1133/EMD-11134 (Umod incubated with FimH_L) and EMD-11134/EMD-11135 (eUmod incubated with FimH_L). Example tomograms of bacterial cells with Umod were also uploaded to the Electron Microscopy Databank with accession numbers EMD-11136 to EMD-11143. All other data needed to evaluate the conclusions in the paper are present in the paper or the supplementary materials.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S21

Tables S1 to S4

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MDAR Reproducibility Checklist

Movies S1 to S7

25 October 2019; resubmitted 22 April 2020

Accepted 18 June 2020

Published online 2 July 2020

10.1126/science.aaz9866

Fig. 1. Umod site-specific glycosylation pattern and filament architecture. (A) Domain organization of mature Umod with four EGF-like domains (I-III, light blue and IV, green), the cysteine-rich D8C domain (blue) and the bipartite ZP module (ZP-N and ZP-C, green) (1). The most abundant N-linked glycan forms identified at each N-glycosylation site are shown schematically (amino acid numbering according to Umod with N-terminal signal peptide). Except for Asn275 and Asn513, all N-glycans were confirmed to be di-, tri-, or tetra-antennary complex type that could be sialylated and/or fucosylated. We observed mixed N-glycan structures at Asn513, comprised of Man6 and, as previously reported, complex type N-glycans (8). The sum of the masses of all identified glycans corresponded well to the average mass difference (20.1 kDa) between glycosylated and deglycosylated Umod. The elastase-digested form of Umod (eUmod = Ser292 through C terminus Phe587) is marked in green and scissors indicate the elastase cleavage site after Ser291. (B to D) CryoET of purified Umod filaments revealed irregular bending and helical parameters. (C) and (D) show magnified views of the two most common orientations, revealing the zigzag core (C) and the lateral arms causing a fishbone-like appearance (D). Shown are 13.8 nm-slices through cryotomograms. Bars, 100 nm in (B), 50 nm in (C) and (D). (E) Different orientations of a Umod subtomogram average (surface renderings). The subtomogram average was low-pass filtered to 27 Å to demonstrate the complete three-dimensional architecture. (F) Different orientations (surface renderings, green) of the eUmod subtomogram average. The superposition of eUmod with native Umod (transparent, light blue), low-pass filtered to the same resolution of 27 Å, demonstrates the absence of the lateral arms in eUmod. The unfiltered averages can be seen in fig. S9. (G) Proposed alternating ZP-N/ZP-C stacking model of the Umod filament architecture [same orientation as in (F, right)]. The two high-mannose type N-glycan structures in each Umod monomer are indicated by green glycan trees, while complex type N-glycans are shown as gray triangles.

Fig. 2. Two FimH_L molecules bind to the high-mannose glycan on the Umod arm. (A and B) Thermodynamics and kinetics of Umod binding and release by FimH_L at pH 7.4 and 25°C. (A) Competitive equilibrium displacement of the fluorescent mannoside GN-FP (21, 22) from FimH_L with increasing Umod concentrations, recorded via the decrease in GN-FP fluorescence anisotropy. (B) Kinetics of spontaneous dissociation of FimH_L from Umod, recorded via binding of excess GN-FP to released FimH_L. The obtained first-order kinetics (solid lines) were independent of GN-FP concentration and thus directly monitored dissociation of FimH_L from Umod ($k_{\text{off}} = 9.1 \times 10^{-5} \text{ s}^{-1}$). Direct determination of the affinity of full-length FimH for Umod filaments proved to be impossible due to the limited solubility of Umod filaments ($\sim 100 \text{ } \mu\text{M}$ monomers). (C and D) Titration of FimH_L with increasing amounts of Umod filaments to determine the stoichiometry of complex formation at pH 7.4 and different temperatures (15°C, 25°C, and 37°C). (C) SEC elution profiles of 10 μM FimH_L incubated with different amounts of Umod filaments (Umod monomer concentrations between zero and 20 μM), detected via protein absorbance at 280 nm. (D) Peak area of free FimH_L, plotted against the Umod monomer: FimH_L ratio, revealing that on average two FimH_L molecules can bind to a single Umod monomer. (E) Different orientations of the subtomogram average (shown as a surface rendering) of Umod filaments that were incubated with a four-fold excess of FimH_L over Umod monomers. Major additional densities are detectable on the filament arms, whereas no extra densities could be identified on the filament core. (F) Overlay of surface renderings of subtomogram averages of native Umod (blue) and Umod incubated with a four-fold excess of FimH_L (orange, transparent), both low-pass filtered to a resolution of 27 Å. The additional densities could accommodate two copies of FimH_L (fig. S12, D to G). Together, the data indicate that up to two FimH_L molecules bind to the high-mannose glycosylation site at Asn275 in the D8C domain of the Umod arm.

Fig. 3. Umod associates with pilated bacteria and can lead to cell aggregation. (A and B) The type 1-pilus deficient strain *E. coli* AAEC189 was transformed with the type 1 pilus expression plasmid pSH2, co-incubated with Umod filaments (1 μM Umod monomers and $\sim 1.6 \times 10^9$ *E. coli* cells/ml) and imaged by cryoET. Shown is a slice (8.6 nm thickness) through a cryotomogram (A) and a segmentation of the same tomogram (B). Pilated bacteria (cell, brown; pili, orange) were always seen in close association with a loose mesh of Umod filaments (blue/arrows). Note the contact site of the pilus tip with the Umod filament. Shown is a partly lysed cell that resulted in higher image quality. See fig. S13, A and B, for more examples. Bars, 100 nm. (C) Quantification of Umod-mediated formation of cell aggregates with light microscopy. The bar graph shows the normalized clump area observed upon incubation (2 hours) at 37°C of a constant amount of type 1-piliated *E. coli* HB101 [FimAra] ($\sim 10^8$ cells/mL) with different concentrations of Umod filaments (0.08-5.0 μM, concentrations refer to the Umod monomer). The presence of 10 mM D-mannose (Man) inhibited aggregation across all Umod concentrations tested. Representative light microscopy images are shown on the right (without and with 10 mM Man). Bars represent the mean of biological triplicates $\pm$ SEM. * $p < 0.05$ difference between groups (two-way ANOVA, post-hoc Tukey test). Bars, 10 μm. (D) Cell aggregates co-localized with Umod filaments. Shown are bright field and TIRF microscopy images of type 1-piliated *E. coli* HB101 [FimAra] cells (excitation at 405 nm, green) that were incubated with fluorescent Umod-Alexa647 filaments (red). Bars, 10 μm. (E) Umod-induced *E. coli* aggregates were plunge-frozen, thinned by cryo-focused ion beam milling, and imaged by cryoET (shown is an 8.6 nm tomographic slice). *E. coli* cells (brown) were always seen embedded in a highly dense meshwork of Umod filaments (blue arrows). For further examples see fig. S19. Bar, 100 nm.

Fig. 4. Urine from UTI patients shows cell aggregation and pilus-mediated Umod association with bacterial cells. (A) DIC light microscopy imaging of urine from a patient with acute *E. coli* UTI revealed clustered bacteria. Bar, 10 μm . (B) CryoET imaging of urine from the same patient showed piliated (orange arrowheads) bacterial cells (labeled “*E. coli*”). All analyzed cells ($n = 27$ tomograms/cells, shown are two representative examples) were surrounded by filaments with the typical Umod appearance (blue arrows). Bars, 100 nm. (C) The cryotomograms revealed multiple contact sites (black arrowheads) between pili (orange arrowheads) and Umod (blue arrows). Bars, 100 nm. Magnified views in insets. Bars, 50 nm.