How Glycosaminoglycans Promote Fibrillation of Salmon Calcitonin

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Glycosaminoglycans (GAGs) bind all known amyloid plaques and help store protein hormones in (acidic) granular vesicles, but the molecular mechanisms underlying these important effects are unclear. Here we investigate GAG interactions with the peptide hormone salmon calcitonin (sCT). GAGs induce fast sCT fibrillation at acidic pH and only bind monomeric sCT at acidic pH, inducing sCT helicity. Increasing GAG sulfation expands the pH range for binding. Heparin, the most highly sulfated GAG, binds sCT in the pH interval 3–7. Small angle x-ray scattering indicates that sCT monomers densely decorate and pack single heparin molecules in the pH interval 3–7. Heparin, the most highly sulfated GAG, binds sCT in the pH interval 3–7. Small angle x-ray scattering indicates that sCT monomers densely decorate and pack single heparin chains, possibly via hydrophobic patches on helical sCT. sCT fibrillates without GAGs, but heparin binding accelerates the process by decreasing the otherwise long fibrillation lag times at low pH and accelerates fibril growth rates at neutral pH. sCT-heparin complexes form β-sheet-rich heparin-covered fibrils. Solid-state NMR reveals that heparin does not alter the sCT fibrillar core around Lys11 but makes changes to Val8 on the exterior side of the β-strand, possibly through contacts to Lys11. Thus GAGs significantly modulate sCT fibrillation in a pH-dependent manner by interacting with both monomeric and aggregated sCT.

Since their first description in 1855 (1), amyloid deposits have been extensively studied both in vivo and in vitro. Besides the major amyloidogenic protein component (e.g. amyloid-β, α-synuclein, or tau), amyloid deposits contain components like inflammatory molecules, metal ions, amyloid P, proteoglycans (PGs),2 and other proteins (2). PGs are present in both systemic

(3), prion (4), and neuritic (5) amyloid plaques and appear directly involved in amyloid deposition (6). The precise role of PGs in amyloid deposition remains to be clarified. The core PG protein is mainly involved in protein and membrane binding (7). In contrast, PG activity in amyloid formation is associated with the anionic polysaccharide chains or glycosaminoglycans (GAGs) decorating the protein core (3, 5). GAGs are structurally diverse polysaccharides consisting of repeating disaccharide units with an amine, a carboxylic group, and widely varying sulfation levels. The GAG heparin has three sulfates in every disaccharide group, whereas chondroitin sulfate A (ChA), dermatan sulfate (DS), and heparan sulfate have lower overall degrees of sulfation (Fig. 1). Heparin is the GAG with the most consistent repetitive disaccharide composition, whereas other GAGs follow a less strictly repetitive sulfation pattern (8). PG functions range from cell signaling over proliferation (9) to structural roles. Aggrecan is the major constituent of connective tissue (9), and perlecans is a heparan sulfate PG that adds charges to the basement membrane in an asymmetric fashion (7). Heparin has been used as a model GAG in the study of the fibrillation of major disease-related amyloidogenic proteins such as amyloid-β (5, 10), α-synuclein (11), tau (12), human muscle acylphosphatase (13), and β2-microglobulin (14). Heparin commonly promotes aggregation by decreasing fibrillation times and affects fibril morphology. The mechanism for fibril enhancement and the resulting fibril morphology seems to be protein-specific (6). Besides promoting fibrillation, GAGs hinder cytotoxicity induced by prefibrillar oligomers of other proteins such as islet amyloid polypeptide (15) and amyloid-β (16). Furthermore, non-fibril-inducing GAG mimetics are a growing field of interest for therapeutic applications (17). Thus it remains unresolved whether GAGs primarily hinder or promote unwanted aggregation in vivo (17–19). Recently GAGs have been suggested to be involved in the storage of protein hormones in granular vesicles by promoting aggregation of protein hormones (20, 21). During granular vesicle formation, the pH decreases from 7.4 in the endoplasmic reticulum to 5.5 in mature granular vesicles (22), making it important to understand how pH affects protein-GAG interactions. Considering the central role played by GAGs in fibrillation, there is remarkably little information available on the molecular basis for these effects and whether and how GAGs incorporate into the highly repetitive cross-β amyloid structure. One of the few reports to
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Address this is a study by Middleton and co-workers (23, 24) on heparin-amyloid β interactions where heparin binds on the outer surface of amyloid-β fibrils, interacting closely with the loop region and the flexible N terminus.

To address the role of heparin and other GAGs in the molecular steps leading to protein fibrillation, here we present a study of how GAGs affect fibrillation of the 32-residue peptide hormone salmon calcitonin (sCT) used to treat osteoporosis in postmenopausal women (25). sCT is more potent in this regard than human calcitonin (hCT) (26), partly attributed to its reduced fibrillation propensity (27). Nevertheless, spherical prefibrillar oligomers formed by sCT at pH 7.4 are neurotoxic (28) like otherspherical prefibrillar oligomer-forming proteins (29). sCT spherical prefibrillar oligomers interact with the cell membrane in “raftlike” domains rich in ganglioside G_{M1} and cholesterol, leading to increased Ca^{2+} influx and cell death (30). Early fibrillation events of sCT (31) and hCT (32) both involve formation of a helical oligomeric complex. At neutral pH, hCT fibrillation is in part driven by hydrophobic interactions among Tyr^{12}, Phe^{16}, and Phe^{19} and a salt bridge between Asp^{15} and Lys^{18} (32). At low pH, hCT fibrillation is slowed by Asp^{15} protonation (32). The decreased fibrillation propensity of sCT is attributed to the increased number of polar amino acids compared with hCT (33). Our results show that this barrier to fibrillation can be overcome by introducing GAG as an electrostatic binding partner for excess positive charges. CD and small angle x-ray scattering (SAXS) were used to investigate the structural changes going from monomeric sCT to sCT-GAG binding complex and we used solid-state NMR spectroscopy to analyze structural details in the sCT fibrils with or without heparin.

Results

sCT and Heparin Form a Binding Complex with α-Helical Structure at Low pH—The basis for our studies is the fact that the interactions of calcitonin with GAGs in vivo take place over a pH range of several units (∼7.4–5.5). Over this pH interval, the charge of sCT will change by up to 1.3 units (see below), and this will affect interactions with the highly anionic GAG molecules. To investigate the role of charge in these interactions, we used the following three variants of sCT: 1) the natural form, which is C-terminally amidated (sCT-NH₂) and goes from a net charge of approximately +3.9 at pH 5.5 to approximately +2.6 at pH 7.4 based on conventional pK_a value calculations; 2) a form with a free C-terminal carboxylate group (sCT-OH), which will be anionic above approximately pH 4, going from a net charge of +2.9 at pH 5.5 to +1.6 at pH 7.4; and 3) the mutant His^{17} → Ala (sCTH17A-OH; with a free C-terminal carboxylate group), which removes the only side chain that titrates over the pH interval 7.4–5.5 so that the net charge of this mutant only decreases by 0.6 unit from +2.1 at pH 5.5 to +1.5 at pH 7.4.

Initially we investigated how heparin affects the structure of monomeric sCT-OH at pH 5.5 and 7.4. Direct evidence for formation of a complex between heparin and sCT-OH at pH 5.5, but not pH 7.4, was obtained by asymmetric flow field flow fractionation (A4F) where large species elute after small species. We used fluorescein-labeled heparin (fl-hep) to detect the heparin component (fluorescence emission at 516 nm) and absorbance at 214 to monitor sCT-OH. At pH 5.5, a complex containing protein and fl-heparin elutes at 20 min, whereas sCT-OH without fl-heparin elutes around 8 min (Fig. 2A). At pH 7.4, there is no 20-min peak, and sCT-OH and heparin elute as separate peaks at 8 and 10 min, respectively (Fig. 2A).

The appearance of the binding complex at pH 5.5 was accompanied by a significant structural change according to far-UV CD. All three sCT peptides by themselves assume a random coil conformation with a far-UV CD minimum around 200 nm at both pH values (shown for pH 5.5 in Fig. 2B). This changes to two minima at 208 and 220 nm, implying a more α-helical structure, when heparin is added at pH 5.5 but not at pH 7.4. The helical structure was confirmed by the BeStSel deconvolution program (Table 1) (34). Because initially no pH difference was observed among the three sCT versions, we next carried out a more extensive pH titration of the interactions with heparin. Binding (measured in terms of the change in CD signal at a 200/220 nm signal ratio) was determined over the pH range 8–3 (Fig. 3). Binding showed a sigmoidal curve where the
visually estimated midpoint of titration changed from $\sim 6.9$ (sCT-NH$_2$) to $\sim 6.5$ (sCT-OH) and $\sim 5.9$ (sCTH$_{17}$A-OH). Thus, progressive addition of negative charge (from sCT-NH$_2$ to sCT-OH) and removal of positive charge (from sCT-OH to sCTH$_{17}$A-OH) shifted the midpoint down. This indicates that to bind heparin a higher level of protonation had to compensate for these electrostatic changes.

**GAG Sulfation Expands the pH Range of sCT Binding**—Although the two titratable groups (C terminus and His$^{17}$) have some impact on heparin binding as demonstrated above, all three sCT versions bound heparin at pH 5.5 but not pH 7.4. Therefore titration of His$^{17}$ and the C terminus on their own could not explain why heparin binds sCT at pH 5.5 but not pH $>7$. To investigate whether other GAGs showed similar pH dependence, we compared sCT-NH$_2$ binding with heparin and removal of positive charge (from sCT-OH to sCTH$_{17}$A-OH) shifted the midpoint down. This indicates that the pH dependence of binding of the three sCT peptides to ChA is similar to that of heparin, only shifted $\sim 2.5$ pH units further down. Thus increased sulfation increases the pH range for GAG-sCT binding.

**SAXS Reveals Heparin to Adopt a Less Extended Conformation When in Complex with sCT**—Having established that heparin and sCT form a defined binding complex at pH 5.5, we used SAXS to obtain information about the shape of the complex. SAXS data for the complex (Fig. 4A) and free heparin (Fig. 4B) were transformed to pair distance functions (Fig. 4C), which revealed the complex to be more compact than heparin alone. We could model the sCT-OH-heparin complex using an ensemble-based method as a saccharide chain decorated with sCT-OH monomers in a coiled up structure with a radius of gyration ($R_g$) around 35 Å and a maximum diameter ($D_{max}$) of

FIGURE 2. Heparin binds sCT only at low pH. A, A4F analysis of sCT-OH-heparin complex at pH 5.5 (red) and pH 7.4 (blue). sCT was detected using absorption at 214 nm for the sCT monomer (black) and sCT-OH-heparin (filled symbols). Heparin was detected using fluorescence (excitation at 485 nm and emission at 516 nm) (open symbols). B, CD spectra of 150 μg/ml heparin with 0.5 mg/ml sCT-NH$_2$, sCT-OH, and sCTH$_{17}$A-OH at pH 5.5 (filled symbols) or pH 7.4 (empty symbols). Neither heparin nor other GAGs investigated gave any significant signal at the relevant concentrations and buffer conditions used. Inset, CD spectra of sCT-NH$_2$, sCT-OH, and sCTH$_{17}$A-OH at pH 5.5 all show random coil structures. Same axis units as main graph. a.u., arbitrary units; deg, degrees; abs, absorbance.

|          | sCT-NH$_2$ | sCT-NH$_2$:Heparin |
|----------|------------|--------------------|
| RMSD$^a$ | 0.19       | 0.19               |
| Helix    | 33.5       | 33.5               |
| Antiparallel | 5         | 5                  |
| Others   | 36.9       | 36.9               |

|          | sCT-OH     | sCT-OH:Heparin     |
|----------|------------|--------------------|
| RMSD$^a$ | 0.13       | 0.13               |
| Helix    | 22.1       | 22.1               |
| Antiparallel | 27        | 27                 |
| Others   | 34         | 34                 |

|          | sCT-H$_{17}$A | sCT-H$_{17}$A:Heparin |
|----------|--------------|-----------------------|
| RMSD$^a$ | 0.17         | 0.17                  |
| Helix    | 31.9         | 31.9                  |
| Antiparallel | 5.8     | 5.8                  |
| Others   | 45.5         | 45.5                  |

$^a$ Reported root mean square deviation (RMSD) for the fit of the entire model.

**TABLE 1** Structural content as percentage for different sCT variants calculated with BeStSel (34) from CD spectra of sCT versions with or without heparin at pH 5.5.
119 Å (Fig. 4A). Free heparin was modeled as a more flexible chain with $R_g$ of 53 Å and $D_{\text{max}}$ of 163 Å (Fig. 4B). Thus CD, A4F, and SAXS all confirm formation of a relatively stable complex between sCT and heparin at pH 5.5 but not at pH 7.4.

**FIGURE 3. Charge and pH affect binding of GAGs to sCT.** sCT binding to GAGs is shown as a function of pH monitored by CD. sCT alone had 200:220 ratios between 2.2 and 3.2 in the entire pH range (solid lines). Shown are sCT-NH$_2$ in red, sCT-OH in blue, and sCT17A-OH in green with heparin (filled symbols), DS (half-filled symbols), and ChA (open symbols). The samples contained 0.2 mg/ml sCT, 0.2 mg/ml GAG, and 20 mM citrate-phosphate buffer at the indicated pH values. The colored numbers in the graph are visual estimates of the pH midpoint of titration for the seven individual titration curves, which transition from random coil (high pH) to highly helical (low pH). The baseline values for the random coil are estimated from the GAG-free sCT variants at high pH, whereas the helix value is estimated to be around ~0.2 based on the convergence of CD values at low pH. These values are merely used for comparative purposes (see main text).

**FIGURE 4. SAXS analysis of sCT-heparin complex and free heparin.** A, SAXS spectra of purified sCT-OH-heparin complex in dots and model with $R_g$ of 35 Å and $D_{\text{max}}$ of 119 Å in line. Inset, stick representation of modeled sCT-heparin complex. B, SAXS data of heparin in dots and model with radius of gyration of 53 Å and $D_{\text{max}}$ of 163 Å in line. Inset, stick representation of heparin generated from the SAXS data. C, comparison of $p(r)$ functions of heparin alone (broken line) and sCT-heparin complex (full line). Error bars represent S.E.
pH 7.4 heparin only increases the elongation rate and does not affect the fibrillation lag time.

**Removal of Histidine in sCT Diminishes the Increasing Half-times Below pH 5**—To follow up on the observed strong pH dependence of sCT-hep binding, we studied heparin-induced fibrillation over the pH range 3–8 for sCT-OH, sCT-NH₂, and sCT-H17A (Fig. 7A). Assuming a standard His pKₐ value of ~6, we expect His¹⁷ in sCT-OH and sCT-NH₂ to be positively charged at pH <5.5, and this coincides with an increase in the half-time of fibrillation. In contrast, sCTH17A-OH, which lacks this positive charge, undergoes rapid fibrillation over the entire low pH range.

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**FIGURE 5.** Heparin is not released from sCT-OH upon incubation. sCT-OH was incubated with fl-hep at pH 5.5 for 22–45 min (dark gray to light) or mixed immediately before injection (black). An sCT-hep binding complex (peak at 20 min) forms immediately and disappears as fibrillation proceeds. The larger complex at 40 min was not consistently detected.

**FIGURE 6.** Heparin induces ThT-positive sCT fibrils and increases the elongation rate at pH 7.4. A, sCT fibrillation followed by ThT fluorescence. Shown are sCT-OH (blue), sCT-NH₂ (red), and sCTH17A-OH (green) without heparin at pH 5.5 (open symbols) and with 1.5:1 hepdisacc:sCT (closed symbols). B, fibrillation of sCT-NH₂ and sCT-NH₂-heparin (1:1.6 molar ratio) at pH 7.4 followed by CD at 206 nm. The two conditions with/without heparin are each shown as duplicates to show the lag time variability. a.u., arbitrary units; mdeg, millidegrees.
ing more full-length sCT than heparin-free fibrils (Fig. 8, top panel, lanes 1–3) but less than the fibrils formed in the presence of heparin (Fig. 8C, bottom panel, lanes 1–3). When treating soluble sCT with trypsin, no peptide was retained in the gel (data not shown), most likely reflecting the small size of tryptic products sCT(1–11), sCT(12–18), sCT(19–24), and sCT(25–32). For sCT-OH, trypsin digestion of fibrils gave results identical to those presented here for sCT-NH2 (data not shown). We conclude that heparin is incorporated into mature sCT fibrils, resulting in partial protection of Arg24 against trypsin cleavage.

Solid-state NMR Suggests That sCT Makes Up the Fibrillating Core whereas Heparin Interacts with Residues on the Outer Side of a β-Strand—We obtained atomic level detail about the interactions between heparin and sCT in the mature fibrils using solid-state NMR. An sCT-NH2 peptide that contained three 13C- and 15N-labeled amino acids (Arg24, Lys11, and Val8) was synthesized. Fibrils of the labeled peptide were made either in the absence of heparin or at a 5:1 heparin disaccharide:sCT molar ratio. Two-dimensional 13C-13C and 15N-13C correlation spectra (Fig. 9A) allowed us to unambiguously assign chemical shifts for all 13C and 15N in Val8 and Lys11 for both samples, whereas only the backbone nuclei for Arg24 were visible in the sCT-NH2-heparin fibril sample, and no signals from Arg24 were found in the sCT-NH2 fibrils formed without heparin. The NMR spectra showed relatively narrow lines, indicating well ordered fibrils, and each labeled site gave rise to only a single signal, which indicates a symmetric packing of the fibrils (35).

The assigned chemical shift values are compared with random coil values in Table 2. A secondary chemical shift analysis,
which exploits the relationship between chemical shift values and secondary structure (36), shows that Val8 and Lys11 are in a β-sheet (for both samples), whereas the analysis remains inconclusive for Arg24 in sCT-NH₂-hep. There are no signals for Arg24 in heparin-free sCT-NH₂ fibrils.

Differences between the fibrils formed with and without heparin may be identified from differences in chemical shift values between the two samples (Table 2). Val8 is influenced by heparin because the chemical shifts differ by more than 1 ppm, whereas Lys11 is unaffected with only minor chemical shift differences. The missing Arg24 signals may be explained by an inhomogeneous, unstructured, or flexible C terminus. To resolve this, we acquired one-dimensional cross-polarization (CP) spectra, which show only rigid parts of the samples, and INEPT spectra, which only give signals from flexible parts. The two-dimensional spectra mentioned above are initiated with CP elements and thus only reveal the rigid parts of the molecules. With strong signals from Val8 and Lys11 in the two-dimensional spectra, the central part of the peptide must be rigid and part of the fibril core. The INEPT spectrum of sCT-NH₂-heparin contains no signals (Fig. 9B), and the signals from the side chain of Arg24 in the fibrils containing heparin are broadened beyond detection. This can be explained by an unspecific interaction between Arg24 and heparin or by an inhomogeneous C terminus. Conversely, for sCT-NH₂, INEPT signals from both Val8 and Arg24 are seen (Fig. 9C), whereas Lys11 gives no INEPT signals. These findings indicate that Lys11 is buried inside the fibril, whereas Arg24 is situated in a flexible C terminus (Fig. 10C). Lys18 and Arg24 are hatched to highlight the expected heparin binding site (Fig. 10C). The basic residues Lys18 and Arg24 could coordinate acid and sulfate groups of heparin, whereas chemical shift differences for Val8 in sCT-NH₂ compared with sCT-NH₂-heparin fibrils indicate that heparin affects this residue.

Serendipitously we also synthesized a sCT-OH Gly10 → Lys/Lys11 → Gly mutant. This mutant underwent a structural change upon heparin addition, similar to that seen for the other sCT peptides (data not shown). This indicates that the exact position of Lys11 is not crucial for heparin binding. However, the sCTG10K/K11G-OH double mutant does not fibrillate within 60 h (sCT-OH fibrillation is observed within 2 h). In practice, this double mutant moves Lys11 from one side of a β-strand to the opposite site. These findings nicely agree with the high level of Lys protection against trypsin digestion and NMR studies, indicating Lys11 to be similar for both sCT-NH₂ and sCT-NH₂-heparin fibrils. We suggest that Lys11 is not crucial for heparin binding but is most likely central to sCT-NH₂ fibrillation and part of the fibrillating core. In a β-strand structure, Lys11 resides on the same side of the strand as Glu15, allowing sCT-NH₂ to form salt bridges between Lys11 and Glu15 on opposing sCT-NH₂ monomers in the core of a fibril strand (Fig. 10C). Val8, whose chemical shifts differ in sCT-NH₂ and sCT-NH₂-heparin, is on the opposite side of a sCT-NH₂ β-strand compared with Lys11. However, Val8 is on the same side as Lys18, which could be a possible electrostatic binding partner for heparin in sCT-NH₂ fibrils (Fig. 10C).
Our data clearly show that sCT fibrillation is affected by GAGs in a complex manner with pH having a large impact on sCT-GAG interactions. The immediate formation of a binding complex at low pH indicates that under these conditions GAGs must affect the formation of spherical prefibrillar oligomers and hence might affect their toxicity (30). Also our data show that chondroitin sulfate A and less sulfated GAGs are not merely less active versions of heparin but have altered pH binding profiles. Understanding these effects is a prerequisite for understanding the delicate balance between the beneficial storage of hormonal peptides in granular vesicles versus disease-related amyloid formation. Our data from a broad range of techniques show that sCT under mildly acidic conditions (pH 5.5), but not under neutral conditions (pH 7.4), forms a semi-stable binding complex with heparin. There is no evidence to suggest that this binding complex dissociates before it starts to form amyloid-like fibrils when agitated at 37 °C. These fibrils show strong ThT fluorescence, twisted rod-like structures with thickness of 5–8 nm, and a strong CD β-sheet signal. We have multiple lines of evidence for heparin incorporation into sCT fibrils. Our data from a broad range of techniques show that sCT under mildly acidic conditions (pH 5.5), but not under neutral conditions (pH 7.4), forms a semi-stable binding complex with heparin. There is no evidence to suggest that this binding complex dissociates before it starts to form amyloid-like fibrils when agitated at 37 °C. These fibrils show strong ThT fluorescence, twisted rod-like structures with thickness of 5–8 nm, and a strong CD β-sheet signal. We have multiple lines of evidence for heparin incorporation into sCT fibrils. Nevertheless, ssNMR data indicate that sCT-NH₂ and sCT-NH₂-heparin fibrils have similar fibrillating cores, providing evidence that Lys¹¹ is in the fibrillating core and experiences similar environments in the two fibril types. NMR data further suggest that Val⁸ points outward from the strand and that Arg²⁴ is present in the flexible C terminus supported by trypsin/MS data showing Arg²⁴ to be accessible for trypsin cleavage. Both Val⁸ and Arg²⁴ are affected by the presence of heparin in sCT-NH₂ fibrils.

### Discussion

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### Charges Determine the pH Range for sCT GAG Binding

We reasoned that His¹⁷ might serve as a pH sensor for sCT binding to GAGs through its positive charge. This is indeed the case because sCTH¹⁷A-OH CD titration curves (Fig. 3) are shifted down to lower pH compared with sCT-OH. This means that this half-binding is achieved around pH 5.9 for sCTH¹⁷A-OH in contrast to sCT-OH (pH 6.5) and sCT-NH₂ (pH 6.9). Thus to compensate for the loss of a positive charge in sCTH¹⁷A-OH, it is necessary to decrease the pH to achieve heparin binding. For amyloid β, a more dramatic effect is seen in that a His → Ser mutant completely loses its heparin binding abilities both at pH 8 and pH 4 (37). Similarly, the reduction in negative charge in the GAG CHA compared with heparin means that electrostatic interactions with sCT are weak, and therefore the positive charge of sCT has to be increased by lowering the pH to achieve efficient binding.

### Combining CD and SAXS Data Provides Structural Information about the Binding Complex

The α-helical structure suggested by CD combined with results from SAXS inspired us to suggest that sCT monomers bind through electrostatic interactions between the basic amino acids present on one side of helical sCT and the negative heparin surface (Fig. 10A), leaving an exposed hydrophobic patch on sCT as a possible interaction site for other sCT monomers in the same conformation. sCT-sCT hydrophobic interactions, which are known to occur in GAG-free sCT dimers (31), may also occur along the heparin surface and would lead to a denser and less elongated structure of the binding complex as suggested here by SAXS. Upon incubation, this complex converts into heparin-containing fibrils.

### Table 2

|         | sCT-Hep⁸ | sCT⁸ | R.c. | (sCT-Hep⁸)-r.c. | Class   | (sCT)⁸-r.c. | Class   | (sCT-Hep⁸)-sCT⁸ |
|---------|----------|------|------|----------------|---------|------------|---------|----------------|
| Val⁸, 15N | 123.4    | 121.8| 123.1| -0.3           | coil     | -1.3       | α-helix | 1.6            |
| Val⁸, 13Ca | 59.1     | 57.4 | 60.2 | -1.1           | β-sheet  | -2.8       | β-sheet | 1.7            |
| Val⁸, 13CO | 171.2    | 170.9| 173.7| -2.5           | β-sheet  | -2.8       | β-sheet | 0.3            |
| Lys¹¹, 15N | 124.4    | 123.7| 121.0| 4.4            | β-sheet  | 3.7        | β-sheet | 0.7            |
| Lys¹¹, 13Ca | 52.7     | 52.5 | 54.2 | -1.5           | β-sheet  | -1.7       | β-sheet | 0.2            |
| Lys¹¹, 13CO | 170.4    | 170.5| 174.6| -4.2           | β-sheet  | -4.1       | β-sheet | 0.1            |
| Lys¹¹, 13Cg | 34.3     | 34.3 | 30.9 | 3.4            | β-sheet  | 3.4        | β-sheet | 0            |
| Lys¹¹, 13Cd | 23.4     | 23.2 | 23   | -3.4           | α-helix  | -          | N.A.⁸  | c              |
| Arg²⁴, 15N | 118.5    | 121.9| -3.4 | α-helix        | -         | N.A.⁸      | c        |
| Arg²⁴, 13Ca | 52.2     | 54   | -1.8 | β-sheet        | -         | N.A.⁸      | c        |
| Arg²⁴, 13CO | 173.8    | 174.4| -0.6 | coil           | -         | N.A.⁸      | c        |

Fibrils formed at pH 5.5 and 37 °C with orbital shaking.
Peaks could not be assigned.
Not applicable.
At pH 7.4, heparin interacts with sCT-NH₂ after self-nucleation, again leading to heparin-containing fibrils.

**sCT-NH₂ Nucleation Is Heparin-independent at pH 7.4**—Our data also provide mechanistic insight into how GAGs affect sCT fibrillation. At pH 7.4, monomeric sCT-NH₂ does not bind heparin and fibrillates rapidly in the absence of heparin. Remarkably, heparin does not affect the fibrillation lag time at pH 7.4 but increases the elongation rate. The simplest interpretation of this is that at neutral pH heparin does not interfere with the ability of sCT-NH₂ to form fibrillation nuclei but rather promotes the subsequent fibril extension process, very likely by binding to the growing fibrils and stabilizing them through interactions with cationic side chains as indicated by our ssNMR data (Fig. 9). This is consistent with our observation that heparin does not interact with monomeric sCT and that fibrillation times do not correlate with GAG concentration at pH 7.4 (data not shown) but that the ThT intensity nonetheless follows GAG concentration in a saturable manner (data not shown). The same considerations likely apply to sCT-OH and sCTH17A-OH. The shorter fibrillation times of sCT-NH₂ compared with sCT-OH indicate that sCT self-nucleation at pH 7.4 is more efficient when the C terminus is amidated, most likely due to the removal of electrostatic repulsions between the C termini. These results agree with our studies of a fragment of human islet amyloid polypeptide where C-terminal amidation also affected fibrillation kinetics (38). As a further corroboration, an inspection of the calculated net charge of the three sCT species as a function of pH (assuming random coil pKa values for the titratable side chains) reveals that the fibrillation times for sCTH17A-OH and sCT-OH start to increase when the overall charge drops below +2 (pH 6 and 6.8, respectively). In contrast, the sCT-NH₂ charge remains above +2 in the pH range investigated (up to pH 8) and does not diminish its fibrillation kinetics over this range.

**sCT Fibrillation Is Slowed by His Protonation under Acidic Conditions**—To understand the lag time increase at low pH seen for sCT-OH and sCT-NH₂, we turn to the structure of the fibrils of sCT-NH₂ deduced from ssNMR and complementary experiments. sCT-NH₂ and sCT-NH₂-heparin fibrils give rise to similar chemical shifts for Lys¹¹, and CP and INEPT experiments reveal the residue to be highly immobilized in both fibril types, suggesting that the side chain is positioned on the inside of the β-strand. Our trypsin digest data reveal that Lys¹⁸ is highly protected from cleavage; hence we suggest it to be

![FIGURE 10. Models of sCT-heparin structures and fibrillation paths. A, helical wheel projection of sCT(8–24) (VLGLSQELHKLQTYPR) using the European Bioinformatics Institute online tool. Hydrophobic residues are shown as blue squares, basic residues are represented as black circles, and polar and anionic side chains are shown as red diamonds. B, proposed model for GAG-inducing fibrillation of sCT at pH 5.5 and 7.4. At pH 5.5, monomeric sCT binds heparin, reducing fibrillation times and leading to heparin-containing fibrils. At pH 7.4, monomeric sCT does not bind heparin, but after sCT oligomerization, heparin interacts with either prefibrillar aggregates or the growing fibrils, forming heparin-containing fibrils. C, schematic representation of sCT in a β-strand conformation with isotope-labeled (black) (NMR) and basic/acidic residues (gray) at the indicated positions. The schematic represents a view down the fibril axis with hatched sides of possible heparin binding sites along the fibril. A disaccharide unit is drawn to scale with the β-strands to visualize size relations.](image)
located in, or close to, the β-strand, leaving His17 most likely placed in the interior of the β-structure just like Lys11. Lys11 has a possible salt bridge partner in Glu15, leaving His17 to pair up with Leu8, a hydrophobic residue not likely to favor a positively charged His. These structural speculations rationalize why (heparin-induced) sCT fibrillation times increase at low pH for sCT-OH and sCT-NH2 but not for sCTH17A-OH. At pH 5.5, 76% of His17 will be protonated (assuming a $K_p$ of 6.0). Fibrillation times start to increase from pH 5.3 where 86% of His17 is protonated.

Residues 11–18 Form the Central Core of sCT Fibrils—Results from ssNMR spectra, ThT binding assays, fibrillation studies, and proteolytic data indicate that heparin-induced fibrils consist of an sCT-NH2 fibrillating core with heparin decorating the surface of the otherwise positively charged protein surface. Because Lys11 and Lys18 are not cleaved by trypsin when sCT-NH2 is in the fibrillar state and the sCT(1–24) peptide remains in a fibrillar/insoluble form after digestion, we suggest that the central part of sCT-NH2 constitutes the β-strand, whereas the C-terminal part containing Arg24 is accessible for proteolytic attack. This agrees well with the NMR data where Arg24 was found to be in a flexible state in sCT-NH2 fibrils but was not detectable in sCT-NH2-heparin fibrils, possibly because unordered heparin interactions lead to line broadening beyond detection. In both fibril types, Lys11 is rigid, whereas Val8 is somewhat flexible in sCT-NH2 fibrils and rigid in sCT-NH2-heparin fibrils. We suggest that Val8 is positioned on the outside of the fibril core, just like Lys18, so that it is affected by heparin binding to the fibril exterior. Similarly, for heparin-free hCT fibrils, the C-terminal part was found to be flexible, whereas the central part was found in a β-sheet (32). Thus for both sCT-NH2 and hCT, the central part of the peptide constitutes the fibrillation core. We suggest Lys11 to be part of the fibril interior, possibly stabilized by Glu15, whereas Lys18 may act as the heparin binding partner on the exterior side of a β-strand. This is in contrast to what is suggested for hCT where Lys18 is thought to interact with Asp15 (32). If such an interaction is also in the fibril interior it would lead to a face-to-back (39) arrangement assuming identical strands. We suggest sCT-NH2 to have Lys11 placed in the fibril interior in a face-to-face arrangement (pairing β-stands are found in the same conformation and arranged with the same residues in the interior of the structure) stabilized by a salt bridge to Glu15.

Biological Implications—sCT is known to be less fibrillation-prone than hCT because of the increased polarity of sCT (33). Lowering the pH increases the polarity of the peptide due to His protonation, and hence both hCT and sCT are expected to be less fibrillation-prone at low pH. We observed GAGs to have the most dramatic effect on fibrillation propensity at low pH compared with the more subtle kinetic effect observed at pH 7.4. In CT-producing cells, the protein is packed into granular vesicles under increasingly acidic conditions, reaching pH 5.5 in the mature granular vesicles. The reduced pH could lead to increased binding (and subsequent aggregation) of sCT to highly sulfated GAGs such as heparan sulfate, which is also known to be present in mature granular vesicles (40). Our study is in good agreement with earlier reports on heparin-promoting fibrillation in numerous other proteins including amyloid-β (10), α-synuclein (11), tau (12), and β2-microglobulin (14) and provides new insight into how GAG-protein interactions can be manipulated simply by altering the pH.

Experimental Procedures

Materials—sCT-NH2 was from Polypeptide (Limhamn, Sweden). Heparin sodium salt and chondroitin sulfate A and were from Sigma-Aldrich. Heparin sulfate and dermatan sulfate were from Iduron (Cheshire, UK). NHS-fluorescein was from Thermo Scientific (Slangerup, Denmark). $^{13}$C,$^{15}$N-Labeled Val, Arg, and Lys were from Sigma-Aldrich.

Peptide Synthesis—All peptides were synthesized utilizing the SPPS Fmoc/tert-butyl methodology (41) on an ABI433A peptide synthesizer (Applied Biosystems). Global deprotection/cleavage was conducted by gently agitating the dried down peptidyl resin in 95% TFA,2,5% trisopropylsilane, 2.5% H2O for 2 h. The crude linear peptide was collected by precipitation with ice-cold tert-butylmethyl ether, dissolved in 20% aqueous acetonitrile, and lyophilized. Formation of the disulfide bridge was effected using trans-$[\text{Pt(en)}_2\text{Cl}_2]^{2+}$ as described by Shi and Rabenstein (42). Briefly, the crude linear crude peptide was dissolved in pH 7 phosphate buffer (0.1 mM) at a concentration of approximately 0.1 mM. The platinate complex (~1.2 eq) was added, and the cyclization was followed by HPLC. Upon completion, the crude cyclized peptide was subjected to purification by means of semipreparative HPLC. Analytical HPLC and semipreparative HPLC were performed on an Agilent 1200 or 1260 system (Agilent Technologies) using eluent A (H$_2$O, 0.1% TFA) and eluent B (acetonitrile, 0.1% TFA). Semipreparative purification was performed on a Phenomenex Luna C$_18$(2) (10 × 250 mm, 5-µm particle size, 100-Å pore size) column (Phenomenex, Vaerloese, Denmark) using a gradient of 10–60% B over 40 min at a flow rate of 5 ml/min (detection at 280 nm).

trans-$[\text{Pt(en)}_2\text{Cl}_2]^{2+}$—To a solution of 0.5 g of dichlorobis(en)Pt(II) in 10 ml, concentrated HCl was slowly added under stirring followed by 10 ml of H$_2$O$_2$ (~30%). The suspension was stirred for 1 h and then filtered. The filtrate was concentrated and dried in vacuo.

sCT-NH$_2$ with $^{13}$C and $^{15}$N Labeling at Val8, Lys11, and Arg24—The peptide was synthesized on a 50-µmol scale using Tentagel XV RAM resin (0.21 mmol/g). Fmoc-amino acids (5 eq) were coupled for 30 min using PyOxim (5 eq) and $N,N$-disopropylethylamine (10 eq) in DMF (3 ml) to obtain the labeled Fmoc-amino acids (3 eq), which were coupled for 60 min. Fmoc removal was performed with 20% piperidine, DMF (2 × 3 min). Semipreparative HPLC yielded the peptide in >95% purity (15 mg). High resolution MS: C$_{128}$H$_{244}$N$_{37}$O$_{48}$S$_2$+ [M + H$^+$]; calculated, 3454.76, found, 3454.77. RP-HPLC: $t_r$ = 7.24 min (Phenomenex Aeries WP C$_{18}$(4.6 × 150 mm, 3.6 µm, 100 Å); 10–60% B over 10 min at 2 ml/min.

cTH17A-OH—The peptide was synthesized on a 100-µmol scale using Tentagel H-Pro-Trt resin (0.21 mmol/g). Fmoc-amino acid couplings were performed using 1H-benzotriazol-1-yl-[bis(dimethylamino)methyl]ene]-5-chlorohexafluorophosphate (1:1),-3-oxide (5 eq), $N,N$-disopropylethylamine (10 eq) in DMF (3 ml) for 10 min. Fmoc removal was conducted
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with 20% piperidine, DMF (2 × 3 min). Semipreparative HPLC yielded the peptide in >95% purity (25 mg). High resolution MS: C_{145}H_{240}N_{43}O_{49}S_{2} [M + H^+]; calculated, 3365.68; found, 3365.71. RP-HPLC: t_R = 7.96 min (Phenomenex Aeris C_{18} (4.6 × 150 mm, 3.6 μm, 100 Å); 5–90% B over 15 min at 2 ml/min).

sCT-OH—The peptide was synthesized on a 200-μmol scale using a H-Pro-2CT PS resin (0.7 mmol/g). Couplings were performed using diisopropylcarbodiimide (10 eq) and 6-chloro-1-hydroxybenzotriazole (5 eq) in DMF (3 ml) for 20 min. Fmoc removal was conducted with 20% piperidine, DMF (2 × 3 min). Semipreparative HPLC yielded the peptide in >95% purity (54 mg). MALDI-TOF MS: C_{145}H_{240}N_{43}O_{49}S_{2} [M + H^+]; calculated, 3431.7; found, 3431.6. RP-HPLC: t_R = 13.7 min (Phenomenex Aeris C_{18} column (4.6 × 150 mm, 3.6 μm, 100 Å); 40–80% B over 15 min at 1 ml/min).

Prior to use, all sCT versions were dissolved in Milli-Q water. Protein concentration was determined via absorption at 280 nm using a molecular mass of 3434 Da and an extinction coefficient of 1615 M^{-1} cm^{-1}.

Fibrillation Assays—Peptide fibrillations were performed in a 96-well clear bottom plate (catalog number 265301, Thermo Fischer Nunc) loaded with one glass bead of 3–4 mm diameter in each well (Glaswarenfabrik Karl Hecht, Sondheim, Germany). The total volume in each well was 150 μl. The buffers used were 20 mm acetate, pH 5.5, with 15 mm NaCl or 20 mm sodium phosphate, pH 7.4, with 15 mm NaCl. For the fibrillation assay spanning pH 2.8–8, the buffer system was 20 mm citrate-phosphate mixed to obtain the desired pH. All samples were mixed in Eppendorf tubes, checked for pH (room temperature), and stored on ice. ThT was added to a final concentration of 40 μM from a 1.2 mM stock in ethanol (final ethanol concentration, 3.3 volume%). Protein concentration was 0.5 mg/ml, and GAG concentration was 150 μg/ml unless otherwise indicated. Fibrillation was performed in a Genius Pro plate reader (Tecan, Männedorf, Switzerland) with the following settings: 37 °C; excitation, 448 nm; emission, 485 nm; gain, 40; and 40-μs integration time. For each measurement, planar orbital shaking for 3 min at 2.5-mm amplitude was applied every 5 min. 10 reads were averaged for each well. ThT traces were fitted to a sigmoidal function using nonlinear least square regression in KaleidaGraph (v4.0; Synergy Software, Reading, PA).

\[ F = y_i + m_i t + \frac{y_f + m_f t}{1 + e^{-\left(\left(t - t_{0.5}\right) / \tau\right)}} \]  
(Eq. 1)

where F is the measured fluorescence intensity; \( y_i \) and \( y_f \) are initial and final ThT intensity levels, respectively; \( t_{0.5} \) is the time at which ThT intensity reached \( y_f / 2 \); \( m_i \) and \( m_f \) are the slopes of the initial and final baselines; and \( \tau \) is the elongation rate. ThT end point intensity was taken as the mean data points after the end of the elongation phase.

Circular Dichroism—CD spectra were recorded on a J-810 spectrometer (Jasco) in a 1-mm quartz cuvette from 250 to 190 nm with 0.2-nm data pitch, 1-s integration time, 1-s bandwidth, and six accumulations. Only data points with detector voltages below 600 V were used. To record spectra of soluble species at pH 5.5 and 7.4, sCT was dissolved to 0.2–0.4 mg/ml and measured in the appropriate buffer. GAGs were present at concentrations mentioned in the text. Aggregated samples were pelleted at 11,000 × g for 30 min at 4 °C. The supernatant was gently removed from each sample and analyzed for monomer content using SDS-PAGE together with an sCT calibration series. The insoluble pellet fraction was resuspended in the appropriate buffer without ThT or GAG. The sCT concentration in the aggregated sample was calculated by subtracting the measured sCT supernatant concentration from the initial concentration. For the pH 2.8–8 binding assay, GAGs were added in a 5:1 heparin_{disac}∶sCT ratio. Negligible CD signals were obtained from GAGs and buffers. For sCT-NH_{2}, GAG binding at pH 3–8 structural conversion is reported as the ratio of ellipticities at 200 and 220 nm. sCT-NH_{2} fibrillation spectra were recorded at 5-min intervals from 250 to 195 nm with 0.1-nm data pitch, 2-nm bandwidth, 4-s response, and 50 nm/min scanning speed at 37 °C. Between reads, the cuvette was incubated in a Vortemp 56EVC (Tehtnica, Zelazniki, Slovenia) Eppendorf thermoshaker modified to hold cuvettes at 59 rpm and 37 °C. Protein concentration was 0.5 mg/ml, and heparin concentration was 150 μg/ml.

Fibril Imaging by Atomic Force Microscopy—sCT fibrils were twice pelleted and resuspended in double distilled H_{2}O to avoid salt precipitates. The washed and diluted fibrils were dried on freshly cleaved mica and analyzed by atomic force microscopy on a 5100 AFM with PicoView 1.12 (Agilent Technologies) using a silicon cantilever (AppNano, ACSTA-50) in tapping mode with a resolution of 1024 × 1024 points in a 3 × 3-μm frame, scan speed of 1.5 μm/s, and integral/proportional gain of 3%. No morphological difference was observed between washed and unwashed fibrils. The height profiles of the fibrils were extracted using the software Gwyddion (v2.30; Free Software Foundation), and all heights reported on twisted fibrils were measured on the highest points in the repetitive profile along a fibril axis.

Fibril Trypsin Digestion and Analysis—sCT-NH_{2} and sCT-NH_{2}hep (2:1, 3:1, or 1:5 sCT:heparin_{disac}) fibrils were formed at pH 5.5 in a plate reader assay, pelleted at 136,000 × g, washed, and resuspended in phosphate buffer at pH 7.4. Trypsin was added to a final concentration of 0, 0.145, 0.725, and 1.45 μM, respectively, and incubated at 37 °C for 1 h with 20 rpm shaking in a Vortemp 56EVC Eppendorf thermosthaker. In all cases, trypsin activity was inhibited by addition of 1 mM phenylmethanesulfonfyl fluoride (PMSF), and samples were reduced and treated with iodoacetamide. 145 μM sCT-OH, 1.5 mg/ml heparin, pH 7.4, fibrils for AFM were pelleted, washed as above, and treated with 1 μM trypsin for 2 h at pH 7.4. Trypsin activity was inhibited by addition of 2 μM PMSF. The digested sCT super-
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nantants and fibrils were analyzed by Tricine SDS-PAGE using a 16% separating gel, 10% overlay gel, and 4% sample gel as described previously (43). Gels were stained with Coomassie Brilliant Blue and toluidine blue for peptide and heparin visualization, respectively.

In-gel Digestion and Micropurification—For each fibril experiment, two bands were subjected to Lys-C in-gel digestion, namely the non-trypsin-treated sCT band and the lower sCT peptide band observed at the highest trypsin concentration. Gel bands were incubated 2 × 15 min in 50% acetonitrile, dehydrated in neat acetonitrile for 15 min, and equilibrated in 0.1 M NH₄HCO₃ for 5 min before an equal volume of acetonitrile was added and further incubated for 15 min. Supernatants were removed, and the gel pieces were lyophilized for 10 min. In-gel digestions were performed by incubating the gel pieces with 50 ng of sequencing grade Lys-C (Promega) in 50 mM NH₄HCO₃ at 37°C for 16 h. The resulting peptides were desalted using homemade microcolumns (P10 tips plugged with C₁₈ material) and stored at −20°C before LC-MS/MS analysis.

Parallel Reaction Monitoring-MS—Parallel reaction monitoring (PRM) analyses were performed using an EASY-nLC II system (Thermo Fisher Scientific) connected to a TripleTOF 5600+ mass spectrometer (AB SCIEX) equipped with a NanoSpray III source (AB SCIEX) and operated under Analyst TF 1.6.0 control. Lys-C-digested and micropurified samples were dissolved in 0.1% formic acid, injected, and trapped on an in-house-packed trap column (2 cm inner diameter) and stored at −3,000,000 cps, thereby preventing MS/MS of precursor ions to be selected for MS/MS in each cycle. The number of precursor ions to be selected for MS/MS in each cycle was measured at concentrations of 2, 0.5, and 0.2 g/liter. Only pure water measurement was used to convert the scattering to ion concentration effects. The 0.2 g/liter sample was used because the others showed clear concentration effects. The 0.2 g/liter heparin sample and its dilution were measured at concentrations of 2, 0.5, and 0.2 g/liter. Only the 0.2 g/liter sample was used because the others showed clear concentration effects. The 0.2 g/liter heparin sample and its reference were measured for 4 h each. The data are displayed as I(q) versus q where q is the size of the momentum transfer $q = 4\pi \sin(\theta)/\lambda$ and 20 is the scattering angle. The pair distance distribution functions $p(r)$ were found using GNOM (47). The modeling was performed using the Ensemble Optimization Method (EOM) (48) on the basis of up to 10 blocks, each containing one copy of sCT (modified from Protein Data Bank code 2GLH) and three heparin disaccharides, each of which consists of N,O-sulfo-dulcosylglucosamine (SGN) and 2-O-sulfido-α-L-idopyranuronic acid (IDS), described as (SGN × IDS)³. The building block was put together in PyMOL (The PyMOL Molecular Graphics System, version 1.7.4, Schrödinger, LLC). In the case of modeling the heparin alone, the basic building block con-
sisted of Cys × (SGN × IDS)³ × Pro where the amino acid caps (cysteine and proline) allow the program to run.

**Fibril Structural Analysis Using Solid-state NMR—sCT-NH₂** with uniform ¹³C- and ¹⁵N-labeled Val, Lys, and Arg²⁴ was incubated at pH 5.5 with heparin at a 1:5 heparin_disac:CT ratio overnight at 37°C with one glass bead and orbital shaking (900 rpm, TS100 Biosan Thermosthaker) to form sCT-NH₂-heparin fibrils. CD showed a strong 218-nm minimum, and AFM verified the presence of fibrils. Fibrils were pelleted at 11,000 × g, and no residual sCT-NH₂ was detected in the supernatant by CD or AFM. Labeled sCT-NH₂ was also incubated without heparin at pH 5.5 in a plate reader assay for 4 days. CD and AFM confirmed fibril content, and fibrils were pelleted by centrifugation at 136,000 × g for 30 min.

The NMR experiments were carried out on 700-MHz Avance III and 500-MHz Avance III Bruker spectrometers (Rheinstetten, Germany) equipped with 4-mm triple resonance Bruker probes. One-dimensional ¹³C spectra were acquired using CP and INEPT techniques with 3072 points, 2048 repetitions, and a relaxation delays of 3 s. The ¹H-¹³C CP experiments used an 80–100% ramp for ¹H and square pulses for ¹³C with average values of 54 (¹H) and 42 kHz (¹³C) and mixing times of 2.5 ms. The INEPT spectra used 180° pulses of 7.6 µs. ¹³C-¹³C correlations were obtained using dipolar assisted rotational resonance mixing (49) with mixing times of 20 and 200 ms, 512 points for spectral widths of 200 ppm in the indirect dimensions, 80 repetitions, and 6 relaxation delays of 3 s. Two-dimensional adiabatic double CP spectra (500 MHz) with 8-ms mixing were used for acquiring ¹⁵N-¹³C correlations with average rf fields of 41/32 kHz (¹³C/¹⁵N) and 100-kHz continuous wave ¹H decoupling during mixing. The ¹⁵N dimension used a spectral width of 120 ppm and 128 increments, and 224 repetitions per increment were applied. All experiments used magic angle spinning with spinning rates of 12 kHz (700 MHz) and 9 kHz (500 MHz) and −100 kHz SPINAL-64 (50) ¹H decoupling during direct and indirect acquisition periods. The samples were kept around 4°C using a precooled gas flow. All spectra are presented in the Supporting Information.

**Author Contributions**—K. G. M and D. E. O. planned experiments. K. G. M, E. H. T. N., M. B., E. T. P., C. M. J., and G. C. performed experiments and communicated experimental methods and results. D. E. O., J. S. P., T. V., T. S., and J. J. E. supervised experiments. K. G. M wrote the manuscript. D. E. O. edited the manuscript.

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