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Self-assembling peptides and proteins for nanotechnological applications

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Photolithography enables the precise construction of nanodevices in two-dimensional formats. However, self-assembly of designed molecules serves as an alternative for the construction of three-dimensional nanoscale systems and is particularly appealing in that material properties can potentially be engineered at the molecular level. Peptides and proteins hold promise as building blocks for self-assembled systems because of their exquisite three-dimensional structures and evolutionarily fine-tuned functions.

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Abbreviations

AFM atomic force microscopy

Introduction

Nanotechnology aims to construct materials and operative systems at nanoscale dimensions. Several potential applications can be envisioned: targeted drug delivery systems, tissue engineering scaffolds, photonic crystals, and micro/nano fluidic and computational devices. The fundamental challenge in nanotechnology is to construct systems with varied functional features and predictably manipulate processes at the nanometer length scale. Conventional construction methods based on photolithography can successfully generate two-dimensional structures using a ‘top-down’ approach, in which patterned surfaces are prepared by etching with light. Feature sizes on the order of 50 nm are easily achieved with commonly available technologies [1]. Advances in specialized lithographic techniques (e.g. scanning probe lithography) have extended the resolution to below 20 nm [2*].

Molecular self-assembly serves as an alternative paradigm for preparing functional nanostructures. The idea of manipulating individual atoms and molecules was pro-

posed by Richard Feynman through his seminal talk in 1959 titled “There’s plenty of room at the bottom” [3]. This approach complements the two-dimensionality of lithography, possibly allowing three-dimensional structures with diverse shapes, sizes and functions to be constructed from self-assembled molecules.

Herein, self-assembly is discussed in the context of using peptides and proteins as building blocks. Specific examples highlighting the mechanisms of assembly and the potential of using proteinaceous materials in nanotechnology are elaborated.

Molecular assembly

Molecular self-assembly is characterized by spontaneous diffusion and specific association of molecules dictated by non-covalent interactions. There are numerous recent examples involving different molecular entities: organic molecules [4–8], proteins [9,10], peptides [11–16], DNA [17] and others [18–21]. Although this is not an exhaustive list, it demonstrates the diversity of building blocks that can be used. Assembly can be biologically inspired where complex nanoscale structures are made to function with precision. For example, cells can be considered as active machines that respond to their environment and are epitomes of advanced *micro*technology. However, the gears of cells are forged from biomolecules such as proteins, lipids and DNA, often self-assembled into functional *nano*structures. Central to mimicking biologically inspired self-assembly is understanding the forces that govern the thermodynamic stability and specificity of naturally occurring self-assembly events. As early as 1981, Eric Drexler [22] proposed that protein design could be used to fabricate devices through a ‘bottom-up’ approach, in which proteins are used as monomeric building blocks for the fabrication of higher order structures via self-assembly. Peptides and proteins are particularly attractive as building blocks because a great deal is known about their folding and stability, and rules governing protein–protein interactions are actively being established. Based on this knowledge, the field of *de novo* design emerged and tests our expertise in answering the simple question: can one build structural and functional proteins from first principles? To date, most *de novo* design efforts have centered on self-assembled proteinaceous structures of finite size. However, the design principles established from these endeavors are now being extended to the fabrication of larger nanoscale devices and materials. The challenge is twofold: first, to rationally design building blocks amenable to self-assembly that

form nanostructured materials and, next, to impart desired functionality. Functionality can arise from either the extremely small feature sizes inherent to the material, such as those needed for photonics applications, or the incorporation of specific functional epitopes into the self-assembled scaffold.

In contrast to irreversible aggregation, equilibrium self-assembly takes place in three distinct steps: molecular recognition followed by reversible association, when self-correction is possible, and ultimately termination [23^{*}]. Self-assembly could, in principle, be applicable to any length scale. Whitesides and co-workers [24] demonstrated mesoscale self-assembly of millimeter-sized objects driven by capillary forces. However, for nanoassemblies, recognition events are governed by favorable thermodynamics, often mediated through weak directed non-covalent interactions such as hydrogen bonding, electrostatic interactions involving dipoles and formal charges, hydrophobic interactions and van der Waals interactions, as well as kinetically labile metal coordination. Although many of these interactions may be small in magnitude (<5 kcal/mol), the large number formed in the final assembled structure is significant. The large entropic cost of ordering molecules is only slightly off-set by the favorable enthalpy gained from these weak interactions, rendering the self-assembled system to exist in a thermodynamic equilibrium. As a result, self-assembly is a dynamic process whereby weak interactions dictate reversible associations to achieve an energetically optimized supramolecular structure.

Non-covalent synthesis of supramolecular structures can be classified as commutative or non-commutative [23^{*}]. Commutative self-assembly describes a process in which

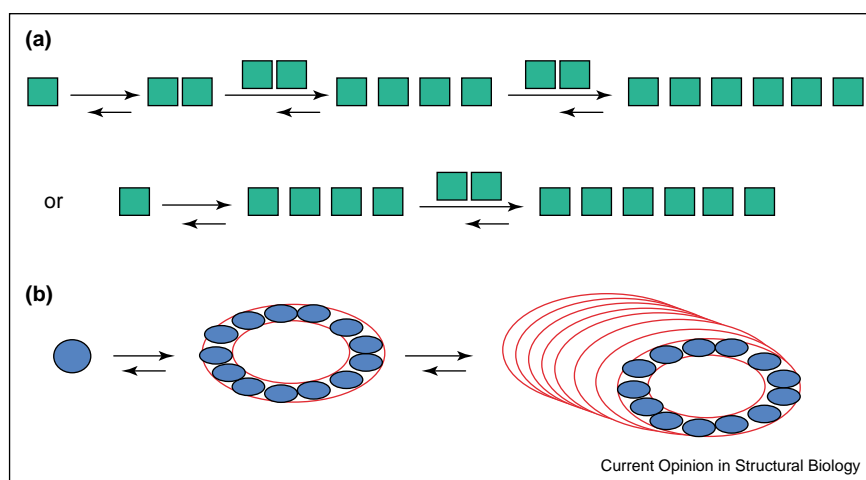
given steps leading to the formation of the supramolecular structure may be interchanged (Figure 1a). Non-commutative self-assembly takes place through a sequence of steps that cannot be interchanged; for example, a hierarchical process in which intermediate structures are formed and subsequently assemble into complex systems (Figure 1b).

Peptides and proteins in self-assembly

One attractive benefit of employing self-assembly to construct materials is that the bulk properties of the resultant material are often dictated by the individual monomeric building blocks comprising the assembly; this allows novel materials with tailored morphologies and functions to be prepared through single-molecule engineering. As detailed structural and functional information is available for an almost endless variety of peptides and proteins, their use as building blocks is very attractive. For example, peptides can be designed to adopt well-defined helical and β -hairpin/sheet secondary structures that can be utilized as topologically defined building blocks. The chemical synthesis of peptides is rapid and allows a near infinite palette of structure and function if non-coded residues are used in addition to naturally occurring amino acids.

Naturally occurring proteins can be used as the starting point for material construction. For example, functionality can be engineered into proteins having an inherent propensity to self-assemble. S-layer proteins make up the crystalline cell surface layers of certain bacteria and are known to self-assemble into diversely shaped objects such as sheets, open-ended cylinders and closed vesicles [10]. Resultant structures are characterized by regular oblique, square and hexagonal nanomorphologies.

Figure 1



Commutative and non-commutative self-assembly processes. (a) Given steps in the sequence of assembly may be interchanged in a commutative process. (b) Non-commutative processes entail hierarchical order in the assembly process.

Although the main function of these proteins is to self-assemble, additional functional attributes can be incorporated by modifying their primary sequence.

Alternatively, soluble proteins displaying a desired function, such as a particular enzymatic activity or binding epitope, can be modified at their surfaces to enable self-assembly; the resultant nanostructure contains a factory of sites whose function is dictated by the monomeric precursor [25]. In terms of production, many proteins can be overexpressed and technologies to incorporate non-coded residues facilitate diversity [26,27]. In spite of these advantages, peptides and proteins have limitations. Devices that must operate in rigorous environments, such as at high temperature, or in strongly acidic and alkaline solutions, may require more robust materials. For electronic applications, limited electrical conductivity is a severe drawback. For applications that necessitate *in vivo* function, enzymatic degradation may be problematic. However, controlled degradation can be advantageous for tissue engineering applications where extracellular matrix substitutes fabricated from self-assembled peptides or proteins may potentially be degraded at a rate similar to tissue regeneration [28].

Nanoscale morphologies from designed peptides

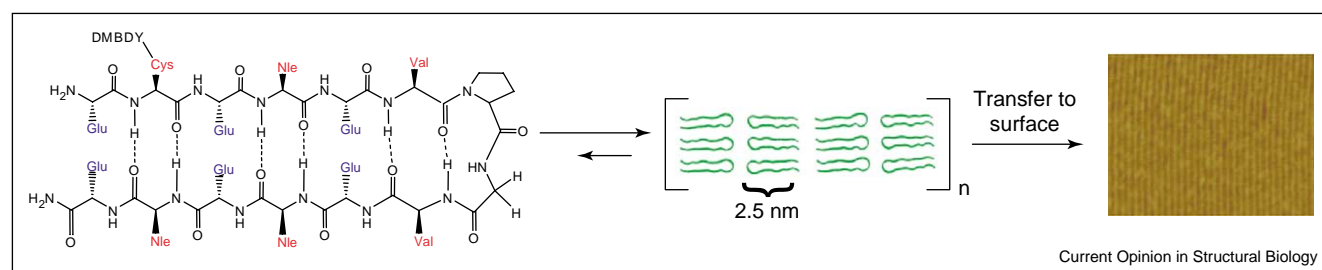
Peptides have been used to construct supramolecular architectures such as ribbons [11,29], nanotubes [30,31–34] and monolayers with nanoscale order [35–37]. These structures are mainly composed of β -sheet motifs at the secondary structure level. Helical-based assemblies are less common, although a few examples of helical fibrils have appeared [38–40]. More ornate structures than ribbons, tubes and fibrils are very rare if not non-existent, but perhaps by combining helical- and sheet-based building blocks, they may soon be realized. Technologically, metallization of peptide nanotubes [32] or protein fibrils [41] leads to conducting nanocircuits, ordered monolayers can serve as templates for minerali-

zation and directed crystal growth [42], and nanofibers have been used as scaffolds for drug delivery and tissue engineering applications [16,43].

De novo designed amphiphilic β -strands and β -sheets have been shown to assemble at the air/water interface or on solid surfaces in a commutative manner, affording extended monolayers of regularly ordered assemblies. Exquisite control was demonstrated by Kelly, Powers and co-workers [44] in the design of β -hairpins that self-assemble at the air/water interface; the resultant assembly could subsequently be transferred to surfaces using Langmuir–Blodgett techniques. Atomic force microscopy (AFM) images of adsorbed monolayers showed the formation of large domains of ordered structure characterized by 2.5 nm ridges and lattice spacings of 0.2–0.3 nm (Figure 2). Importantly, the ridge width and lattice spacings can be predictably altered by modifying the strand length of individual hairpins, demonstrating that bulk material properties can be altered at the molecular level.

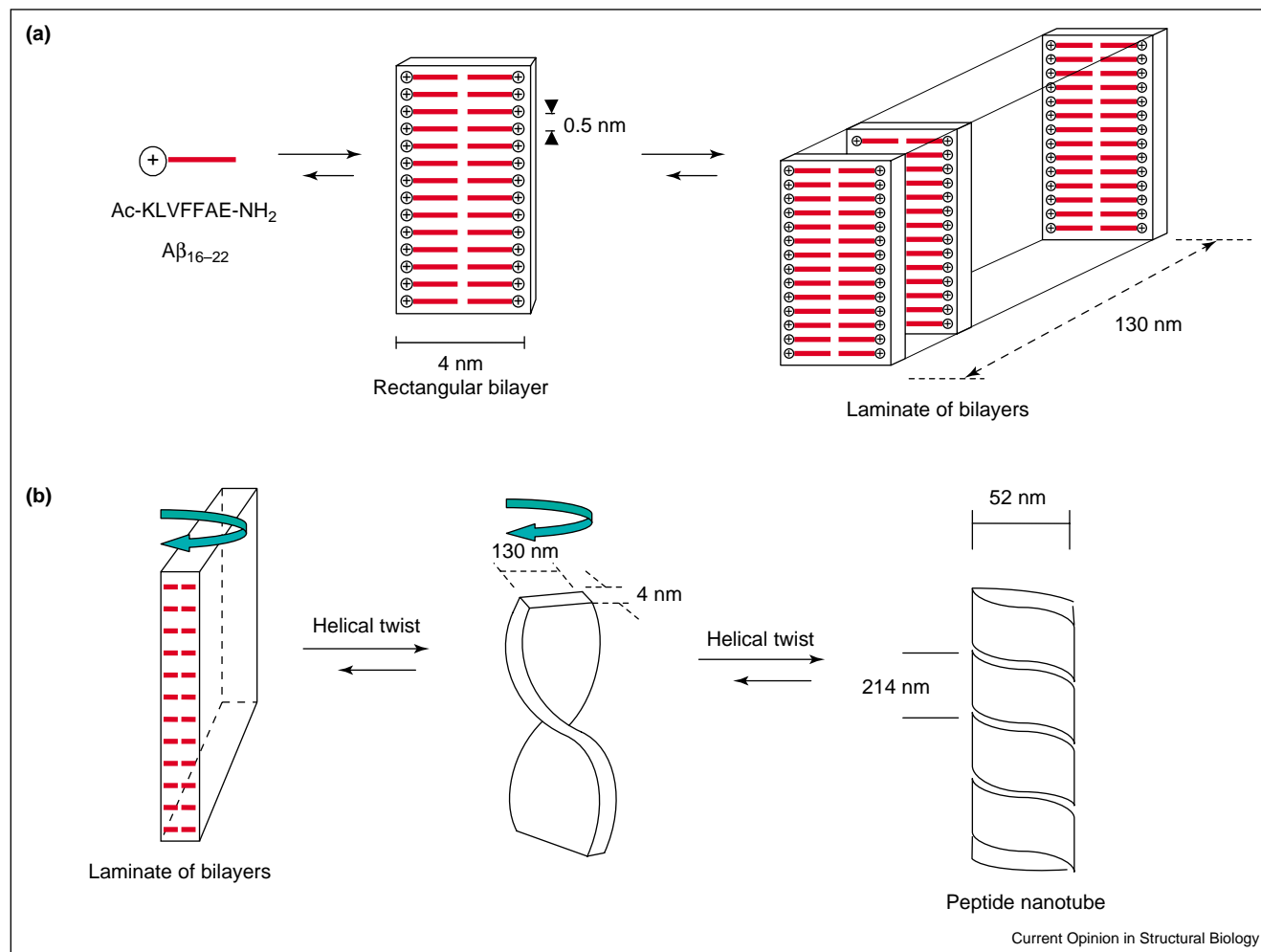
A particularly well characterized example of non-commutative self-assembly involves the $A\beta_{16-22}$ peptide, as reported by Thiyagarajan, Conticello and Lynn [30]. This peptide first self-assembles into parallel β -sheets that undergo subsequent defined lamination. Resulting laminates twist into helical ribbons whose edges fuse to afford hollow nanotubes of uniform diameter (Figure 3). More complex non-commutative assemblies can be found in living systems. Cells have been argued to be an example of a dynamic self-assembled system. Although not purely ‘self’-assembled, cells illustrate the complexity to which we can aspire to understand and replicate. Non-commutative hierarchical strategies are inherently more difficult to design because one needs to consider not only the assembly of monomeric building blocks into intermediate structures but also predictive mechanisms that allow these intermediates to correctly assemble — a current challenge in the field.

Figure 2



Use of peptides to form nanoscale-ordered monolayers. An amphiphilic β -hairpin undergoes ordered self-assembly at the air/water interface. Resultant Langmuir–Blodgett films can be transferred to mica for AFM characterization using single-wall carbon nanotube tips. The image shows the expected 2.5 nm spacing consistent with ordered hairpin assembly. DMBDY is a dimethyl derivative of BODIPY, a fluorescent label used for imaging.

Figure 3



Hierarchical self-assembly of A β ₁₆₋₂₂ into nanotubes. **(a)** Although the exact mechanism of their formation is under study, the peptides self-assemble into rectangular bilayers that form laminates (130 bilayers). **(b)** The laminate twists to form a helical structure whose edges ultimately fuse, affording a nanotube.

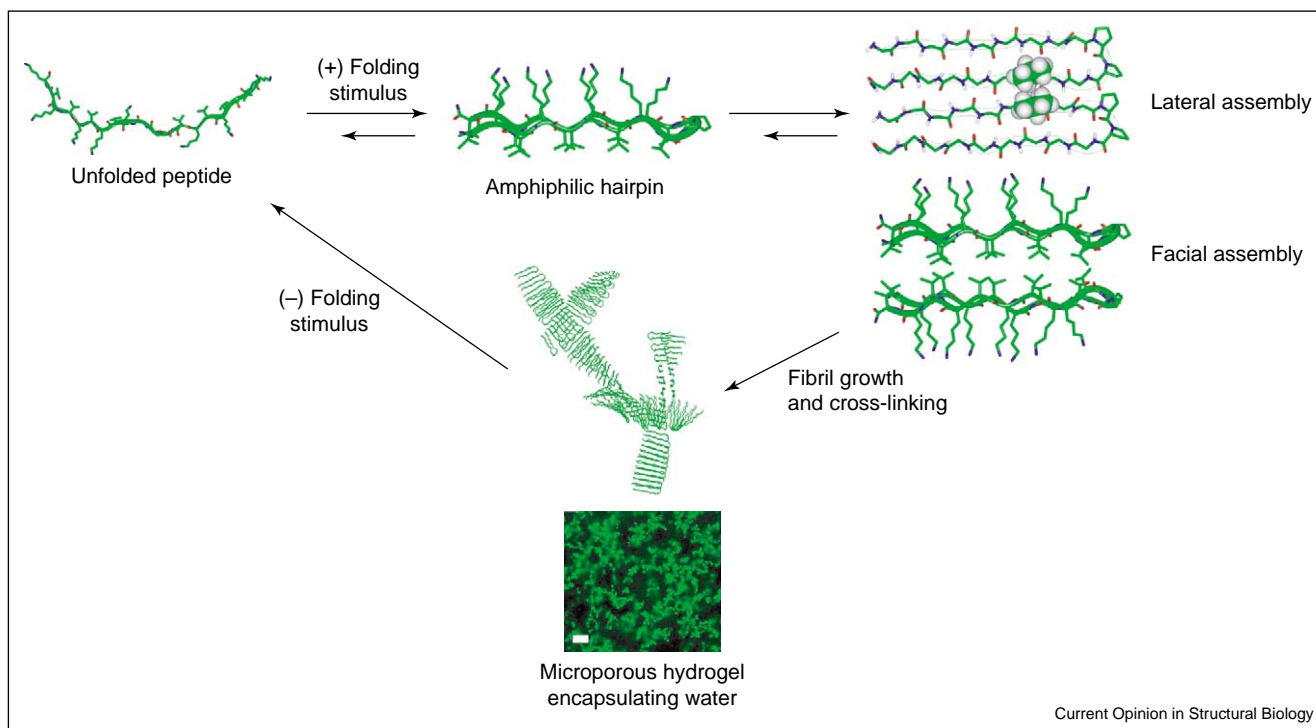
Responsive materials from designed peptides

A major thrust in materials design is the preparation of smart materials whose morphologies and associative functions can change in response to their environment. β -Strand peptides are a rich source for the construction of self-assembled materials. However, they often self-assemble slowly, affording β -sheet-rich materials that may be very thermodynamically stable and, as such, display limited responsiveness to changes in environmental conditions.

Alternatively, Schneider and Pochan envisioned that responsive materials could be prepared from peptides whose ability to self-assemble into a targeted morphology is directly dependent on their intramolecularly folded conformational state. Peptides were designed to fold in response to a distinct external stimulus, adopting a con-

formation that is amenable to self-assembly. The resultant self-assembled materials are environmentally responsive; removing the folding stimulus results in peptide unfolding and material dissolution. Peptides have been designed that undergo triggered folding in response to pH and/or temperature changes, adopting a β -hairpin conformation (Figure 4). These hairpins self-assemble into responsive hydrogel materials [45,46]. Twenty-residue amphiphilic hairpins were prepared containing strand sequences of alternating hydrophobic and hydrophilic residues flanking an intermittent tetrapeptide β -turn. Optimal turn construction, typically type I' or II' in these designs, ensures that, under folding conditions, only correctly folded hairpins take part in the self-assembly process that leads to hydrogelation. Folded hairpins are amenable to self-assembly both laterally (via the formation of intermolecular hydrogen bonds and van der Waals

Figure 4



Triggered folding of a designed peptide affords an amphiphilic β -hairpin that is amenable to facial and lateral self-assembly. Ultimately, a hydrogel is formed whose nanostructure is consistent with a network of cross-linked short fibrils (10–200 nm in length, as determined by cryo-transmission electron microscopy). Laser scanning confocal microscopy was used to determine the microstructure; the image at the bottom shows the assembled peptide (green) surrounding large water-filled pores (~20 μm in diameter) and channels (black) that permeate the gel. Material formation is reversible — simply unfolding the peptides that comprise the assembly dissolves the hydrogel.

contacts) and facially (via the burial of the hydrophobic face of distinct hairpins).

Depending on the peptide sequence and the folding stimulus used, the hydrogelation process can be very fast and fully reversible. For example, heating a 2 wt% solution of unstructured peptide results in hairpin folding and self-assembly; cooling the resultant hydrogel results in hairpin unfolding and material dissolution [46].

Detailed structural characterization at the nanometer length scale indicates that these gels are composed of a network of short fibrils (10–200 nm) rich in β sheet. Fibrils are physically cross-linked by non-covalent hydrophobic interactions between the hydrophobic faces of the assembled hairpins (Figure 4). In this working model, fibrils are composed of a twisted bilayer of intermolecularly hydrogen-bonded hairpins. Bilayer formation within a given fibril is driven by the ordered packing of the hydrophobic faces of individual hairpins. However, because the hydrophobic face of each hairpin is topologically smooth, non-ordered association also occurs, providing a nucleation site for nascent fibril growth in a different direction and ultimately forming a heavily cross-linked

network of short fibrils. In terms of the strict definitions outlined in this review, these peptides undergo nanoscale non-commutative self-assembly during fibril formation. However, the formation of interfibril cross-links is most likely a non-specific aggregation process.

Hairpin-based hydrogels are composed of >98% water and are microporous yet display significant rheologically defined material rigidity, presumably due to their nanoscale fibril content. These peptides are currently under study for use in tissue engineering applications; hydrogelation can possibly be triggered *in vivo*, affording injectable extracellular matrix substitutes with defined nanoscale structure. Rational modification of the hairpin sequence allows direct control over the nanoscale features present within the fibril matrix. Although it was known that cells can modulate their behavior in response to micrometer-scale structure, recent studies indicate that nanoscale features displayed within a matrix impact cell adhesion, morphology and proliferation [47].

Conclusions

Supramolecular chemistry aimed to construct defined structures is 'information science' [23^{*}]. The manner in

which molecules self-assemble and their interaction energies, shapes and ultimate functions can be programmed at the molecular level. Examples illustrated herein show that peptides and proteins, coupled with the power of self-assembly, can be used to construct nanoscale structures. However, it is very difficult to design, *a priori*, molecules that predictably self-assemble into targeted three-dimensional supramolecular structures, especially via non-commutative mechanisms. Many examples of peptides and proteins that are found to self-assemble are discovered serendipitously, and the rules by which they assemble are then sought. This is a perfectly valid approach to discovery and establishing the guidelines for self-assembling systems. In fact, with respect to peptides and proteins, self-assembling systems are often overlooked. How many times have NMR samples been prepared that precipitate or gel and the immediate reaction of the investigator is 'how am I going to solubilize this mess' and not 'maybe we should study this'. Irrespective of how the rules are established, the next step is to design molecules based on these rules that can assemble in a predictive manner into a targeted morphology. With the ability to fabricate three-dimensional assemblies with spatial precision in hand, one can then look forward to incorporating function. Intense interest in the fabrication of nanoscale devices and demand for miniaturization in both academia and industry necessitate progress in this area.

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