THE STRUCTURE OF PROTEOGLYCAN AGGREGATE DETERMINED BY ATOMIC FORCE MICROSCOPY

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INTRODUCTION:
The proteoglycan aggregate (PGA) is one of the largest proteins made by living organisms. The PGA is formed through proteoglycan (PG) interaction with hyaluronic acid (HA). The structure is stabilized by a glycoprotein known as link protein. The PG consists of a linear core protein to which is attached 50-100 side chains of the glycosaminoglycans (GAG). This special structure of PGA is closely related to its mechanical function. In cartilage, this aggregate is the primary one responsible for resisting joint compression by mutual electrostatic repulsion on the glycosaminoglycan molecules.

Traditionally, electron microscopy (EM) techniques have been used for imaging the PG monomers and aggregates, but the sample preparation for EM requires complicated fixation, dehydration, staining and coating processes. The newly developed Atomic Force Microscopy (AFM), on the other hand, provides an alternative approach for visualization of molecule structures at Angstrom level. Besides offering high resolution imaging, AFM can also minimally disturb the molecule during sample preparation and imaging, and therefore, provide a better technique for imaging many important biological molecules.

The purpose of this study was to demonstrate the feasibility of using the AFM to determine the PGA ultrastructure, and to discuss various techniques used during the image process.

METHODS:
The principle of AFM is to determine a surface geometry by scanning the entire surface with a mechanical probe. Briefly, the mechanical probe tip (~tens of nm in radius) is attached to a flexible cantilever arm. In the simplest mode of operation, contact mode, the tip is brought into contact with the surface of interest and raster scanned. The deflection of the cantilever on which the tip is mounted is monitored and used as a feedback mechanism to control the interaction between the tip and the sample. By maintaining a constant deflection of the cantilever throughout the scanning process, a highly accurate topography can be generated. In tapping mode AFM, a stiff cantilever oscillates at a certain distance from the sample surface while scanning laterally. When the tip approaches the surface due to sample feature or protrusion, the oscillating frequency of the cantilever is forced to change and its amplitude at a given frequency is reduced. The feedback system then raises the position of the cantilever to resume its original amplitude of oscillation. The topography of the sample is recorded as the changes in the input voltage which corresponds to the movements of the cantilever. Operating in the tapping mode of the AFM can dramatically reduce the contact and lateral forces to the sample.

Mica surface is one of the most popular surfaces used for biological material AFM scanning. The scanning process requires a smooth surface for the specimens. The layered structure of mica supplies a large area of flatness in each layer. Freshly cleaved mica surface eliminates possible contamination from the process and environment and is a good substrate material for AFM biological material visualization.

In our imaging method, PGA (25 µg) (Sigma, St. Louis, MO) was dissolved into 20 ml of distilled water. The freshly cleaved mica was treated with 3-aminopropyltriethoxysilane for 30 minutes. The PGA solution (10 µl) was dropped on freshly cleaved mica, then another 10 µl of distilled water was added on the mica for 1 minute. The specimen was carefully rinsed and dried with pressured air. An image was taken using the tapping mode of the AFM (Synergy ESPM, Novascan, Ames, IA). A tip with a cantilever length of 130 µm and a resonance frequency of 145 KHz was used (NSC12, Mikromasch USA, Portland, OR). The image was recorded with 2Hz scan rate and a 1024X1024 resolution. The measured test dimension was calculated using Image Analysis software.

RESULTS:
Over a dozen specimens were investigated. The images illustrated the aggregates with the individual PGs and the core HA. In a typical molecule shown in Figure 1, the PGA was about 1.5 µm long and 700 nm wide. The HA had ~35 PGs linked to it. Each individual PG was about 400 nm long and 80 nm wide. The individual bright spots along the core HA were likely the attached linked proteins. The space of PG binding sites in HA was about 40 nm. The PGs were attached on all the possible binding sites of HA.

DISCUSSION:
The AFM images clearly demonstrate the PGA ultrastructure in a nearly natural state. The HA chain and individual PG were clearly distinguishable. Because the sample preparation for AFM does not require fixation or stain for the specimens, the molecules were kept and observed in the most natural condition possible.

Tapping mode AFM imaging provides clear images of the PGAs with minimal contact with the samples. This unique feature is especially suitable for visualization of soft materials such as biological molecules.

In summary, we demonstrated the feasibility of using the AFM for visualization of the ultrastructure of PGAs. Because the AFM does not require sample fixation, staining, coating, and other routine procedures for EM imaging, this technique could be a simple alternative approach for future analysis of PGA and its assembly.