The measurement of biomechanical properties of porcine articular cartilage using atomic force microscopy*

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**Summary. We have recently demonstrated that indentation-type atomic force microscopy (IT-AFM) is capable of detecting early onset osteoarthritis (OA) (Stolz, 2009). This study was based on biopsies, using a desk-top commercial atomic force microscope (AFM). However, cartilage analysis in the knee joints needs to be non-destructive to avoid new seeding points for OA by the taking of biopsies. This requires bringing the probe tip in contact with the articular cartilage (AC) surface inside the joint. Here we present our recent progress towards a medical instrument for performing such IT-AFM measurements for in-vivo knee diagnostics. The scanning force arthroscope (SFA) integrates a miniaturized AFM into a standard arthroscopic sleeve, and is used for direct, quantitative, in situ inspection of AC (Imer et al., 2006). The stabilization and the positioning of the instrument relative to the surface under investigation were performed by means of eight inflatable balloons. An integrated three-dimensional, piezoelectric scanner allowed raster scanning and probing of a small area of cartilage around the point of insertion. An AFM probe with an integrated deflection sensor was mounted at the distal end of the instrument. Using this instrument, several measurements were performed on agarose gel and on porcine cartilage samples. The load-displacement curves obtained were analyzed and the dynamic elastic moduli $E'$ were calculated. A good correlation between these values and those published in the scientific literature was found. Therefore, we concluded that the SFA can provide quantitative measurements to detect early pathological changes in OA.

Introduction

With the increase of life expectancy and changes in social habits, more and more people will be affected by degenerative joint diseases in the next few decades. Despite the high prevalence of osteoarthritis (OA) in people over 50, currently there exist no treatments that stop or reverse the degeneration of articular cartilage (AC). Pathological changes in cartilage are initiated at the molecular (i.e. nanometer) scale, from where they spread to higher levels of the cartilage hierarchical architecture and cause progressively irreversible structural and functional damages. There are several non-invasive (Burstein and Gray, 2006; Li et al., 2007; Qazi et al., 2007) and invasive (Saarakkala et al., 2003; Hattori et al., 2004; Kleemann et al., 2005) methods suggested for

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evaluating the joint surface. The different techniques and instruments currently available on the market for evaluating the cartilage state of health can only diagnose structural changes at an advanced stage of the disease, usually at a point too late for treatment.

For an early diagnosis of OA, as well as for the development of drugs and effective therapies, more sensitive diagnostic tools are needed. Since AC is a load-bearing structure that provides the mechanical function of the joints, measuring the mechanical properties of AC is of key importance and provides advanced diagnostic information on the 'health' or quality of AC compared with biochemical and structural analysis. We have recently demonstrated that the AFM is sensitive for measuring the biomechanical properties of AC (Fig. 1) in aging and early onset OA when assessed at the nanometer level, but not at the micrometer- to millimeter levels (Stolz et al., 2009).

The atomic force microscope (AFM) has a sharp probing tip which scans a sample surface while monitoring the force between the tip and sample surface. It has been used as a versatile tool for imaging, measuring and manipulating soft biological tissues at the nanometer level. The functionalization of its tip offers a wide range of future possibilities. For example, Thalhammer et al. (1997) used an AFM tip as nano-scalpel, which may be adapted to manipulate cartilage tissue. Moreover, Hug et al. (2005) presented a hollow cantilever probe suited for administering small amounts of drugs to a cartilage defect site. Obviously, the development of this new class of therapeutic instrument requires a highly interdisciplinary approach involving clinicians, biologists, physicists and engineers.

In the present study, we used the scanning force arthroscope (SFA) for the measurement of the physical properties of AC. This medical instrument integrates a miniaturized AFM into a standard arthroscopic sleeve, and is used for direct, quantitative, in situ inspection of AC. We will show the potential of the AFM as a sensitive diagnostic tool for joint diseases.

Materials and Methods

Scanning force arthroscope

The SFA (Fig. 2) is a medical instrument with dimensions comparable to those of classical arthroscopic
The piezoelectric tube scanner allowed the establishment of a two-dimensional map of cartilage stiffness. Depending on the voltage applied between the inner and outer electrodes, the piezoelectric tube contracts laterally or longitudinally, thereby providing an XYZ motion. This 3D scanner was able to probe a surface of about $1 \times 40 \mu m^2$ with a z-range of 40 $\mu m$. A grid of data was achieved by combining a raster scan in the XY plane with an indentation movement in the z-direction.

The complete indentation stage contained the AFM cantilever used for measuring the biomechanical properties of cartilage. The microfabricated chip was glued on a small PEEK connector, which allowed the quick exchange of the probe in case of cantilever failure.

The AFM probe consisted of a rectangular silicon cantilever on which a 10 $\mu m$ high pyramidal tip was displacement of the tool tip in a plane perpendicular to the cartilage surface.

**Fig. 2.** Picture of the SFA. The handle (1) facilitates the manipulation of the instrument. The electrical and pneumatic connections are embedded into a rigid shaft (2). The inset shows a magnified image of the distal end of the instrument containing—from left to right—the stabilization stage (3), the scanning stage (4) and the indentation stage (5).

**Fig. 3.** Picture of the configuration used during the measurements. The handle of the instrument (2) was held by a clamp (1), whereas its distal end was stabilized inside a PVC tube (3). A three-axis stage (4) facilitated the fine positioning of the tool. A vat (5) kept the sample moist during the measurements.
generating the indentation and recording the cantilever deflection signal.

**SFA calibration**

For the measurements, each piezoelectric tube scanner and each cantilever spring constant was measured separately. The instrument was fixed onto a 3-axis tilt platform stage which allowed alignment of the instrument relative to a reference surface placed on a 3-axis translation stage. An optically reflective surface was added to the top of the indentation stage in order to allow optical probing. This device was then placed under a Wyko NT1100 optical profiler (Veeco). For calibration and measurements voltages from $-100$ to $+100$ Volts were applied to the electrodes of the scanner. For each value of voltage we recorded a three-dimensional position profile. The Z-displacement of the tube as function of the applied voltage was constructed using these data.

The calibration of the piezoresistive cantilevers was performed by placing the chip into the Nanoscope commercial AFM. An approach curve was taken on a hard stainless steel substrate. During these measurements, the signals from the SFA piezoresistor and from the
Gels with 0.75%, 2.0%, 2.5%, and 3.0% w/w agarose in deionized water were prepared. The solution was heated to 90°C and mixed for 10 min using a magnetic stirrer. O-rings (inner ring diameter = 4 mm; outer ring diameter = 8 mm; thickness = 2 mm) were glued onto stainless steel disks (diameter = 10 mm). A droplet of the melted gel was deposited in the center of the o-ring and allowed to cool to ambient temperature. Once the gel solidified, the whole sample was immersed in the deionized water to avoid dehydration.

**Knee model**

For some experiments, we employed a plastic reproduction of a human knee joint also mimicking the bones and ligaments.

**Agarose gels**

Gels with 0.75%, 2.0%, 2.5%, and 3.0% w/w agarose in deionized water were prepared. The solution was heated to 90°C and mixed for 10 min using a magnetic stirrer. O-rings (inner ring diameter = 4 mm; outer ring diameter = 8 mm; thickness = 2 mm) were glued onto stainless steel disks (diameter = 10 mm). A droplet of the melted gel was deposited in the center of the o-ring and allowed to cool to ambient temperature. Once the gel solidified, the whole sample was immersed in the deionized water to avoid dehydration.

**Porcine articular cartilage**

The lower leg of a pig was obtained from a slaughter house. The femur, the tibia and the patella were delicately extracted and stored in Phosphate Buffered Saline (PBS) solution until testing (2.6 mM NaH2PO4, 3 mM Na2HPO4, 155 mM NaCl, 0.01% NaN3 w/v, pH 7.0). During measurement PBS was regularly doused over to the sample to keep the cartilage wet.
Oliver and Pharr model assumed that the dynamic elastic modulus $E^*$ was proportional to the reduced modulus of elasticity $E_r$ provided by the Hertz model (Hertz, 1882; Ikai, 2007; Vlassak et al., 2003):

$$E^* \approx E_r,$$

which in turn is related to the stiffness as:

$$E_r = \frac{1}{S},$$

thus:

$$|E^*| \approx \frac{\sqrt{\pi}}{2} (1-v^2) \frac{S}{\sqrt{A}},$$

where $v$ is Poisson's ratio, $S$ the unloading stiffness, and $A$ an area function related to the effective cross section of the indenter.

A schematic load-indentation curve obtained during a complete cycle of loading and unloading is shown in Figure 4.

Depending on the rate and the duration of the applied load, AC changes its stiffness. In short and cyclic loading, the dynamic elastic modulus depends on the loading frequency. For AC testing, the applied loading should be close to the frequencies in natural joint loading (Shepherd and Seedhom, 1997). For that reason, we applied a 3 Hz sine wave signal with amplitude of 20 $\mu$m to the $z$-axis of the piezoelectric tube during indentation testing. During data analysis, the precise localization of the initial point
of contact between the tip and the sample becomes the most difficult problem. The irregular surface and the low stiffness of the samples explain the lack of any abrupt increase in load that usually marks the point of contact between the tip and the sample. This problem can be solved by calibrating the instrument on a reference material such as an agarose gel just before measuring. The disadvantage of this technique is that the calibration curve obtained is only valid for exactly the same set of parameters. Every time a parameter changes, a new calibration curve must be determined.

**Results**

**Knee model**

The first experiment was performed on the plastic femoral condyle of a flexible, anatomic knee model. The model was placed upside down into the vat. The SFA was inserted and stabilized in the PVC tube. The coarse approach was performed using the 3-axis scanning-stage and the pneumatic system. Several load-displacement curves were recorded on the femoral condyle model. Each consisted of an average of 5 consecutive indentation cycles. The dynamic elastic modulus |E'| derived from the unloading curves (Fig. 5), using the Oliver and Pharr model, was about 2 GPa. This value is in good agreement with the 2.4 GPa (CRMPC, 1985) found in the literature for acrylonitrile butadiene styrene (ABS) plastic ((C8H8·C4H6·C3H3N)n).

**Agarose gel**

The main goal of this second experiment was to validate SFA measurements by comparing the results for the dynamic elastic modulus |E'| with literature values. We selected agarose gels as a test sample because these materials have been widely studied and exhibit well known mechanical properties (Chen *et al.*, 2004; Stolz *et al.*, 2004). For measurements, the samples were fixed under the distal end of the SFA as described in the Materials and Methods section. Several load-displacement curves (Fig. 6) were recorded on agarose gels of different concentrations, each consisting of an average over 5 consecutive indentation cycles. Unloaded curves recorded on agarose gels with different concentrations could be directly converted into corresponding dynamic elastic moduli |E'| by means of calibration curves. We used a method was originally established by Stolz *et al.* (2004), who measured the macroscale agarose gel stiffness in unconfined compression employing a compression tester (Zwick Z010; Zwick GmbH, Ulm, Germany) and a standard protocol for polymer testing. The resulting modulus values for the different concentrations of agarose gel are shown in Table 1. The comparison of these values with those known from the literature (|E'|Stolz (Stolz, 2004), |E'|Chen (Chen *et al.*, 2004) showed a good correlation above a gel concentration of 0.75%.

**Porcine articular cartilage**

The last set of measurements was performed on porcine knee AC samples. Several load-displacement curves were recorded at different sites: on the femur (Fig. 7), on the tibia (Fig. 8), and on the patella (Fig. 9). For each set of measurements we derived the nanoscale dynamic elastic moduli |E'| from corresponding unloading curves using the Oliver Pharr model. These values are shown in Table 2.

As described in the data analysis section, the elastic modulus can also be obtained by calibrating the instrument on a reference material just before measuring. Since we used exactly the same parameters for the measurements on agarose gel rather than those on porcine cartilage, we could use data on agarose gel as a calibration curve for calculating the elastic modulus |E'|cal of porcine cartilage.

We found an average value of 0.241 MPa for the measured nanoscale dynamic elastic modulus |E'|mes. This value was in good agreement with the 0.235 MPa given by the calculated elastic modulus |E'|cal, but different from those of Stolz *et al.* (2009), who obtained a value of 2.3 MPa.
Table 1. Comparison of the dynamic elastic moduli of agarose gels with concentration of 0.75%, 2.0%, 2.5%, and 3.0% resp. measured with the SFA (\(\mathbf{\text{E}}^*_{\text{mes}}\)) and those known from the scientific literature (\(\mathbf{\text{E}}^*_{\text{Stolz}}\) (Stolz et al., 2004) and \(\mathbf{\text{E}}^*_{\text{Chen}}\) (Chen et al., 2004)).

| Sample | Slope | \(\mathbf{\text{E}}^*_{\text{mes}}\) in kPa | \(\mathbf{\text{E}}^*_{\text{Stolz}}\) in kPa | \(\mathbf{\text{E}}^*_{\text{Chen}}\) in kPa |
|--------|-------|-----------------|-----------------|-----------------|
| Gel 0.75% | 0.047 | 1.0     | 19       | -               |
| Gel 2%   | 0.329 | 10.3    | 14       | 15              |
| Gel 2.5% | 0.575 | 28.4    | 22       | -               |
| Gel 3%   | 0.654 | 39.8    | 29       | 35              |

Table 2. Comparison of the dynamic elastic modulus of porcine cartilage computed using the Oliver and Pharr model (\(\mathbf{\text{E}}^*_{\text{mes}}\)) and those obtained by using the calibration curve (\(\mathbf{\text{E}}^*_{\text{cal}}\)).

| Sample | Figure | Site | Slope | \(\mathbf{\text{E}}^*_{\text{mes}}\) in MPa | \(\mathbf{\text{E}}^*_{\text{cal}}\) in MPa |
|--------|--------|------|-------|-----------------|-----------------|
| Femur  | 6      | A    | 0.948 | 0.391           | 0.386           |
|        |        | B    | 0.837 | 0.128           | 0.108           |
| Tibia  | 7      | A    | 1.079 | 0.289           | 0.287           |
|        |        | B    | 0.821 | 0.093           | 0.096           |
|        |        | C    | 0.708 | 0.052           | 0.051           |
| Patella| 8      | A    | 1.040 | 0.557           | 0.546           |
|        |        | B    | 1.140 | 1.140           | 0.171           |

Discussion

In the present study, the SFA has demonstrated its ability to quantitatively measure the mechanical properties of a plastic knee model and agarose gels as well as authentic porcine AC. However, some technical details of the instrument need to be improved and scrutinized to fulfill the requirements for use in an operating theatre. Moreover, special attention must be given to the design in terms of weight and shape to allow the surgeon ergonomic handling.

The values obtained during measurements on porcine AC were a factor 10 lower than those obtained ex-vivo by Stolz et al. (2009). This difference could be partially explained by the shape of the AFM tips used for the measurements. Stolz et al. (2004) demonstrated that the biomechanical properties of AC highly depend on the size and the shape of the indenter. Since large, non-elastic stains are induced when indenting with a sharp, pyramidal tip, the Hertz model (Hertz, 1882) is not appropriate for analyzing such indentation data. To compare our results with those obtained by Stolz et al. (2004, 2009) we need to replace the 10 \(\mu\)m high pyramidal tip of our cantilevers (Gautsch, 2002; Parrat, 2007) by spherical nanoscale tips as used by Stolz et al. (2004, 2009).

At the moment, no treatment is available for OA. Early detection and monitoring the progression of the disease in patients is a prerequisite for developing effective prophylactic therapies and drugs. Precise measurements of the cartilage functional properties will enable a rapid assessment of disease-modifying therapies in clinical trials as well as providing the surgeon with insights into whether a therapy is effective in the patient.

Tissue engineering strategies often employ biodegradable polymer scaffolds seeded with cartilage cells (chondrocytes) to promote the growth of cartilage in bioreactors suitable for surgical implantation and are able to repair cartilage defects in the joints (Langer and Vacanti, 1993; Marsano, 2006). Quantitative data may facilitate the design and production of engineered cartilage that exhibits biomechanical properties close to those of normal cartilage.

In conclusion, a direct, in situ inspection of AC morphology and biomechanical properties at the nanometer scale, may be used for the early detection of OA; it may also be used for the development of anti-OA drugs and to improve treatment strategies by permitting the surgeon to rapidly and reliably assess the health status of AC or control the intervention after cartilage transplantations. Moreover, as stated in the corresponding “News and Views”, by Aigner et al. (2009): “...integration of the AFM into an arthroscope — as a minimally invasive way to examine cartilage — would be a big step towards a ‘real life’ application. This will have an impact on diagnosis and patient management as well as leading to a better understanding of the disease.”

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