Nano-mechanical properties of starch and gluten biopolymers from atomic force microscopy

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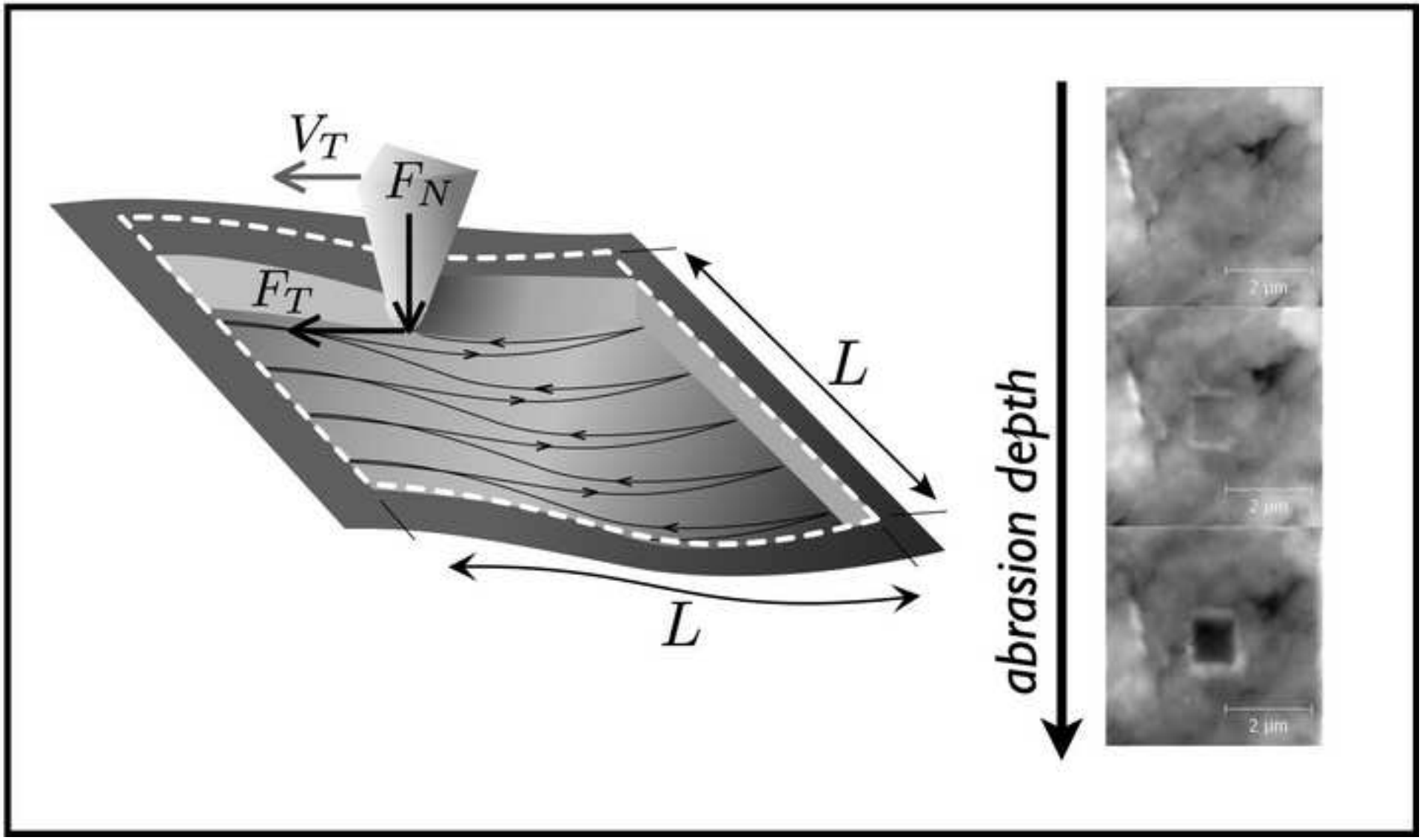
*Graphical Abstract (for review)



***Highlights (for review)**

Highlights

- Scratching with AFM tip was used to screen polymer resistance
- A tribological model was developed to determine polymer hardness
- The methodology is used to reveal hardness contrasts in biomaterials at nanoscale
- Wheat starch was found to display higher hardness and shear strength than gluten

Nano-mechanical properties of starch and gluten biopolymers from Atomic Force Microscopy

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Abstract

An original method based on atomic force microscopy (AFM) in contact mode was developed to abrade progressively the surface of tablets made of starch or gluten polymers isolated from wheat. The volume of the material removed by the tip was estimated from the analysis of successive topographic images of the surface, and the shear force was measured by keeping a constant normal force. Our data together with a simple tribological model provide clear evidence for a higher hardness and shear strength of starch compared to gluten. Gluten appears to have mechanical properties close to soft materials, such as talc, whereas starch displays higher hardness close to calcite. Our results are in a better agreement with structural properties of gluten (complex protein network) and starch (granular and semi-cristalline structure) than earlier studies by micro-indentation. This work shows that the AFM scratching method is relevant for the characterization of any polymer surface, in particular in application to materials made of different polymers at the nano-scale.

Keywords: AFM, Biopolymer, Hardness, Friction, Scratch test

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1. Introduction

Wheat is a major cereal crop for both food and non-food industries. The starchy endosperm, which is the main constituent of wheat grains (80-85%), contains two important biopolymers, which display unique rheological properties [1, 2, 3] : starch (80-90% of dry mass) in the form of granules which are embedded in a gluten matrix, mainly made of the storage proteins [4].

Food products, such as bread, biscuits or pasta, are made of flour and semolina which are obtained by the isolation of wheat grain endosperm and its reduction by dry fractionation in successive steps of grinding and sieving. In turn, further processing of flour and semolina can be undertaken to isolate starch and gluten biopolymers which are used in food industry, e.g. as additives to adjust food rheology, and in non-food applications to replace petroleum-based packaging materials [5, 6, 7], coating agents in paper industries [8, 9] or adhesives [10, 11] due to their renewability, physical properties and biodegradability. Purified starch is also employed as a starting material in petrochemical processes [9].

Therefore for a better control of the endosperm product quality, it is necessary to better characterize the fractionation behavior of the wheat endosperm which is clearly related to its structure and mechanical properties. The endosperm structure could be related to a granular cemented material and a numerical model was built in order to identify key factors, which could play a role in its mechanical behaviour [12, 13]. The model was based on actual knowledge of the endosperm structure and organization and took into account the described mechanical properties of starch and protein that were reported to be identical based on micro-indentation assays [14, 15]. The model showed that the fracture propagates differently depending on both the protein content and the starch/protein matrix adhesion. However, lack of information about the polymer mechanical properties at a nano-scale constitutes a major limitation for the modeling construction.

In this paper, we investigate the mechanical properties of isolated unmodified wheat starch and gluten with an original AFM nanoscratch approach. Since its invention [16], AFM has proved to be a powerful tool for topographical imaging at the nanoscale and with minimal sample preparation, of various biomolecules such as nucleic acids, proteins, polysaccharides, but also for the measurement of their mechanical properties [17, 18]. AFM has already been employed to characterize the surface topography of wheat starch and to compare its structure to starch isolated from other plant resources

[19, 20]. Similar topographic studies were also reported for some of the storage proteins forming the gluten network. Gliadins (α, ω) interactions in different solvent conditions [21] , as well as non-covalent interactions between glutenins of high molecular weight [22], have been investigated by means of AFM but with slight modifications of the molecules by either immobilisation or reduction and alkylation. Wheat endosperm was also tentatively observed using AFM to compare the endosperm structure in wheat grains differing by their hardness, but the method was unable to discern between starch and gluten polymers even if some differences in surface morphology were observed depending on the wheat genetic origin [23].

Indentation or scratching assays are generally used to probe the mechanical properties of different polymers from the nano to micro-scale [24, 25]. Recently, Kurland et al. [26] reviewed how AFM could be used for nanoindentation under native conditions to access the Young modulus of globular, fibrous and filamentous proteins. New developments have also been reported on the use of an AFM tip to abrade a target sample surface for the measurement of cohesive energy in a biological material [27]. Similar scratching tests on polymers by means of a nanoindenter were used for friction analysis [28] and determination of shear strength [29]. But, to our best knowledge, AFM has never been used as a tool to characterize the mechanical properties, i.e. hardness and shear stress of such biopolymers as starch and gluten. In the following, we first present our materials and AFM nanoscratch method. The data will then be analyzed for starch and gluten samples and used to determine their hardness and apparent friction coefficients. A simple tribological model will be used to approach the shear strength of both polymers. Finally, we conclude with the most salient findings and perspectives of this work.

2. Experimental

2.1. Materials

All commercial products, wheat starch (Fluka $N°85649$, $\leq$ 0.5% ash, 10.5% water content) and gluten biopolymer (Sigma$G5004$, 80% protein, 7% fat, 7.5% water content) were purchased from Sigma-Aldrich Co (St-Louis, MO, USA). Starch (98% purity, 11.5% moisture content) and gluten (65% purity, 9.5% moisture content) were also purified from wheat grains displaying distinct hardnesses (hard and soft common wheat cv. Glasgow and Dinosor, respectively) using a previously described method [30]. Polymethyl methacrylate (PMMA, Fluka 183350, $T_g = 124°C$, of average molecular

weight $1.2\,10^6$g/mol), was used as reference material with known mechanical properties at the nanoscale [31, 25, 32].

2.2. Sample preparation

Several tablets of commercial or extracted starch and gluten powders were prepared with approximate weight of 1 g in a pelletizer (Specac Inc, Smyrna, USA) by applying a pressure of 0.5 MPa during one minute using a laboratory press (Hydraulische Press, Perkin-Elmer, USA). Tablets were stored before analysis under controlled conditions of temperature and humidity ($20°C$, 30% RH).

2.3. Microscopy

Environmental Scanning Electron Microscopy (ESEM, Fei Quanta 200 FEG, FEI Co, Hillsboro, OR, USA) without sputter coating was used to check the homogeneity and absence of defaults in the samples as well as for imaging AFM tips before and after abrasion.

2.4. AFM assays

AFM assays were performed with a Nanoscope V atomic force microscope (Bruker instruments, Madisson, WI, USA), operating in the contact mode under controlled conditions of temperature and humidity ($20°C$, 30% relative humidity). Commercial Si_3N_4 tips (Bruker) mounted on a rectangular cantilever with stiffness in the range between 1 and 5 N/m were chosen to preserve reasonable measurement sensitivity and to exert sufficiently large forces to abrade the samples. Before each measurement, the normal and tangential forces F_N and F_T, respectively, were calibrated by means of a hard silicon wafer in order to convert the values measured in volts to force units.

The calibration of the normal force was performed through displacement-force plots whereas vertical cantilever stiffness measurements were calibrated by means of the thermal fluctuation method [33]. The friction forces were calibrated using Coulomb's friction law and the value of the friction coefficient for the silicon wafer was fixed ($\mu_{Si-Si} = 0.1$) according to the studies of Morton et al. [34]. Since AFM calibration was made on hard silicon wafers, the tips are expected to be partially flattened. Several ESEM observations were made, as illustrated in Figure 1a, in order to check the tip geometry before and after calibration. The contacting areas of the tips were characterized through reverse imaging obtained with AFM (Figure 1b) on a calibrating

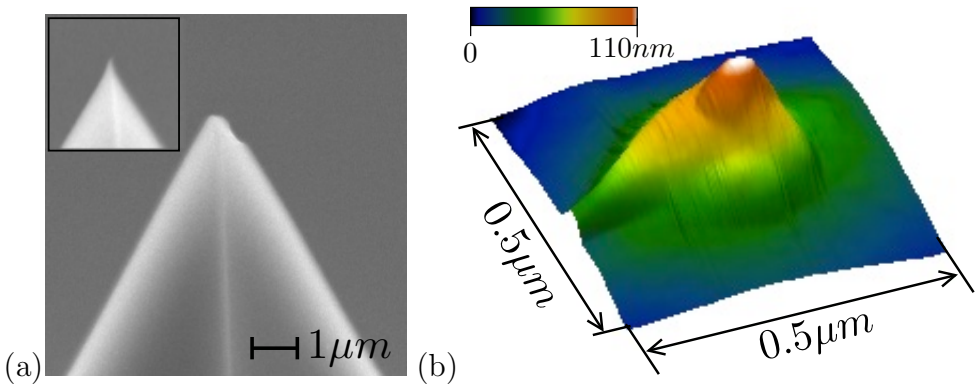

Figure 1: a) ESEM pictures of an AFM tip before (insert) and after calibration; b) Picture in false colors of an AFM tip obtained by mirror imaging with a calibrating grid.

grid of equally-spaced sharp points of apex radius $\simeq 10$ nm (TGT01, Mikromasch, Inc., Estonia). These measurements clearly showed that the AFM tip apex can be well fitted after calibration by a sphere from the extremity to 20 nm high, with an average radius for the set of tips $R = 82 \pm 32$ nm.

The AFM assays were conducted, as schematized in Figure 2a, by following a procedure inspired by a previously described method [27]. It consists of successive steps of topographic image acquisition on a large scale and abrasive scans on a predefined area by setting the force applied on the AFM cantilever to the desired value. First, a large ($L \times L > 10 \times 10$ μm^2) topographic image is acquired as the tip scans the sample surface at a low applied normal force ($F_N = 100nN$) in order to select the appropriate working area for polymer abrasion, i.e. the center of a starch granule or a homogeneous gluten area. Then, a smaller topographic image ($5 \times 5\mu m^2$) at a scan tip velocity V_T of 10 μm/s (512×512 pixels) is acquired (step 1) inside the selected area in identical conditions, that will serve as reference image of the undamaged surface.

The abrasion process (step 2) is initiated on the central area ($L \times L = 1 \times 1$ μm^2) with an increase of the applied normal force ($F_N > 200$ nN) and a decrease of the scan velocity ($V_T = 2$ μm/s, 256×256 pixels). Both the trace and retrace F_T force maps are acquired (respectively scanning from left side to right side of the image and from right side to left side) to determine the average force sustained by the sample in the direction of displacement. Thereafter, the normal force is decreased back (step 3) to its initial value (100 nN) and a second topographic image (5×5 μ m^2) is recorded at $V_T = 10$

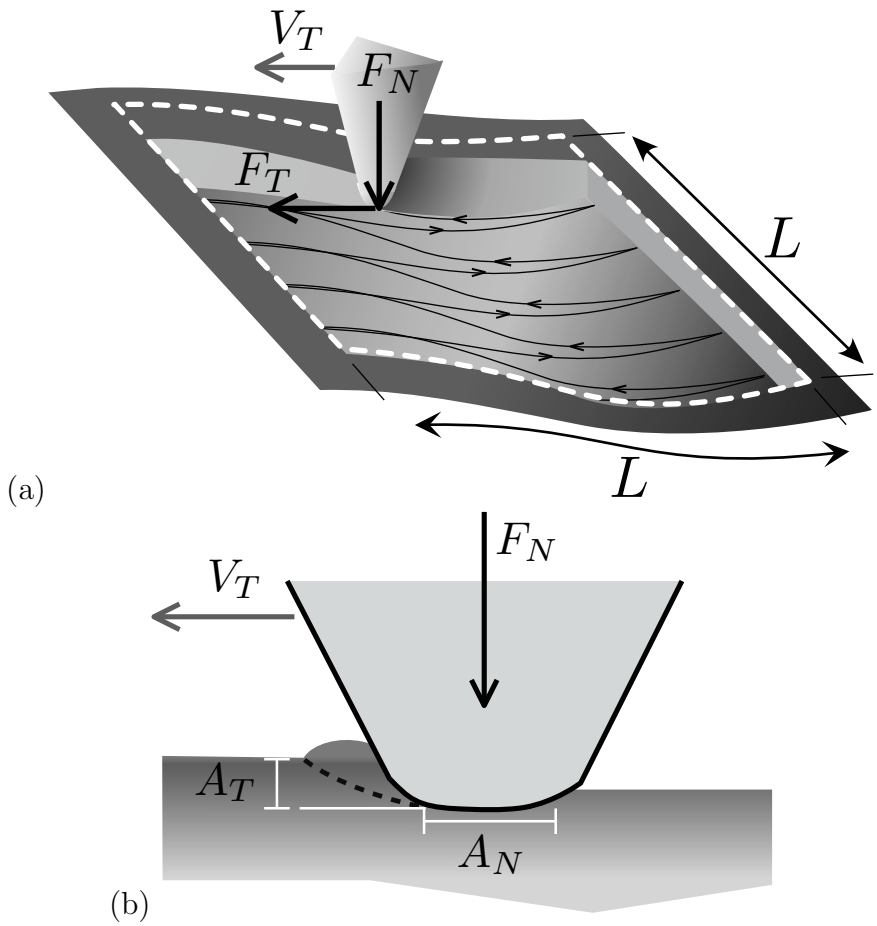

Figure 2: a) Schematic representation of the AFM procedure where F_N is the applied normal force, V_T is scan tip velocity, F_T is friction force and L is the length of the abraded area; b) Schematic description of the abrasion zone where A_N is the projected contact area of the tip on the sample surface and A_T is the projected area in front of the tip in the direction of displacement.

μm/s before increasing again the normal force to further abrade the material. A progressive and controlled abrasion of the polymer sample was ensured by repeating up to ten times the abrasion step (step 2), interrupted by regular acquisitions of larger topographic images (step 1) after $N = 1, 4, 7$ and 10 abrasive scans, respectively. This abrasion process was undertaken at least on ten distinct independent locations for each analysed polymer. The acquired AFM images were visualized and analyzed by means of the software Gwyddion 2.26 (Department of Nanometrology, Czech Metrology Institute, Brno, CZ) in order to evaluate the abrasion depth and friction force F_T.

2.5. Nano-indentation assays

Nano-indentation assays were performed using a diamond Berkovich indenter (CSM Instruments, Switzerland, ultranano indentation tester) at an angle of 141.9°. In the first step, the indenter is placed on the sample surface by a rough approach at a speed of 2000 nm/min until a contact force (set to 15 μ N) is detected. Then, force-displacement curves are acquired under imposed linear load/unload conditions. For gluten and starch, the maximum load was set to 50 μN, the loading/unloading rate to 25 μN/min and the pause time between loading and unloading to 20 s. These parameter values were chosen to be as small as possible in order to avoid the sliding of the indenter. For bulk PMMA, these parameters were respectively set to 100 μN, 50 μN/min and 30 s for most accurate measurements. The hardness H is defined as the ratio between the maximum force F_{max} just before unloading and the projected contact area A_N determined by the tip geometry. The indentation assays ($n = 20$) were performed by displacements of 10 μm.

3. Results and Discussion

3.1. Evolution of abrasion depths

The AFM abrasion tests were performed on tablets prepared by powder compression to avoid slipping of the starch granule or the protein polymer along abrasion and also to avoid resin inclusion which may interact with the analyzed material and influence their mechanical properties. Due to potential variability of the polymers, reflecting their wheat origin or isolation method, the tablets were made of either commercially purchased purified starch and gluten or were extracted in the laboratory from wheat grains of different genetic background. The abrasion experiments were performed by a methodology derived from that of Ahimou et al. [27]. The AFM tip was

placed on a homogeneous gluten area or inside a starch granule, as shown in Figure 3 (a-b, a'-b'), and a square of 1 μm^2 area was scraped. The abrasion area was analyzed by means of 5×5 μm^2 topographic images taken before and after N abrasion scans. As shown in Figure 3 (c and c'), the abrasion depth in gluten is higher than in starch for a similar applied normal force (F_N = 480 nN), which indicates a higher resistance to abrasion of starch compared to gluten.

Two different methods were used to measure the volume of the removed material. In fact, the potential lateral drift between successive acquired images and the roughness of the scanned area makes it difficult to determine the total depth of the abraded area after ten abrasive scans. We used the differences between topographic images to measure the depth along the abrasion path. The depth was obtained by averaging only in the central part of the path as the removed material is pushed to the edges. Therefore, the depth reached after N abrasion scans was calculated either as the difference between each topographic image and the initial image (before abrasion) or by cumulating the differences between successive images.

The results of the two methods for depth measurement are presented in Figure 4 for starch and gluten as well as an example of image subtraction. Values obtained by both methods almost coincide. However, the error in the first method (subtraction of each topographic image from the initial image) remains around 10% for the total depth after ten abrasive scans, which is below the 30% error in the second method. A linear increase of the depth was observed at the rates of 11.3 ± 4 nm and 1.57 ± 0.9 nm per abrasive scan for gluten and starch, respectively. Increasing the applied normal force from F_N = 480 nN to F_N = 2600 nN led to an increase of the depth after ten abrasive scans to 111 ± 28 nm and 15 ± 4 nm for gluten and starch, respectively.

The above depth data show clearly that gluten and starch have very different mechanical properties irrespective of their genetic origin. This observation is in strong contrast with earlier studies [14, 15]. It is also important to note that, as the abrasion depth and removed volumes are independent of the scan number, the mechanical behavior in the samples may be considered to be homogeneous.

3.2. Hardness starch and gluten determination

Due to the employed methodology, the abrasion process by the AFM tip can be interpreted as a linear scratching test usually performed to test the

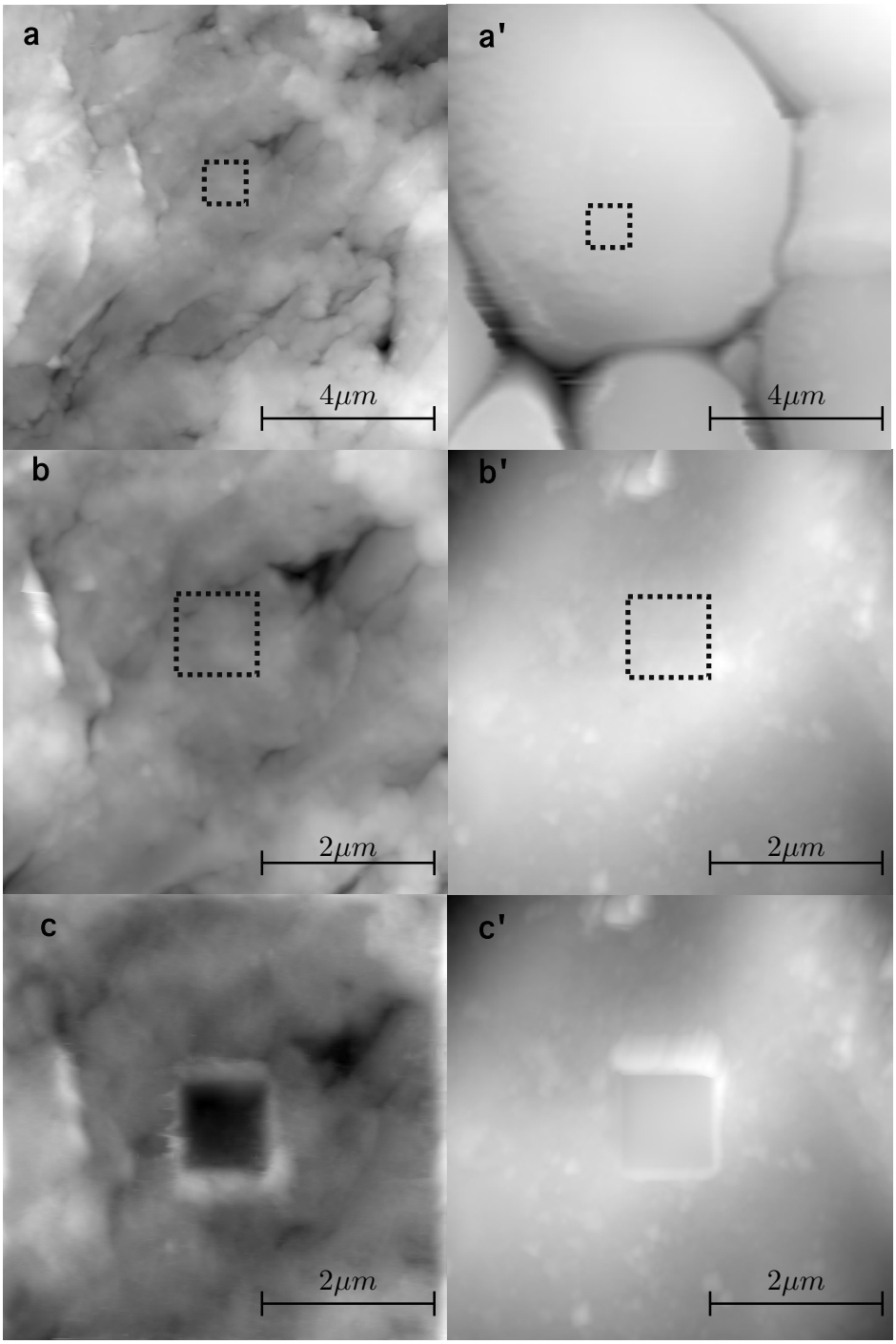

Figure 3: Examples of gluten (a-c) and starch (a'-c') AFM topographic images taken before abrasion at 10×10 μm^2 (a, a') and 5×5 μm^2 (b, b') and after 10 abrasive scans (c, c') with an applied normal force of 480 nN. The abrasion zone is marked into the figure with a square

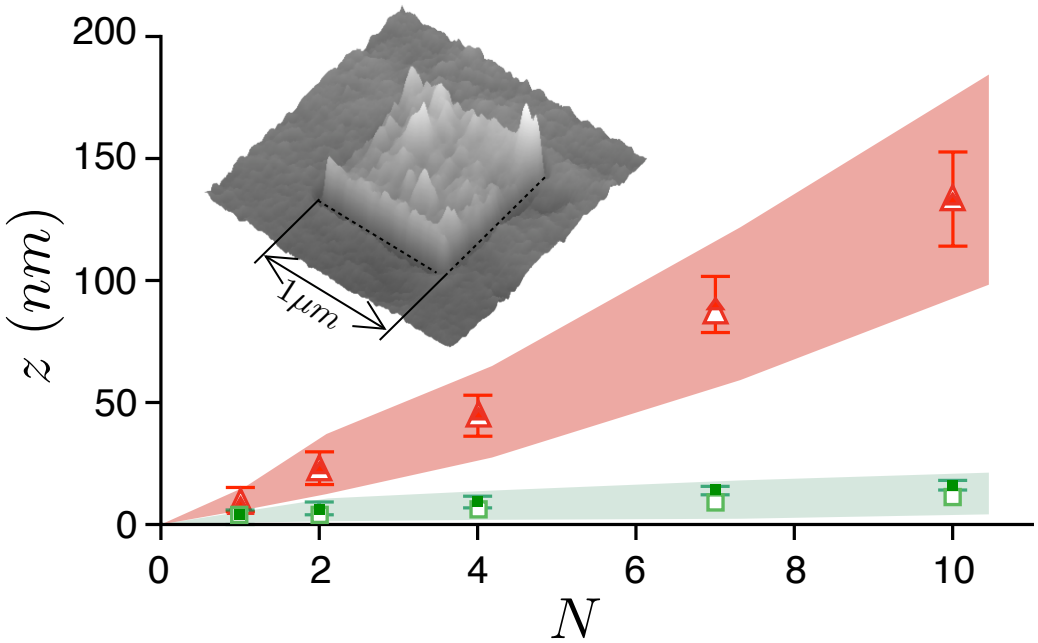

Figure 4: Illustration of different methods of depth (z) measurement as a function of the abrasive scan number (N) in gluten (red triangles) and starch (green squares) subjected to a normal force $F_N = 480$ nN. For each biopolymer, the filled symbols and error bars correspond to the total difference between the scratched area and the initial topographic image whereas the empty symbols correspond to the cumulate of differences between successive images. The colored area corresponds to the maximum error obtained with this last method. The insert shows an example of a 3D image after substraction between two images.

resistance of materials. Considering the trace and retrace tip displacement over the abraded 1 μm^2 square area, the volume V of removed polymer after N iterated abrasive scans may be expressed as

$$V = 2n_\ell NLA_T \tag{1}$$

where n_ℓ is the number of scan lines in the acquired topographic image (256 scanning lines), N is the number of scans, L is the length of the abraded area (1 μm) and A_T is the projected frontal area in contact with the tip as schematized in Figure 2b. The validity of the above expression was checked on PMMA by measuring the removed volume as a function of n_ℓ, which was changed from 128 to 1024 scanning lines (data not shown). As expected for a linear scratching test, an increase of the removed volume was observed with the number of scanning lines. Therefore, the cohesive energy determined by [27] from the measured volume does not reflect only the intrinsic properties of the analysed material although the data may still be sufficient to determine mechanical properties such as the hardness.

The measurement of hardness requires the geometry of the indenter. The apex radius of the AFM tip was given by the manufacturer to be below 10 nm but the tip wears off with calibration and reached a steady radius R value (see experimental section) that remains stable during probing process. With this spherical tip apex, the projected contact area A_N can be related to the frontal area A_T by the following relation:

$$A_N = \pi(2RA_T)^{2/3} \tag{2}$$

By definition, the hardness H of a material is the ratio between the applied normal force F_N and the area A_N under the tip (Figure 3b). Hence, we get the following relation between H and F_N:

$$F_N = H\pi(2RA_T)^{2/3} \tag{3}$$

The data points for A_T for different values of F_N are plotted for starch and gluten, as well as for PMMA, in Figure 5. The data are correctly fitted by Eq. (3) allowing for the determination of hardness H for each polymer (Table 1).

The measured hardness of PMMA is consistent with previous data obtained with distinct approaches yielding a value between 0.3 GPa and 0.6 GPa [31, 25, 32]. The hardness of gluten is found to be of the same order of

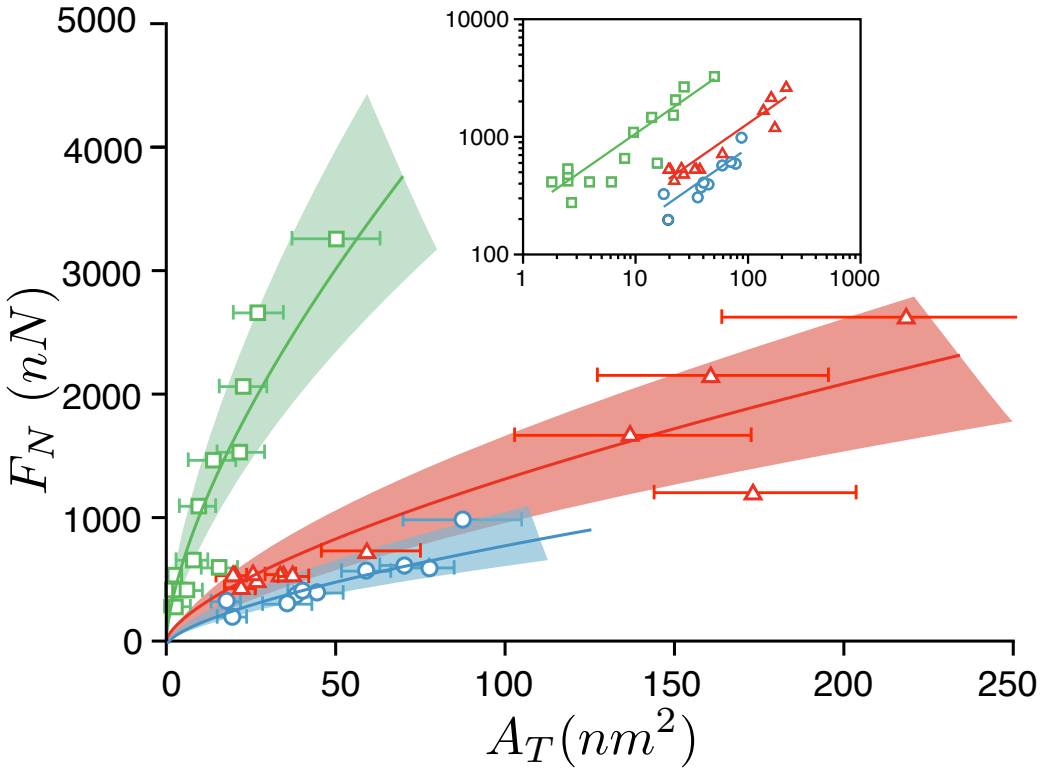

Figure 5: AFM data of the projected area A_T in front of the tip as a function of the normal force F_N for starch (green squares), gluten (red triangles) and PMMA (blue circles). Error bars on A_T represent measurement errors on the depth z and thus on the removed polymer volume V during abrasion. The full lines represent the predicted behavior by equation (3). The colored area around the fitting curve represents the error resulting from the tip radii variability. Insert: log-log representation of the same data.

magnitude as PMMA but four times lower than that of starch. The hardness of gluten at the nanoscale is thus close to that of soft materials, such as talc [35], and similar to other biopolymers such as wheat straw or other crop stalks [36], whereas the starch hardness is closer to that of calcite [35] and the values measured for core shells [37].

The observed hardness for starch and gluten is in a better agreement with the differences in the structure of the two biopolymers than earlier evaluations by micro-indentation [14, 15]. In fact, the gluten is characterized by a complex protein network [39] whereas the starch has a granular and semi-cristalline structure [43] . This discrepancy between our results at the nano-scale and those of earlier measurements by micro-indentation may be due to the scale of measurement or the orders of magnitude of the applied forces, as already pointed out in the literature [35, 24, 37]. In fact, a close look at the previous results obtained for wheat grains [15] or for purified starch and gluten dispersed in a polyester resin [14] indicate that the indentation depth was generally above 10 μm for applied forces of several mN. Therefore, at this resolution, the polymers in wheat grains are difficult to distinguish, and hence the measurement reflects in practice the hardness of the softer polymer. Furthermore, in Barlow et al. [14], the purified isolated polymers were included in a resin which was prone to modify the polymer properties. Indeed, the measured hardness of a single component in a composite material was found to be highly dependent on the dimensions of indenter [38].

Table 1: Hardness H and apparent friction coefficient μ_{app} obtained by AFM for gluten, starch and PMMA.

	H (MPa)	μ_{app}
Gluten	640 ± 170	0.39 ± 0.05
Starch	2400 ± 600	0.32 ± 0.05
PMMA	400 ± 100	0.59 ± 0.05

The mechanical properties of PMMA, as a bulk material, as obtained with AFM were also compared with the data obtained by indentation at the nanoscale. The nanoindentation assays confirmed the PMMA hardness even with a better accuracy at 420 ± 30 MPa, which is similar to those reported in previous studies [31, 32]. But gluten and starch, which respectively display a complex protein network and a granular structure [39, 3] were more difficult to explore. A number of indentation tests on these two polymers had to be discarded due to the sliding of the indentor during the assay. However, the

data obtained confirmed the difference between the two biopolymers with a hardness between 400 and 760 MPa for gluten and between 1000 and 2600 MPa for starch. Nevertheless, we observed a higher variability of the measurements for this type of polymers in nanoindentation assays.

3.3. Shear strength determination

The friction coefficient μ_{app} at the interface between two solid bodies at the nanoscale can be evaluated from the friction force F_T, recorded during AFM nano-scratching test, and the applied normal force F_N [40, 25]:

$$\mu_{app} = \frac{F_T}{F_N} \tag{4}$$

Figure 6 shows the mean value of F_T versus F_N for each polymer. The data collapse on a straight line passing through the origin. The apparent friction coefficient μ_{app} is given by the slope and its values are presented in Table 1. The measured values of μ_{app} are quite close for starch and gluten and about 1.5 to 2 times below that of PMMA[2].

It is worth noting that this apparent friction coefficient μ_{app} measured at the nanoscale can not be directly interpreted as Amonton's friction coefficient measured at the macroscopic scale, where multi-asperity contact is assumed [29]. In fact, in scratching of a soft material at the nanoscale, the apparent friction occurs at a single asperity from the addition of two effects:

- Interfacial shear in a small layer of the material;
- Visco-elastoplastic flow of material around the scratching tip.

In all models described in the literature, the interfacial shear was characterized by adhesion or a real friction coefficient μ_{true}, which is of special interest as it is linked to the mechanical hardness through equation:

$$\mu_{true} = \frac{\tau}{H} \tag{5}$$

[2]The vanishing of the friction force with normal force means that F_N is the real contact reaction force including both the compressive force exerted vertically on the tip and the cohesive van der Waals force exerted by the surface on the AFM tip. This is because the reference state for the deflection of the cantilever is the force-free state, so that the deflection of the cantilever is the resultant of both the attraction force and compressive forces acting at the tip.

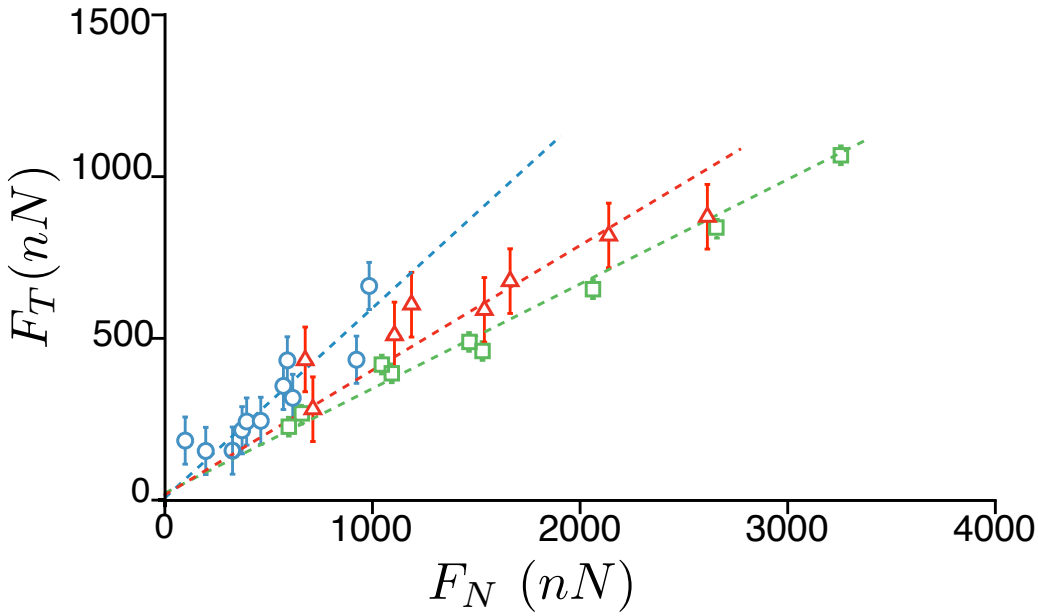

Figure 6: Friction force F_T plotted against normal force F_N for starch (green squares), gluten (red triangles) and PMMA (blue circles). The error bars represent the standard deviation of F_T for ten independent abrasive scans. The data are fitted by a straight line crossing the origin.

where τ is the shear strength of the material. The measured apparent coefficient is thus comprised between the real friction coefficient and a higher value [41], which depends on the behavior of the scratched material under experimental conditions (applied forces, tip geometry, strain velocity, temperature).

If the polymers were totally elastic, we would have $\mu_{app} = \mu_{true}$ and the shear strength could be estimated by equation (5). In this case, the values of the shear strength τ for gluten, starch and PMMA would be equal to 250 MPa, 768 MPa and 236 MPa, respectively. However, during the test, the polymers are not in the elastic domain because of scratching and plastic deformation leaving a track as observed in Figure 3, c-c'. Therefore, the apparent friction coefficient μ_{app} should be modified by subtracting the effect of the front created ahead of the moving tip with a ploughing coefficient μ_{plough}:

$$\mu_{true} = \mu_{app} - \mu_{plough} \tag{6}$$

The coefficient μ_{plough} may be evaluated from the rear contact angle ω in front of the tip along the scratching assay and the radius of contact determined by in-situ measurement on a homogeneous and transparent material [29]. However, in our AFM conditions, the measurement of those parameters were not possible. But for PMMA it has a value between 0.1 and 0.2 according to previously reported data [41, 42]. Taking into account the ploughing correction, μ_{true} is found to be comprised between 0.39 and 0.49 ± 0.05, and thus the shear strength τ is found to be between 156 ± 59 MPa and 196 ± 68 MPa. These values of μ_{true} and τ are consistent with the previous scratching studies developed on PMMA using similar conditions of temperature, strain rate and contact pressure but different method of mesurement [29].

Assuming that gluten and starch have a similar elasto-plastic behavior, we may use the same value of μ_{plough} in equation (6) to estimate μ_{true} for starch and gluten. The resulting shear strength is then comprised between 122 ± 64 MPa and 185 ± 80 MPa for gluten, thus close to that of PMMA, and between 288 ± 192 MPa and 528 ± 248 MPa for starch. In all cases, starch shows two to three times more strength than gluten in scratching tests, supporting once more the difference in mechanical behavior of these two biopolymers as it was already discussed with hardness measurements.

4. Conclusions

In this paper, AFM scratching assays were performed with two important biopolymers, starch and gluten. Our findings reveal a higher resistance to fracture and a less friction coefficient for starch compared to gluten, the later being closer to our reference material PMMA. These data will serve to refine a numerical model of the starchy endosperm fractionation process. Given the broad use of these two biopolymers in food and non-food products, the described method also appears helpful in order to further explore the mechanical properties of starch and gluten in a wide range of conditions of temperature, relative humidity and stresses. In contrast with nanoindentation, this AFM scratching assay also allows to map the local mechanical properties and assess the potential heterogeneity of the material at a nanoscale level. This method thus appears an interesting alternative to characterize any type of polymers. It also opens the way to determine the mechanical properties of each of the components in a composite material and possibly the polymer interface.

5. Acknowledgement

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