

Technical note

Nanoindentation and storage of teeth

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Abstract

This study determined changes in nanomechanical properties of dentin and enamel during storage in deionized water, calcium chloride buffered saline solution and Hank's balanced salts solution (HBSS). Atomic force microscopy based nanoindentation showed that storing teeth in deionized water or CaCl₂-solution resulted in a large decrease in elastic modulus and hardness. At 1 day a decrease in the mechanical properties values of up to 20% and 30% was observed for enamel and dentin, respectively. After 1 week, mechanical properties dropped below 50% of their starting values, which is attributed to a demineralization process during storage. In contrast, storing teeth in HBSS did not significantly alter the mechanical properties for a time interval of 2 weeks. The use of HBSS for storage of samples from teeth is recommended. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Teeth; Nanoindentation; Atomic force microscopy; Mineral dissolution; Storage

1. Introduction

Nanoindentation has become a common technique for the determination of local mechanical properties of structural features in biological hard tissues (Habelitz et al., 2001; Kinney et al., 1996; Rho et al., 1997; Zysset et al., 1999). Although nanoindentations only examine a thin surface layer (<1 µm), the mechanical properties obtained are assumed to be representative of the bulk material. Any change in the surface layer of mineralized tissues resulting from storage solutions is an important consideration for accurate determination of nanomechanical properties.

Dentin and enamel are highly mineralized tissues. Enamel is about 85 vol% mineralized and the hardest tissue in the human body (Ten Cate, 1994). Its unique microstructure consists of keyhole shaped rods, which are aligned parallel and run from the dentino–enamel junction (DEJ) towards the surface of the tooth. Each rod (4–5 µm diameter) consists of protein-covered carbonated apatite fibers of 30–80 nm diameter. The fiber alignment within the rod results in anisotropy of

mechanical properties (Habelitz et al., 2001; Katz and Ukraincik, 1971; Xu et al., 1998). Dentin is a complex hydrated biological composite containing approximately 50 vol% mineral, 30 vol% organic components (mostly type I collagen) and 20 vol% fluids. Its distinct microstructure is characterized by tubules (1–2 µm diameter) that run from the DEJ towards the pulp (Marshall et al., 1997; Ten Cate, 1994). A 1 µm thick layer of higher mineralization, the peritubular dentin, surrounds each tubule, and has different properties than the intertubular dentin.

Tooth samples prepared for mechanical testing are usually stored in an aqueous solution to maintain hydration. For nanomechanical tests, storage of samples can become a critical issue since chemical reactions such as etching and dissolution may affect the surface layer. Mechanical properties of calcified tissues are sensitive to their mineral content (Currey and Brear, 1990; Gustafson et al., 1996; Lee and Lin, 1997). Gustafson et al. (1996) studied the effect of CaCl₂-solutions on the elastic modulus by bending tests and recommended the use of a CaCl₂-concentration of 57.5 mg/l, which did not affect the elastic response of bone.

This study is based on the hypothesis that nanomechanical properties of tooth specimens can be affected

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by storage in aqueous solutions. We used nanoindentation to determine the reduced elastic modulus and hardness of dentin and enamel when stored in deionized water, CaCl_2 -buffered saline solution and Hank's buffered salts solution (HBSS) for times of 0, 1, 7 and 14 days.

2. Materials and methods

Human third molars with documented history were extracted according to protocols approved by the UCSF Committee on Human Research. Teeth were sterilized by low dose gamma radiation, which did not affect their mechanical properties (Currey et al., 1997), and stored in deionized water at 4°C until prepared (White et al., 1994). Three teeth were sectioned longitudinally into three slices (one for each storage solution), ground and polished with water-based diamond suspensions to $0.25\ \mu\text{m}$. Samples were cleaned ultrasonically for 10 s. Nanoindentation was applied immediately after final surface preparation. Specimens were subsequently immersed in 30 ml of various storage solutions: (1) water, deionized by a water purifier (Millipore), (2) normal saline solution containing 900 mg/l NaCl buffered with 57.5 mg/l CaCl_2 (CaCl_2 -solution recommended by Gustafson et al., 1996) and (3) HBSS (UCSF cell culture facility; 400 mg/l KCl, 60 mg/l KH_2PO_4 , 8000 mg/l NaCl, 1000 mg/l glucose, 90 mg/l $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 350 mg/l NaHCO_3 , 140 mg/l CaCl_2 , 100 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 100 mg/l $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$). Specimens were studied by atomic force microscopy (AFM; Nanoscope III, Digital Instruments, Santa Barbara, CA) with the standard head replaced by a Triboscope indenter system (Hysitron Inc., Minneapolis, MN), as described elsewhere (Balooch et al., 1998). A cube corner diamond indenter ($r \approx 20\ \text{nm}$), calibrated on fused quartz, was used for indentation and imaging. Indentation loads of 1500 and $750\ \mu\text{N}$ were applied to polished enamel and dentin surfaces, respectively, resulting in indentation depths between 300 and 400 nm. Loads on stored specimens were decreased if the specimen was affected by the solution to maintain the indentation depth approximately constant. A minimum of 12 indentations was performed on enamel and dentin of each specimen in ambient air. On dentin, indentations were placed exclusively on the intertubular dentin. Indentations on enamel were made without respect to rod orientation and location. Load–displacement data were analyzed according to the method of Oliver and Pharr (1992). Repeated measure ANOVA was used to determine significant differences in hardness and reduced elastic modulus at storage times of 1, 7 and 14 days with a confidence level of $p < 0.05$. The pH of the solutions was measured with a pH-meter (Accumet 1003, Fisher Scientific, USA).

3. Results

Storage of the specimens in deionized water or CaCl_2 -buffered saline solution significantly altered the elastic modulus of enamel, while storage in HBSS did not (Fig. 1a). The reduced elastic modulus of human dental enamel had average values between $74 (\pm 4.0)$ and $80 (\pm 9.1)$ GPa prior to storage. Storage in deionized water caused the hardness of enamel to decrease by about 10% within 1 day ($p > 0.05$). The hardness was significantly different after 7 days and as low as 25 GPa after 14 days. Storage of enamel in the CaCl_2 -solution affected the elastic modulus similar to deionized water. Within 2 weeks the elastic modulus decreased to about 35% of

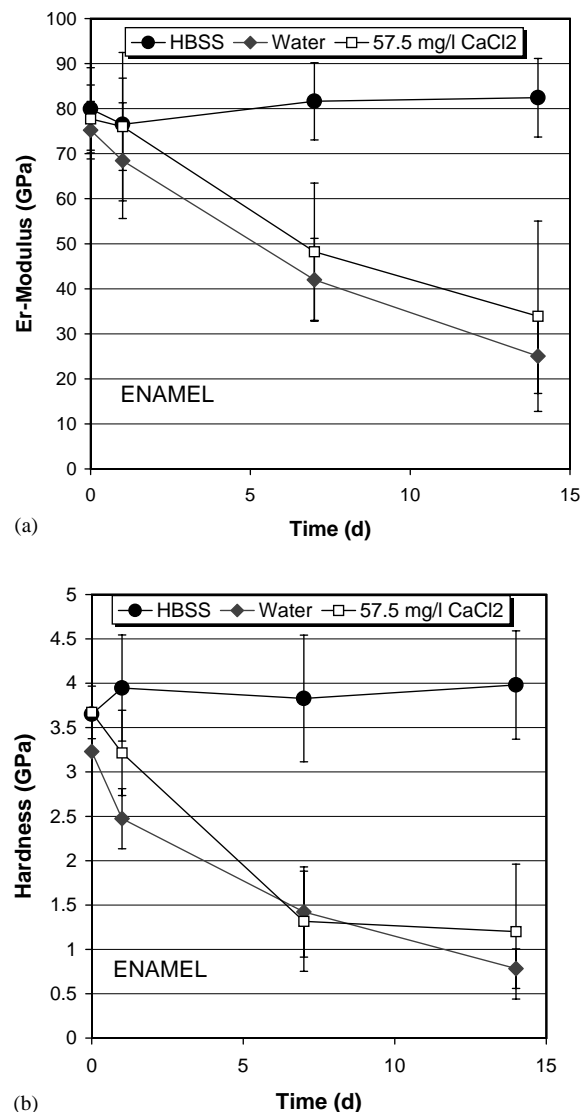


Fig. 1. (a) Reduced elastic modulus of human enamel versus time stored in HBSS, CaCl_2 -solution and deionized water (error bars are standard deviations); (b) nanohardness of human enamel versus time stored in HBSS, CaCl_2 -solution and deionized water (error bars are standard deviations).

the starting value. In contrast, storing enamel in HBSS did not significantly alter its elastic response. Within the two weeks of storage a non-significant ($p > 0.05$) increase from 79 to 82 GPa was observed.

Storage of the specimens in deionized water or CaCl_2 -buffered saline solution also significantly altered the hardness of human enamel, while storage in HBSS did not (Fig. 1b). The baseline hardness of enamel had average values between $3.2 (\pm 0.4)$ and $3.7 (\pm 0.5)$ GPa prior to storage. When stored in deionized water, the hardness decreased significantly, by about 24%, within 1 day and was as low as 0.8 GPa after 14 days. Storage in the CaCl_2 -solution also resulted in a decreased hardness of enamel and was as low as 1.2 GPa after 2 weeks. In contrast, hardness of enamel did not change significantly ($3.7\text{--}4.0$ GPa) within 2 weeks of storage in HBSS.

Similar results were found for dentin. Storage in deionized water or CaCl_2 -buffered saline solution significantly altered its elastic modulus, while storage in HBSS did not induce significant changes (Fig. 2a). The reduced elastic modulus of human dentin had average values between $22.8 (\pm 2.5)$ and $24.5 (\pm 1.5)$ GPa prior to storage. If stored in deionized water, the modulus dropped by 15% after 1 day, which was a significant change. It decreased to 5 GPa after 14 days. Storage of dentin in the CaCl_2 -solution resulted in a significant drop of the elastic modulus to about 18.5 GPa after 1 day and continued to fall to 40% after 14 days. Storing the specimen in HBSS, however, did not significantly alter the elastic response of dentin.

Storage of the specimens in deionized water or CaCl_2 -buffered saline solution significantly altered the hardness of dentin, while storage in HBSS did not (Fig. 2b). Dentin had average hardness values between $1.0 (\pm 0.1)$ and $1.1 (\pm 0.1)$ GPa prior to storage. When stored in deionized water, the hardness decreased significantly by about 25% within 1 day of storage and was as low as 0.35 GPa after 14 days. Storage in the CaCl_2 -solution had a similar effect and resulted in a decrease of the hardness to 0.38 GPa with 14 days. Storing dentin in HBSS, however, did not significantly alter its hardness over time. The pH values of the storage solutions were 6.5, 5.9 and 8.0 for deionized water, CaCl_2 -solution and HBSS, respectively.

4. Discussion

The experiments support the hypothesis that storage of dental tissues can alter their mechanical response when nanoindentation is used. To relate properties measured by nanoindentations to the bulk material, the surface layer must not be altered by storage. In this study, we observed that dentin and enamel showed major changes in their elastic–plastic response when exposed to deionized water or CaCl_2 -solution. The

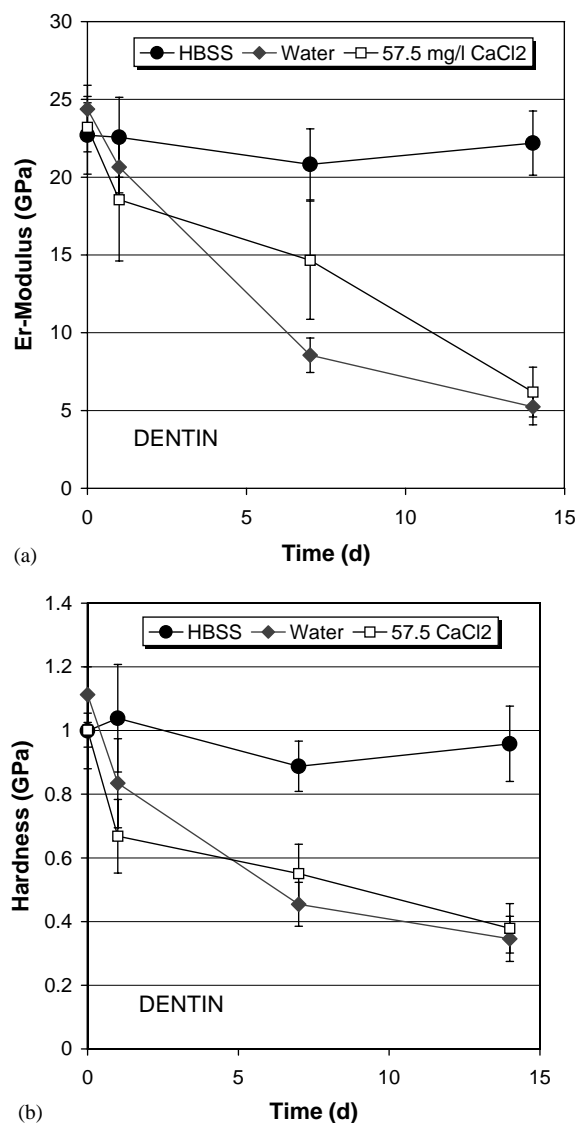


Fig. 2. (a) Reduced elastic modulus of human dentin versus time stored in HBSS, CaCl_2 -solution and deionized water (error bars are standard deviations); (b) nanohardness of human dentin versus time stored in HBSS, CaCl_2 -solution and deionized water (error bars are standard deviations).

reduced elastic modulus and hardness of all specimens decreased within 1 day of storage in either deionized water or CaCl_2 -solution. After 2 weeks in deionized water or CaCl_2 -solution, hardness and elastic modulus of all specimens was decreased by at least 50%. In contrast, storage of dentin and enamel in HBSS for up to 2 weeks did not induce significant changes to the mechanical behavior of the tissues. The observation by Gustafson et al. (1996) that a saline solution with 57.6 mg/l of CaCl_2 maintains the elastic modulus of bone is not supported by this study for dentin, but might relate to the different tissues and method used.

The decrease in modulus and hardness of dentin and enamel when stored in deionized water or CaCl_2 -solution is due to demineralization. Deionized water

had a pH of 6.5 and its effect on demineralization by acidic etching is negligible. Moreover, deionized water lacks calcium and phosphate ions and therefore the chemical potential for dissolution of the mineral phase of dentin and enamel is high and is assumed to be the major reason for demineralizing and softening of the tissues. Addition of calcium chloride to a saline solution did not prevent demineralization. This might be due to the fact that the addition of calcium ions to the aqueous solution does not compensate for the absence of phosphate ions, the other major component of calcified tissues. Thus, the chemical potential for dissolution remains high. Furthermore, the pH of the CaCl_2 -solution was slightly more acidic (pH=5.9) and thus more likely to dissolve the calcium phosphate minerals. HBSS is slightly basic (pH=8.0). It is highly concentrated in Ca^{2+} , Mg^{2+} , Na^+ , PO_4^{3-} and Cl^- ions and has a composition comparable to the dental mineral phases. Therefore, the chemical potential of HBSS to dissolve the calcium phosphate phases in teeth is low and surface demineralization is prevented.

According to this study, human enamel of third molars shows an average reduced elastic modulus and hardness of 77 (± 10.1) and 3.5 (± 0.5) GPa, respectively, prior to storage. Human dentin of third molars had an average reduced elastic modulus and hardness of 23.7 (± 2.6) and 1.0 (± 0.15) GPa, respectively, prior to storage. These values are in good agreement with most of the data in the literature, where nanoindentation was applied to dental tissues in ambient atmosphere (Fong et al., 2000; Habelitz et al., 2001; Marshall et al., 2001; Willems et al., 1993). In order to maintain the physical properties of dental tissues we recommend short-term storage of the specimens in HBSS.

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