Supporting Information

Ferumoxytol nanoparticles target biofilms causing tooth decay in the human mouth

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Experimental Section

Study design

The purpose of this study was to evaluate effects of a clinically translatable approach to control cariogenic biofilm development and enamel demineralization using an FDA-approved iron oxide nanoparticle formulation (FerIONP). This study was approved by the Indiana University Institutional Review Board (IRB#1802077294). In this randomized, crossover, single-blinded *in situ* study, we recruited 16 subjects (Indiana University IRB #1407637739) who met the study requirements for wearing customized lower partial denture-type appliance. We used lower partial denture because 1) it provides consistent sites with sufficient area for placement of the enamel specimen holder assemblies, and 2) it is ideal for modeling dental caries with similar salivary clearance pattern of caries prone areas of the mouth, while avoiding direct contact with the tongue. Furthermore, based on our experience working with both palatal and partial denture caries models, there is much better subject compliance with wearing an appliance that is replacing function (missing teeth), especially over a 14-day study period. Moreover, the lower partial denture also does not interfere with normal speech pattern and is more comfortable. All subjects provided informed consent and agreed to use assigned products according to instructions. The participants were randomized into 3 treatment groups and the sequence of the three treatments was also randomized for each subject (Figure 1C): (1) Control: water (A) + water (B), (2) FerIONP/H_2O_2: 1.5% FerIONP (A) + 3% H_2O_2 (B), and (3) H_2O_2: water (A) + 3% H_2O_2 (B). The subjects wore these intra-oral appliances with enamel specimens in place for three consecutive 14-day study periods (Figure S1A). FerIONP alone was not included because it is inactive as assessed previously.[1] The treatments were applied by dripping one drop from bottles labeled bottle A and bottle B for each treatment group. The study dentist, specimen/sample analysis technicians

and the statistician were blinded to the product allocation of subjects. The subjects and product technicians were not blinded to product allocation as the products may be visibly distinguishable, since the FerIONP is slightly brown in color and H_2O_2 is effervescent. A person not directly related to the study was responsible for determining the identity to the codes of the product. This person maintained the code identity in a locked cabinet. This person corresponded directly with the pharmacy making the products.

Inclusive and exclusive criteria

We selected subjects on the basis of several inclusion and exclusion criteria: 18-85 years of age; willing and capable of wearing the lower partial denture 24 h a day throughout the study periods; normal salivary flow rate (stimulated and unstimulated flow of ≥ 0.8 mL/min and ≥ 0.2 mL/min, respectively); absence of significant oral soft tissue lesions and dental caries; and adequate oral hygiene. Exclusion criteria included: unstable or uncontrolled medical conditions; severe acute or chronic medical or psychiatric conditions; multiple drug allergies or hypersensitivity to iron products; taking or have ever taken bisphosphonate drugs; using blood thinning medications; use of antibiotics within two weeks prior to each treatment visit; severe marginal gingivitis or moderate/advanced periodontitis; alcohol or substance abuse; scheduled for head region MRI within three months after study participation; pregnancy or lactation; and use of chewing tobacco.

Clinical procedures

The clinical study involved 10 visits (Figure S1B). At each visit, the study dentist examined oral soft tissue (OST). Oral hard tissue (OHT) was only examined at Visit 1, 2 and 10. At the first visit (Screening visit), informed consent was obtained, and lower partial denture for each subject was assessed to determine if it met the study requirements. Both stimulated and unstimulated salivary flow were assessed during the first visit, and unstimulated salivary flow was assessed at any visit when a new medication known to decrease salivary flow was reported. For the unstimulated saliva collection, subjects were required to sit quietly for five minutes before beginning the test. During the five-minute test time, they were told to allow their saliva to pool, emptying into a collection cup whenever they feel the need to swallow. For the stimulated saliva collection, subjects were asked to chew unflavored gum base for one minute and then swallow any pooled saliva. Then, they chewed the gum base for two minutes, during which time they emptied any pooled saliva into a collection cup. The samples were weighed and the salivary flow rates were determined. The stimulated saliva sample were stored at 4 °C and sent to the University of Pennsylvania in frozen gel packs within 24 h of the day of collection for *S. mutans* analysis. At the second visit (Prophy visit), the subjects received dental prophylaxis and were randomized to product usage order. At Visit 3 (Treatment Period #1; two to three days following Visit 2), two bovine enamel specimen holder assemblies were mounted in the subject's lower partial denture (Figure 1A) and the assigned study products were dispensed to the subjects. Starting from this visit, all participants discontinued all regular oral hygiene practices and were instructed to use only the study fluoride-free toothpaste and toothbrush provided, with the exception of interdental cleaners, e.g. dental floss, if that was their routine practice. At Visit 4 (End of Treatment Period #1; 14 days ± 1 day), the two bovine enamel specimen holder assemblies were removed from lower partial dentures and shipped to the University of Pennsylvania under hydrated conditions for biofilm analysis. Procedures outlined under Visits 2 through 4 were repeated for the subjects who continued to qualify for the study. At the start of each treatment period, subjects were provided with a diary. Each day the subjects recorded the time of the treatment and sucrose challenge and any deviation from the procedures. In addition, subjects also recorded any changes in their health or medications that had occurred since their last visit. Subjects were required to turn in the completed diary at visits 4, 7 and 10. Study staff reviewed the diary with the subjects to confirm test compliance and clarify listed medical conditions and medications.

Safety assessment

Complications and adverse events were assessed and recorded during study period for all subjects. OST examination was performed at each visit, and OHT examination was performed at Visits 1, 2 and 10. OST examination included the labial mucosa, buccal mucosa, mucogingival folds, gingival mucosa, hard and soft palate, tonsillar and pharyngeal areas, tongue, sublingual area/floor of mouth, submandibular area, major salivary glands, head and neck and temporomandibular joint. OHT examination included assessing for enamel irregularities, tooth fracture, pathologic tooth wear, cavitated lesions, residual roots, faulty restorations and implants.

Treatment regimen

In this study, we compared the bioactivity of topical applications of FerIONP/H_2O_2 with vehicle control and H_2O_2 alone. FerIONP alone was not included because it is inactive as determined both in vitro and in vivo models (1). FerIONP was obtained commercially as 3% Feraheme (AMAG Pharmaceuticals, Waltham, MA). The hydrogen peroxide (H_2O_2) was purchased commercially as Hydrogen Peroxide 3% USP (Henry Schein Inc., Melville, NY). Baxter Sterile Water for Injection (NDC#0338-0013-04, Deerfield, IL) was used to adjust the concentration of the FerIONP. An extra-oral application of approximately 100 μL of the assigned study treatment was dripped onto each enamel specimen assembly twice daily (after breakfast and before bed) using a two-bottle dispensing method. The treatment and sugar challenge were provided as follows: (1) After Breakfast: 50 μL solution A (water or FerIONP) + 50 μL solution B (water or H_2O_2), followed by sucrose challenge (100 μL 20% sucrose solution), (2) After Lunch and Dinner: sucrose challenge (100 μL 20% sucrose solution), and (3) Before Bedtime: 50 μL solution A + 50 μL solution B, followed by sucrose challenge (100 μL 20% sucrose solution). The final concentration of FerIONP in solution A is 1.5% and H_2O_2 in solution B is 3%. The FerIONP dosage is based on our preliminary studies showing that (twice) topical applications of 50 μL of 1.5% FerIONP achieved the best results in disrupting biofilms formed on hydroxyapatite surface. The subjects were instructed to drip one drop from treatment bottle A onto each specimen and wait for 5 min and then immediately drip one drop from treatment bottle B onto each specimen and wait for an additional 5 min. This was followed by applying a 20% sucrose solution onto the specimens for 3 min, which could dilute and wash the study treatment from the specimens and provide a cariogenic challenge in addition to the subject's diet. The subjects dripped 20% sucrose solution onto the enamel specimens four times a day (after breakfast, lunch, dinner and before bed) before placing their lower partial dentures back in their mouth. We use 20% sucrose because this is the sugar concentration that induces cariogenic biofilm development in the *in situ* model, including biochemical changes (similar to that of the *in vitro* model) associated with dental plaque cariogenicity, while taking into consideration the diluting effects in the mouth due to saliva.[2] The purpose of dripping one drop from solution A and waiting for 5 min is to allow FerIONP penetration and binding into the biofilms that were formed on enamel blocks. Based on our previous *in vitro* studies[1,3,4] and the multiple treatment design in the current study, 5 min is enough for FerIONOP binding. In each 14-day study period, subjects brushed their natural teeth with a study provided, marketed, fluoride-free toothpaste twice daily in their normal manner. After breakfast and before bedtime, the subjects were instructed to brush their partial denture outside their mouth with water avoiding the enamel specimen sites and to rinse specimen sites gently with water. They were also instructed not to use any denture cleaner products during the study. We recruited a total of 16 participants for this study, and distributed 5 subjects in Group 1, 5 subjects in Group 2 and 6 subjects in Group 3. All the data were collected from 15 participants since one participant lost the partial denture. Because the current study is a crossover design, each participant completed all three different treatments during the test periods. Thus, the sample size for each treatment is 15. Each partial denture contains 2 enamel specimens, one for biofilm imaging, and the other one used for microbiological and enamel demineralization analysis.

Biofilm analyses

For cell viability, the biofilms were removed and homogenized by sonication, and the number of total viable cells and *S. mutans* were determined by colony forming units (CFU) using blood agar and Mitis Salivarius Agar Bacitracin (MSB; a selective medium for *S. mutans*), respectively.[1,3] The total bacteria data were from all the participants (one participant was removed from the data collection due to the loss of enamel specimens); however, the *S. mutans* counting was collected from 6 participants who had *S. mutans* at the screening visit (baseline) since not all the participants have this bacterium detected in their oral cavity. For spatial organization, intact/undisturbed biofilms were fixed with 4% paraformaldehyde (in PBS, pH 7.4) at 4 °C for 4 h. After fixation, the biofilms were washed twice with PBS, then transferred into 50% ethanol (in PBS, pH 7.4) and stored at -20 °C. Fluorescence *in situ* hybridization (FISH) was used for biofilm analysis as detailed previously.[5]

Fluorescence *in situ* hybridization

The following FISH oligonucleotide probes were used in this study: EUB338, 5'-GCTGCTCCCGTAGGATG-3' with Cy3 for all bacteria; MUT590, 5'-ACTCCAGACTTTCCTGAC-3' with Alexa Fluor 488 for *S. mutans*; and STR405, 5'-TAFCCGTCCCTTTCTGGT-3' with Cy5 for Streptococcus. The sample in the hybridization buffer (30% formamide, 0.9 M NaCl, 0.01% sodium-dodecyl sulphate (SDS), 20 mM Tris·HCl, pH 7.2) with the probes was incubated at 46 °C for 4 h. After incubation, the hybridized cells were washed with washing buffer (0.2 M NaCl, 20 mM Tris·HCl pH 7.5, 5 mM EDTA, and 0.01% SDS) and incubated for another 15 min at 46 °C.[5] The biofilm was acquired using Zeiss LSM 800 with a 20× (numerical aperture = 1.0) water immersion objective. The biofilms were sequentially scanned using diode lasers (488, 561, and 640 nm), and the fluorescence emitted was collected with a GaAsP or multi-alkali PMT detector (490 to 550 nm for Alexa Fluor 488, 565 to 620 nm for Cy3, and 645 to 700 nm for Cy5). Amira 5.4.1 software (Visage Imaging) and Zen software (Carl Zeiss) were used to create 3D renderings to visualize the biofilms.

Enamel demineralization analysis

A core of enamel 4 mm in diameter was prepared from each bovine tooth by cutting perpendicularly to the enamel surface with a hollow-core diamond drill bit. Specimens were ground and polished to create planar parallel dentin and enamel surfaces. The dentin side was ground flat using 500 grit silicon carbide paper, followed by grinding and polishing of the enamel side. The enamel specimens were then implanted in the lower partial denture appliance as shown in Figure 1. Following each treatment period, the surface microhardness analysis (SMH) was used to assess changes in the mineral status of enamel slabs after biofilm removal using Wilson 2100 Hardness Tester. Five baseline indentations spaced vertically 100 µm apart were placed with a Knoop diamond under a 50-gram load in the center of a flattened, polished sound enamel specimen. SMH was determined by measuring the length of the indentations using Clemex CMT HD version 6.0.011 image analysis software. After 14 days of intra-oral exposure, the SMH of enamel specimens were tested by placing five indentations 200 µm to the left of the baseline indentations (Figure S5). The extent of demineralization was calculated as the percentage change in SMH:

$$\% \text{ SMH Change} = [(D1-B)/(B)]*100$$

B = indentation length (µm) of sound enamel specimen at baseline

D1= indentation length (µm) after *in situ* demineralization

FerIONP binding to oral streptococci

Streptococcus mutans UA159, a cariogenic pathogen, *Streptococcus oralis* J22, *Streptococcus gordonii* DL1, and *Streptococcus sanguinis* SK36, commensal oral streptococci, were grown in ultra-filtered (10-kDa cutoff; Millipore) tryptone-yeast extract broth (UFTYE; pH 7.0) at 37 °C and 5% CO_2 to mid-

exponential phase. Subsequently, 1 mL of each bacterial cultures were centrifuged and resuspended in 1 mg/mL FerIONP and incubated for 30 min with rocking at 37 °C. After incubation, the bacteria pellets were washed twice and then dissolved in 1 mL of 70% HNO_3 for iron quantification using inductively coupled plasma optical emission spectroscopy.[1,6] In a separate experiment, *S. mutans* UA130, *S. mutans* UA130 Δ*gbpA*, *S. mutans* UA130 Δ*gbpC* (gift from Jeffrey Banas, University of Iowa), and *S. mutans* UA159 Δ*gtfBC* were used to evaluate the role of surface protein in mediating FerIONP preferential binding to *S. mutans*.

Bacterial killing by FerIONP/H_2O_2

The binding of FerIONP to different bacteria was performed as described above. After incubation and washing, the bacterial pellets were exposed to 1.5% H_2O_2 in 0.1 M NaAc (pH 4.5) for 10 min. Then, the bacterial cells were washed, serially diluted and plated for CFU counting. To visualize *in situ* ROS generation and bacterial killing, *S. mutans* or *S. oralis* was labelled by SYTO 60 (652/678 nm; Molecular Probes). Hydroxyphenyl fluorescein (HPF, 490/515 nm; Molecular Probes) was used to detect hydroxyl radicals.[6] Micrographs were acquired in the same field of view using Zeiss LSM800 with a 40× (numerical aperture = 1.2) water immersion objective. Images were analyzed by Zen software (Carl Zeiss).

To visualize distribution of live and dead bacteria, intact bacteria were labelled with SYTO 60 (652/678 nm; Molecular Probes), and propidium iodide (PI, 535/617nm; Molecular Probes) was used to determine non-viable cells. After bacterial labeling, the bacterial cells were resuspended in 1 mL 0.1 M NaAC (pH 4.5) containing 1 mg/mL of FerIONP, 10 μM of HPF, and 30 μM PI in the presence of 1% H_2O_2. 20 μL of the mixture was immediately placed onto glass slides for confocal microscopy.[6]

***In vitro* biofilm model and colorimetric assay**

The single or mixed-species biofilm method was performed as detailed previously.[1,3,6] Briefly, hydroxyapatite discs (surface area: 2.7 ± 0.2 cm^2; Clarkson Chromatography) or human teeth were coated with filter-sterilized, clarified human whole saliva for 1 h at 37 °C. *S. mutans* and *S. oralis* were grown until mid-exponential phase and inoculated with 10^6 CFU/mL of *S. mutans* and/or 10^2 CFU/mL of *S. oralis* to each disc placed in 24-well plate containing UFTYE with 1% or 0.1% sucrose. The culture medium was changed daily until the end of the experimental period (43 h). For the human teeth model, *S. mutans* single-species biofilm was formed with an inoculum of 10^6 CFU/mL of *S. mutans* in UFTYE with 1% sucrose as described above. Subsequently, the biofilms were treated with 1 mg/mL of FerIONP for 10 min and exposed to a solution containing 3,3′,5,5′-tetramethylbenzidine (TMB) and 1% of H_2O_2 for 5 min. The colorimetric reaction was measured at an absorbance of 652 nm.

Data and statistical analysis

We used an ANOVA model including terms for treatment and study period, and random effects to account for the within subject correlations among the three treatments and the enamel specimens within each treatment. A treatment sequence effect was examined as an indicator of a carryover effect and removed from the model when not significant. All comparisons employed two-sided tests, and differences were considered significant when $P < 0.05$. For in vitro studies, quantitative data were analyzed using ANOVA with post-hoc Tukey's test or Kruskal-Wallis Test with post-hoc Dunn's test for multiple comparisons, whereas Student's *t*-test was conducted for pairwise comparison. Differences between groups were considered statistically significant when $P < 0.05$. The data are presented as mean ± standard deviation (SD). At least three independent experiments were performed unless otherwise stated. Statistical analyses were performed using GraphPad Prism 8.

References:
(1) Liu, Y.; Naha, P. C.; Hwang, G.; Kim, D.; Huang, Y.; Simon-Soro, A.; Jung, H. I.; Ren, Z.; Gubara, S.; Alawi, F.; Zero, D.; Hara, A. T.; Cormode, D. P.; Koo, H. Topical ferumoxytol nanoparticles disrupt biofilms and prevent tooth decay *in vivo* via intrinsic catalytic activity. *Nat. Commun.* **2018**, *9*, 2920.
(2) Cury, J. A.; Rebelo, M .A.; Cury, A. A. D. B; Derbyshire, M. T.; Tabchoury, C. P. Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. *Caries Res*. **2000**, *34* (6), 491-497.
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(4) Naha, P. C.; Liu, Y.; Hwang, G.; Huang, Y.; Gubara, S.; Jonnakuti, V.; Simon-Soro, A.; Kim, D.; Gao, L.; Koo, H.; Cormode, D. P. Dextran-coated iron oxide nanoparticles as biomimetic catalysts for localized and pH-activated biofilm disruption. *ACS Nano* **2019**, *13* (5), 4960-4971.
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Supplementary Figures

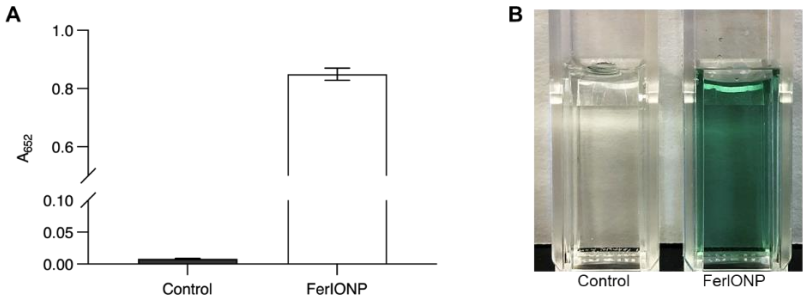


Figure S1. Catalytic activity of FerIONP at acidic pH (pH 4.5). (A) Absorption at 652 nm of FerIONP. The catalytic reaction of 3,3',5,5′-tetramethylbenzidine (TMB, which serves as a peroxidase substrate) in the presence of H_2O_2 produces a blue color (n = 3). All values are reported as mean ± SD. (B) Representative images of FerIONP after colorimetric reaction.

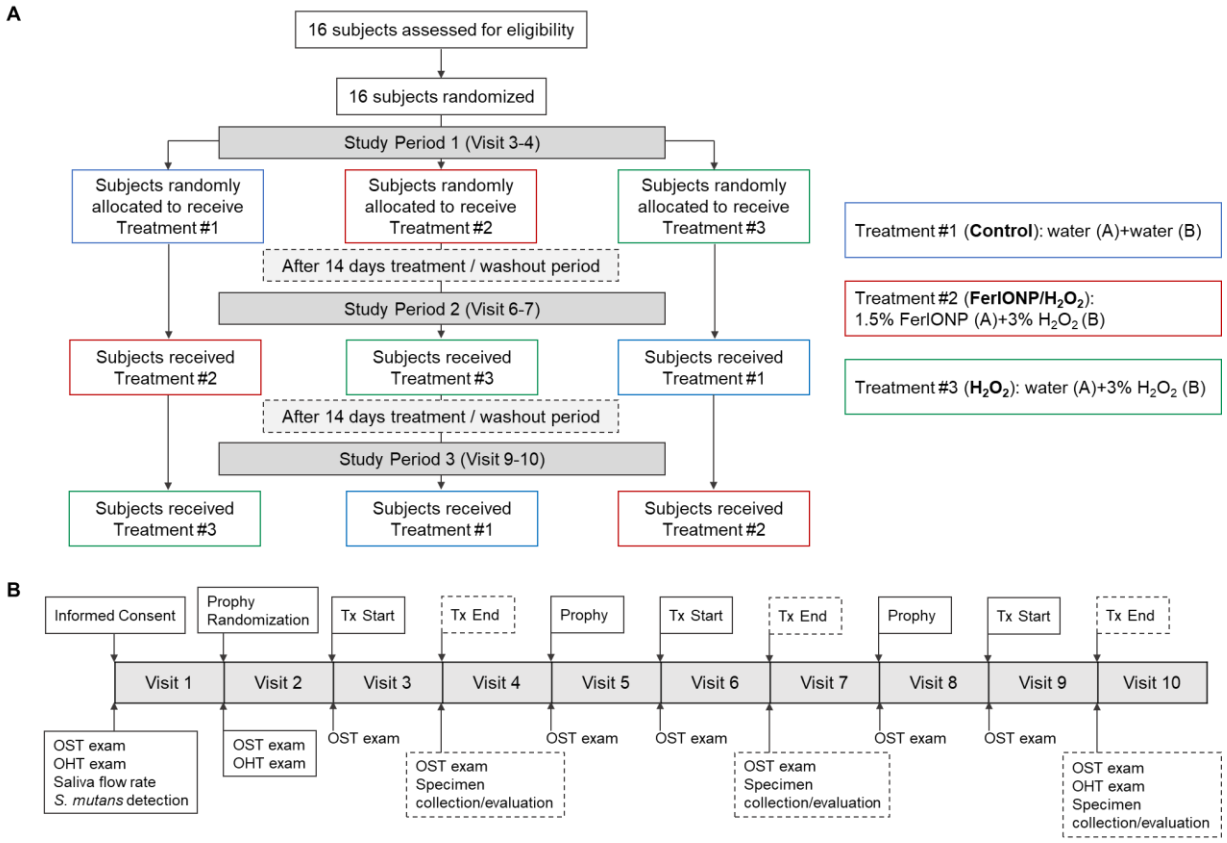


Figure S2. Clinical study design and procedures. (A) Design of clinical study to evaluate anti-biofilm effects of FerIONP using *in situ* biofilm model. (B) Study events and visit of procedures and testing. OST, oral soft tissue; OHT, oral hard tissue; Tx, treatment.

Subject ID	Adverse Event	Related to treatment	Related to Device
1002	Erythema on the right buccal mucosa (2 mm x 3 mm) due to cheek bite	No	No
1004	Caries lesion on tooth #6 disto-lingual surface, asymptomatic	No	No
1004	Fracture of tooth #6 disto-lingual surface, asymptomatic	No	No
1008	Swelling on the middle of the neck	No	No
1008	Worsening of high blood pressure	No	No
1011	Erythema on mandibular edentulous posterior ridge (2 mm x 3 mm), left side, mild	No	No
1015	Erythema on the labial mucosa, scattered, mild due to lip bite	No	No

Figure S3. Side effect observed in the study.

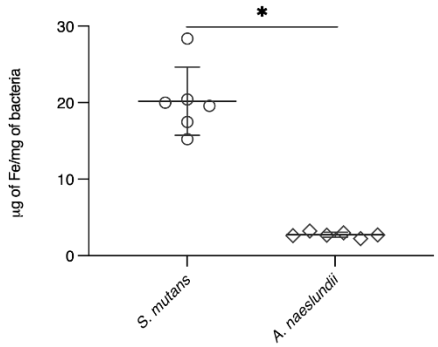
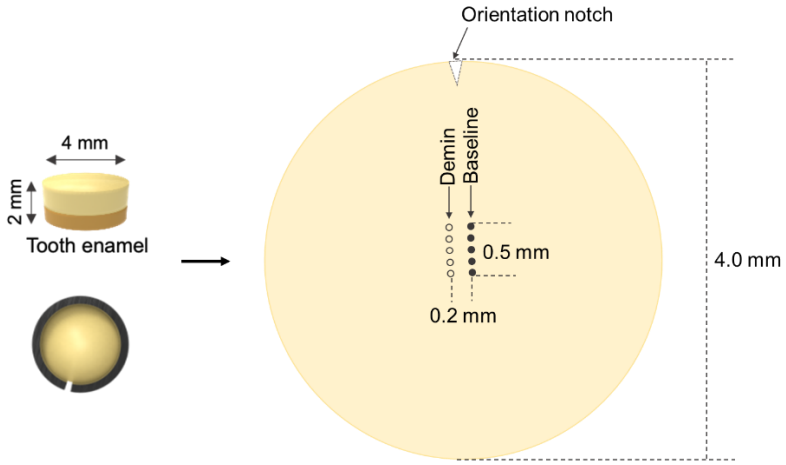


Figure S4. FerIONP binding to non-streptococci and non-H_2O_2 producing species. FerIONP bound poorly to *Actinomyces naeslundii* compared to *S. mutans* (* $P < 0.0001$).



Enamel specimen (top view) and Knoop indentations.
Surface Microhardness Change (%SMHC)

The extent of demineralization as the percentage change in SMH.
% SMH Change = [(D1-B)/(B)]*100
Baseline (B) = indentation length (µm) of sound enamel specimen at baseline
Demin (D1) = indentation length (µm) after in situ demineralization

Figure S5. Enamel specimen with orientation cut and illustration of the Knoop indentations. Demin, demineralization.