The application of cellulose acetate replication sheets in enamel wear investigations

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INTRODUCTION

Replication is basically a non-destructive recording of surfaces of interest. This technique will copy the surface details for later indirect microscopic examination. This is valuable when the surface of interest cannot be further manipulated by cutting or polishing to fit directly under a microscope. Replication by acetate sheets is one of the techniques applied in industrial, metallographic and anthropological wear/examination. Enamel is the outermost protective layer of human teeth and is subjected to mechanical stresses due to the masticatory function; thus, wear is inevitable in human teeth. This relatively inert hard tissue has been reported to possess some properties of both metals and glasses due to its unique delicate microstructure. This study investigated the applicability of replication sheets in registering wear induced features on human enamel surfaces. The sheets replicated wear features successfully with compatibility to use with multiple microscopes. Acetate sheets have a potential in enamel wear replication.

MATERIALS AND METHODS

Samples preparation for impact sliding wear testing (ISWT)

Twenty flat enamel specimens were embedded in acrylic resin within acrylic rings. The surface of the rings was polished to produce enamel windows with a glossy finish. Samples were subjected to “enamel to enamel” ISWT conditions (ISWT K655-07, Tokyo Giken, Tokyo, Japan). The test was set to direct a vertical enamel hammer; a 5 mm ball-shaped enamel specimen attached to a metal stylus, to drop from a height of 1 mm with a force of 30 N. The hammer will then slide 1 mm horizontally while contacting the enamel surfaces and back to its position, before rising up to the beginning of the cycle. A total of 20,000 cycles were performed with a frequency of 0.23 Hz (14 cycles per min). The entire impact sliding test took place below the water level in the tank of the ISWT.
machine to prevent burning of enamel due to friction heat generation\textsuperscript{9). Water temperature and level were checked for heating and evaporation at 37°C. During the test, samples were covered by a slurry of PMMA powder (Non-plasticized, Polymethylmethacrylate particles (spherical, size: 50 µm); Techpolymer MBX-50, Sekisui Plastics, Tokyo, Japan) mixed with tap water at a 1:1 weight ratio to act as a substrate between the contacting surfaces during ISWT. Every 5,000 cycles, enamel samples were rechecked in case of displacement and the slurry was washed off and renewed.

**Samples preparation after ISWT and replication using CARS**

After ISWT, all samples were washed with distilled water for 20 s, dried with gentle air spray without polishing as it might reduce the size of microcracks, or create new features as scratches\textsuperscript{9} even with minimal mechanical preparation as shown on Fig. 1. Samples were then immersed in a hydrochloric acid solution (0.005 M for 10 s), washed for 60 s, and then stored back in distilled water for 15 min to halt any remaining effect of the HCl solution. For surface replication, specimens were re-dried by gentle air spray. Acetone was dropped on the enamel surfaces (Two drops, 0.05 mL each), 1×1cm pieces of CARS (Replicating Material AGG255, Cellulose Acetate, 35 µm, 150×100 mm, Agar Scientific, EM Japan, Tokyo, Japan) were placed immediately over the specimens’ surfaces with gentle pressure using a cotton bud. Acetone was allowed to evaporate for 10 min inside an incubator at 23°C. Sheets were then gently stripped off the dry specimen’s surface, fixed upside down on a glass slab by scotch tapes around the corners. The process of sheet replication was conducted twice. The First set of sheets was marked as ‘extraction set’ and discarded while the second was marked as ‘proper set’. Samples were then stored back in distilled water to prevent excessive dehydration (Fig. 2).

**Imaging of the specimens and CARS**

Both samples and its corresponding CARS replicas were examined using multiple microscopic techniques. Scanning confocal laser microscope SCLM (SCLM; 1LM 15 W, with Nikon lenses, Lasertec, Tokyo, Japan) was used first to take images of the wear-induced features on each sample/replica set with magnifications of ×5, ×10, ×20, and ×50. Samples and replicas were then examined utilizing a 3D colored laser microscope and Profilometer.
(VK 250 series, Keyence, Tokyo, Japan) to examine wear zone shape and measurements, obtain the profile of the surface, and compute surface’s microroughness parameters. The parameters computed were \( S_a \) (arithmetical mean height of the surface; the difference in height of each point compared to the center plain of the surface), \( S_z \) (the sum of the largest peak height value and the largest pit depth value within the defined area of a surface), and \( S_{dr} \) (the percentage of the definition area’s additional surface area contributed by the texture as compared to the planar definition area). Three dimensional (3D images) buildup of the samples and CARS were also constructed with the same microscope. A stereo-microscope with transillumination and multiple fiber-optic illuminations (Leica MZ10 F, LEICA, Tokyo, Japan) was used after to obtain multiple images with different illumination/ transillumination directions for each specimen and replica. Images were taken with both dark field and light field, with and without illumination. For SEM imaging, sheets were carefully cut smaller, fixed on SEM stumps by double-sided carbon tape, directly spattered with gold-palladium (40 nm) and imaged by SEM (H-4500, Hitachi High-Technologies, Tokyo, Japan). At the end of the aforementioned, each sample/replica had a set of multiple microscopic images to capture and analyze most of the wear microfeatures as microcracks, wear crater, pits, furrows, scratches, and roughness.

**Images analysis**

The quality of replication depends on dimensional accuracy and resolution. Since both are difficult to quantify, the “completeness of replication”\(^2\) was calculated for each replica. For this, an image analyzer (Image J 1.51 p, Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/, 1997-2016) was used. The number of microcracks radiating from the wear/impact zone was considered as the “feature” to be counted on samples’ images. Thereafter, the same procedure was conducted on the images of the CARS replica. Images were also superimposed to compare microcracks on each set. The difference between the two readings is the number of features lost.

\[
\text{Percentage of Replication Completeness} = \frac{\text{Feature Count} - \text{Deviation}}{\text{Feature Count}} \times 100\%
\]

Where

\[
\text{Deviation} = \frac{\text{Feature Count on sample}}{\text{Feature Count on Replica}}
\]

**Statistical analysis**

The data obtained by computerized measurement comprised wear zone microroughness measurements of \( S_a \), \( S_z \), and \( S_{dr} \), enamel window microroughness measurements of \( S_a \), \( S_z \), and \( S_{dr} \), wear zone cross-sectional area, and total enamel window volume. Due to the small sample size, absence of both homogeneity of variance and normality in the results, statistical analysis was conducted using Wilcoxon signed-rank test (non-parametric=distribution-free test) with the significance level set at 0.05 by a computer software (IBM® SPSS® Statistics for Windows, version 24, IBM, Armonk, NY, USA).

**RESULTS**

**The effect of ISWT on the enamel surfaces**

The ISWT had its effect on the initially smooth enamel surface. All samples showed fine scratching marks caused by the slurry particles. The initial impact zone with the upper hammer suffered the most extensive loss of substance as pits and craters formed. The sliding movement had its effect in the formation of a shiny wear zone surrounding the pits/craters. Microcracks were radiating from the crater corners or surrounding the wear area with different orientation on both enamel surface and subsurface (Fig. 3).

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Fig. 3 Effects of impact sliding forces on dental enamel.

A: the scanning laser microscope view of an enamel window. B: the same view on scanning confocal laser microscope with a shiny appearance of the wear zone, the differentiation between scratching marks and microcracks is possible. C: a simplistic drawing of the different wear microfeatures present on the enamel surface.
Fig. 4  Inversion between the specimens’ images and its corresponding sheets.
The sheets images were the reverse of specimens’ images in both directionality and topography. Notice the position of the microcrack (marked with * changing from left to right), and the change in depth measurements as depths on the specimens surface became heights on the sheet surfaces, the crater area (marked with arrows) on the specimen change of the depth’s indicator color. Images were taken by the 3D laser microscope.

Fig. 5  Three dimensional (3D) imaging construction of a sample and its sheet replica, the depth change (profile inversion) is demonstrated.
The profile of the center of the surface is displayed beneath each 3D image. The difference in the roughness of the two surfaces is presented in the smoother profile line in the sample profile graph.

**CARS images vs. enamel surfaces images**
CARS replicas had inverted images of the replicated enamel surfaces. The inversion depended on the way of fixation on the glass slap with either a vertical or horizontal inversion (Fig. 4). The inversion was also present in the profile images with depths of the wear features registered as heights in the sheets images (Fig. 5). CARS had a 100% completeness of the microcracks replication as all the microcracks were registered. The resemblance in both the shape and dimensions of the replicas and the samples was detected.

**Scanning confocal laser microscope images**
Sheets were directly examined under SCLM. This revealed capturing of enamel microstructures and wear micro-details. Enamel rods, microcracks pathway, and Fine scratching marks were all registered on the sheets images (Fig. 6). The effect of HCL immersion on the enamel surface was also revealed by this microscopic technique with the ability of CARS to replicate etched enamel microstructures (Fig. 7).

**3D colored laser microscope/profilometer images**
Sheets were directly examined under this microscope. Many images had hazy circles on the surface. The
Fig. 6  Imaging of the sheets by scanning confocal laser microscope. Sheets had a vertically inverted image of the enamel sample. Sheets captured not only the wear features but also enamel microstructures as rods and etching patterns. A: sample wear zone. B, C, and D: sheet images at different magnification. Enamel rods are shown in image D.

Fig. 7  The effect of acid etching on the sample surface. Immersion of the samples in HCl caused damage to wear shallow details as fine scratch marks and micro-polished surfaces. The etching solution did not affect deeper features as microcracks or craters and enhanced the view of enamel microstructures. The enamel rod boundaries are registered on the sheets surface successfully along with the microcrack and crater boundaries due to the removal of smear layer. BF: before HCl immersion, AF: after HCl immersion, SH: sheet image (A bubble is present in the deepest portion of the sheet material).

Fig. 8  A three-dimensional (3D) image of a replicated microcrack. 3D profile image of a microcrack is shown as heights due to the inversion, enamel rods boundaries are also present as semicircular projections on the surrounding surface. The two-dimensional image of the same microcrack as the width was measured.

clarity of those circles changed at different focus depths. Surface roughness measurements and analysis of the dimensions of the image were performed. Measurement of fine details such as microcracks’ width on the CARS surface was also checked by this microscopic view (Fig. 8).
Fig. 9  Stereo-microscopic images of the sheets.
The change of the illumination/transillumination directions resulted in different views of the sheets on the same
magnification (Lens ×3.2). T: transillumination, T-A: transillumination with angled direction of light, D-S: dark field
with a single fiberoptic source of illumination. D-M: dark field with multiple fiberoptic light sources.

Fig. 10  SEM images of a sample and its sheet replica.
SEM images did not capture the details of wear zones. The crater details, microcracks and scratch marks were
captured successfully. Tearing of the sheets materials occurred in some portions of the replica (indicated by the
white arrows).

Stereomicroscope images
CARS were directly imaged by the stereomicroscope. Darkfield background with external illumination
conditions had the best view of the sheets. Scratches, furrows, pits, craters, microcracks and wear zones were
all captured. Background transillumination produced different images which depended on the angulation of
the transillumination, presence or absence of external illumination (Fig. 9).

SEM images
SEM was conducted after samples and sheets preparation. The imaging on high magnifications with secondary
electrons had to be conducted quickly otherwise burning of the sheet occurred especially in the high mode of
magnification. Microcracks and fine scratching marks were clear as whitish shiny lines. This was not the case
in viewing wear zones boundaries. Craters on the other hand inside the wear zone were viewed successfully.
Some tearing was noticed in the sheets deepest portions of the craters or deep microcracks (Fig. 10).

Surface measurements of samples/sheets images
As shown in Fig. 11, the surface microroughness measurements of enamel windows of the replication
sheets were significantly higher than samples’ parameters; Sa (p=0.028), Sz (p=0.003), and Sdr
(p=0.002) values (Table 1). On the other hand, wear area roughness measurements were significantly
higher in the sheets parameters of Sz (p=0.005) and Sdr
Fig. 11  Surface profile of a sample/sheet set.
The difference in the surface microroughness was detected and computed using the 3D scanning laser microscope/profilometer. The sample surface is smoother when compared to the sheet surface. The Line profile (Ra) of the surface sector is shown beneath each surface. The line profile of the sheet diameter shows more irregularities with increase in the heights and depths of the profile.

Table 1  The enamel window microroughness comparisons between the samples group and its corresponding sheets replicas group

|          | Sa (mm) |          | Sz (mm) |          | Sdr      |
|----------|---------|----------|---------|----------|----------|
|          | Sheet   | Sample   | Sheet   | Sample   | Sheet    | Sample   |
| Minimum  | 0.013821| 0.002883 | 0.27695 | 0.034855 | 4.686131 | 0.605471 |
| Maximum  | 0.069542| 0.089237 | 0.68076 | 0.46533  | 73.24075 | 1.866987 |
| Median   | 0.041958| 0.011192 | 0.56328 | 0.158303 | 13.61627 | 1.372017 |
| *-value  | 0.028   |          | 0.003   |          | 0.002    |          |
| Significance Level | *   |          | **      |          | **       |          |

*p<0.05, **=p<0.01

Table 2  The wear zone microroughness comparisons between the samples group and its corresponding sheets replicas group

|          | Sa (mm) |          | Sz (mm) |          | Sdr      |
|----------|---------|----------|---------|----------|----------|
|          | Sheet   | Sample   | Sheet   | Sample   | Sheet    | Sample   |
| Minimum  | 0.010032| 0.001953 | 0.16585 | 0.019575 | 2.901654 | 0.508493 |
| Maximum  | 0.115984| 0.089237 | 0.68076 | 0.46533  | 73.24075 | 1.866987 |
| Median   | 0.051981| 0.011192 | 0.56328 | 0.158303 | 13.61627 | 1.372017 |
| *-value  | 0.06    |          | 0.005   |          | 0.002    |          |
| Significance Level | NS |          | **      |          | **       |          |

**=p<0.01, NS=p>0.05

(p=0.002) only (Table 2), no significant differences in Sa readings were detected (p=0.06). Although no significant differences in the wear zone dimensions were detected (p=0.875) between the samples and its corresponding replicas, the computed enamel volume was significantly greater in the replicas (p=0.012, Fig. 12).

DISCUSSION

The aim of this study was to investigate the use of CARS in capturing wear features on human enamel surfaces. Not only CARS replicated surface microcracks, wear pits, wear craters, scratches, and furrows successfully with high completeness, it was compatible
to use with multiple microscopic techniques. Although microroughness parameters of the sheets surfaces were significantly greater than enamel surfaces, except in Sa of the wear zone \( (p=0.06) \), wear zones/areas were registered successfully using the sheets with no differences detected in the actual measurement of the zones on the samples' surfaces directly \( (p=0.875) \). The aforementioned confirmed our hypothesis as CARS can be applied in enamel wear replication.

The initial microscopic examination of the enamel specimens was obscured with some deposits smeared on the surface. This smear layer comprised the byproducts of enamel breaking under pressure and collapsing of the acrylic particles\(^{11,12}\) during the ISWT. Samples immersion in HCl of 0.005 M for 10 s was sufficient to remove the smear layer and enhance the surface topography of the enamel surfaces to be examined without compromising the micro-details of the enamel wear. Chemical treatment by acid etching enhances the topography of enamel, changing it from a low-reactive surface to a surface that is more susceptible to adhesion, increasing the surface energy so low viscosity fluids \( i.e. \) acetone are attracted to the interior of microporosity\(^{13}\).

Although acetone is an organic solvent, acrylic resin surfaces were not substantially dissolved, and the sheets were stripped off easily. In this study, acrylic surfaces were not the surfaces of interest, yet we recommend precaution in studies involving dental materials with resin components such as composite restorative materials, acrylic dentures, and resin-modified glass ionomers. Using replication twice has decreased the amount of impurities and artifacts between the extraction and proper replication sheets. Some studies used the initial extraction sheets in collecting microorganisms especially in bacterial contamination of enamel surface cracks\(^{10}\).

In this study, the microscopic imaging of enamel surfaces and its replica showed polished wear zones with stripes and striations in addition to various furrows of variable sizes, fine scratch marks, microcracks and pits\(^{12}\). This is consistent with a study\(^{15}\) reported striations on the occlusal surface to be the main feature of occlusal microwear and pits to present in the force application point with microcracks propagating from these zones.

The sheets images taken by the scanning laser microscope showed some hazy circles, these circles did not interfere with the registration of the wear features on the enamel surface as changing the depth of focus on the microscope let these circles disappear. These circles are caused by the roughness of the back surface of the acetate sheets\(^9\). We recommend using a flat, firm but soft object in applying pressure to CARS. This initial pressure would allow maximum adaptation and reduction of bubbles entrapment. A study found no differences in the completeness of registration of metal cracks associated with different pressures applied during the replication process\(^1\). The manufacturer instruction did not recommend any pressure as sheets are allowed to set by gravitational forces alone to prevent distortions.

SEM imaging with secondary electrons detection was recommended by other studies\(^{6,15}\) over backscattered in CARS examination. Microcracks appeared as shiny whitish lines since electrons concentrated in these projections on the sheets surfaces. Our findings indicated that the use of secondary electrons was useful in examining fine-linear details rather than wide-flat details. Wear zones were not detected on the SEM images of sheets or specimens. This might be attributed to the SEM instrumentation on feature visibility and effects of magnification level choices in most microwear studies\(^{16}\). SEM has limitations in defining surface topography, the electron beam technique does not allow visualization of three-dimensional surface texture as its contrast relies on the different emission of electrons, these cannot give contrast on flat homogeneous surfaces. A two-step replication technique is mentioned in the literature\(^1,3,6,17\).

After replication with CARS, the sheet is used as a mold to produce a “positive replica”. Imaging of the positive replicas has the advantages of the absence of inversions and finer details. These replicas can be made by coating negative replicas with carbon and then dissolving away the acetate sheet in acetone. The carbon replicas can be examined under the transmitting electron microscope TEM. Alternatively, the sheet; a negative replica of the surface can be covered with a conductive coating and examined by SEM. In this study, SEM was chosen...
instead of TEM as increasing the number of steps of the replication process might cause less completeness of registration, decreased accuracy, and complicating what is considered a simple procedure.

Surface roughness of dental biomaterials can be assessed by qualitative methods such as optical and SEM and/or quantitative methods such as contact diamond and non-contact laser surface profilers. A study reported using the optical instrument as the most appropriate method for surface roughness characterization of metallic biomaterials. This method does not require any particular preparation of the surfaces nor will cause any deterioration. Since surface topography is three-dimensional in nature, the measurement of 3D surface topography can represent a more realistic and natural characterization of any surface. The development of sophisticated surface characterization parameters suggests that the surface characteristics of dental materials and other related surfaces should be described using more than one surface measurement parameter. Surface parameters should be chosen which can both quantify the surface roughness and provide information on the shape of the surface under investigation. Measurement of the surface texture by using a non-contact laser-scanning microscopy can reveal a more detailed definition of the surface texture in comparison to the examination under SEM. Accordingly, this study assessed the surface microroughness by computing multiple parameters; Sa, Sz, and Sdr with a 3D scanning laser microscope and profilometer; a procedure with both quantitative and qualitative approaches. Those approaches indicated CARS are more suitable for twodimensional analysis of features such as width, length, and area (Fig. 8) rather than three-dimensional analysis such as depth, volume, and surface microroughness (Fig. 11). Some 3D construction images had blackened voids. These voids were reported to represent torn parts of the sheets due to the entrapment of the replication materials in undercuts or deep portion of the surface of interest.

Cellulose acetate sheets replication process was a simple procedure. Sheets are the most common replication material applied in nondestructive surface evaluation. The application of CARS is considered simple, inexpensive, durable, nondestructive and compatible to use directly with SEM since sheets can be spattered directly with a conductive coating. Replication with sheets allows indirect analysis of components, which can be preserved for sequential documentation or registration of surfaces. However, CARS have limitations. These limitations include the inability to examine subsurfaces, the chemical reactivity and toxicity of acetone solvents, and the verification of replication flaws. A study reported replicated microcracks were shorter by 22% when compared to actual surfaces (280 µm vs. 350 µm), the verification of microwear details was highly subjective as the explanation or verification of replica flows from the true specimen was without easiness. Our future research includes the application of a biocompatible solvent and/or modification of the cellulose acetate sheets to be softened with such solvents in the process of replication. A previous study applied cellulose cement instead of acetone to replicate surface wear. Pre-wetting cellulose sheets with acetone might be considered an alternative to the direct application of acetone to enamel. This might broaden the scope of CARS clinical applications especially in the detection of enamel cracks on the microscopic level or “microwear” and/or its progression.

In conventional wear studies, a series of impressions is taken for the materials of interest i.e. tooth substances or restorative materials, over designated time periods. The casts/replicas produced are compared to a standardized series of casts with simulated wear conditions (a series of 0.1 mm gradual step wear of the original surface). Despite the provision of visualized representation of the wear progression, changes in enamel wear could be detected at only a few weeks interval, without the ability to capture microscopic details. CARS are inexpensive, petite in size, easy to store, capable of capturing fine details, and very durable when compared to the conventional elastomeric impression/casts techniques. These properties are quite valuable in the process of early detection of enamel microcracks/wear, indirect microscopic examination of teeth, sequential recording of wear progress, and mass reservation of patients’ records.

CONCLUSIONS

Within the scope of this study, the following conclusions can be drawn: CARS are found to be suitable for replicating wear induced features on enamel surfaces. However, CARS are more accurate in the analysis of two-dimensional wear features/parameters than the three-dimensional analysis.

ACKNOWLEDGMENTS

We highly appreciate the efforts of Dr. Makoto J. TABATA for his guidance and consultation. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICT OF INTEREST

None.

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