Title
Functional Remineralization of Dentin using Polymer Induced Liquid Precursors (PILP) and Biocem

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Functional Remineralization of Dentin using Polymer Induced Liquid Precursors (PILP) and Biocem

by

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THESIS

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in the

Dedication and Acknowledgements

I would like to acknowledge my thesis committee Dr. Stefan Habelitz, Dr. Thuan Le, and Dr. Ray Stewart. Additionally, acknowledge Margot Bacino and Dr. Jean Calvo. I am very grateful for their mentorship and help in this extensive project.

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I would like to dedicate this manuscript to my family. Thank you for your unconditional love and support through everything.

Abstract:

Purpose: Functional remineralization (FR) of carious dentin requires recovery of the mineral content and mechanical properties. FR by the polymer-induced liquid-precursor (PILP) process has been demonstrated in the laboratory by using polyaspartic acid (pAsp). This study compares the ability of Biocem cement (NuSmile, Houston, TX, USA), Biocem modified with pAsp (20% and 40%), Fuji I and Fuji IX glass ionomers (GC Corp., Tokyo, Japan) in their ability to restore the mechanical properties of demineralized dentin matrix in artificial lesions. **Methods**: Artificial lesions were created by solution consisting of 0.05 M acetate buffer containing 2.2 mM calcium phosphate and adjusted to pH 5.0 for 66 hours. The lesions were rehydrated and treated in 5 groups: Biocem only, Biocem + 20% pAsp, Biocem + 40% pAsp, Fuji I, and Fuji IX (n=3) and then left to remineralize for 2 weeks. A group consisting of Biocem only, Biocem + 20% pAsp, Biocem + 40% pAsp was also remineralized for 6 weeks. The shrinkage after dehydration (indicating remineralization) was measured and compared. **Results**: For 2 weeks, the shrinkage was not reduced by Biocem only treatments, but was reduced by 54% ($P<0.01$) and 75% ($P<0.01$) with 20% or 40% pAsp added respectively. Fuji I and Fuji IX reduced shrinkage by 24% ($P>0.05$) and 40% ($P<0.05$), respectively. For 6 weeks, the shrinkage was not reduced by Biocem only or Biocem + 20% pAsp treatments, but Biocem + 40% pAsp reduced shrinkage significantly by 31% ($P<0.02$). **Conclusions**: Results from 2 and 6 weeks of remineralization show addition of pAsp may enhance Biocem cement functionality by inducing FM. Further research is needed to demonstrate FM of dentin lesions from the laboratory to the clinic.

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Introduction:

Dental caries (tooth decay) remains the most common chronic disease in children and adolescents. It affects the health of 60-90% of school-aged children worldwide.[1] Dental caries occurs when bacteria, like *Streptococcus* mutants and *Lactobacilli*, form a biofilm on the surface of the tooth, primarily on enamel. The bacteria produce acids which dissolve the apatite mineral in the tissue, leading to the formation of a cavity. Current trends in conservative and minimally invasive dentistry emphasize the reversal and repair of the active caries process as a first step to restoring the damaged tissue.[2] Remineralization of enamel, which is comprised of 95% mineral, is an accepted phenomenon with established mechanisms.[3] Although the remineralization of shallow lesions in enamel has been proven through well-established mechanisms, the remineralization of dentin has not.

The “Polymer-Induced Liquid Precursor” (PILP) approach has been shown as an effective and efficacious method to reintroduce mineral into collagen fibrils, the major structural component of dentin. The restoration of the mechanical properties of the tissue is critical for regaining its function. Studies have shown that when localized, demineralized dentin is immersed into a remineralizing solution composed of a calcium phosphate solution, a process-directing agent like polyaspartic acid (pAsp) is able to functionally recover its nanomechanical properties. Elastic modulus and hardness of the fully demineralized outer zone was recovered to about 60% of normal dentin values with PILP applications of two weeks, while calcium phosphate solutions alone did not produce any significant mechanical recovery. [4-7]

Based on FR observed in solution, this study focused on developing PILP-releasing restorative materials that can be utilized clinically. This novel treatment aims to be integrated with a conservative minimally restorative treatment facilitating long-term collagen FR and repair of the natural tissue while in place as a restoration. FR of dentin requires recovery of the mineral content and mechanical properties. FR by the polymer-induced liquid-precursor (PILP) process has been demonstrated in the laboratory by using pAsp.[8-10]

This study compares the ability of Biocem cement (NuSmile, Houston, TX, USA), Biocem modified with polyaspartic acid by addition of pAsp powder at 20% or 40% by weight of Biocem cement. In addition to the resin-modified glass-ionomer Biocem, two pure glass ionomer cements, Fuji I and Fuji IX (GC Corp., Tokyo, Japan), were tested in their ability to restore the mechanical properties of demineralized dentin matrix in artificial lesions (Table 1).

Methods:

Permanent, fully-formed human third molars were extracted as part of a clinical treatment plan and obtained from the UCSF dental hard tissue specimen core according to protocols approved by the UCSF Committee on Human Research. After extraction, the teeth were sterilized with gamma radiation and stored intact in de-ionized water and thymol at 4 degrees Celsius. Dentin blocks measuring 6.8 mm in diameter and 3-4 mm in thickness were cut from the mid-coronal region of the selected teeth perpendicular to the tubule direction using a 9.5mm drill bit. The specimen surfaces to be exposed to

artificial caries formation and remineralization were ground with SiC abrasive papers from 320 to 1200 grit, and then polished with aqueous diamond suspensions (Buehler, Lake Bluff, IL) of 6.0, 3.0, 1.0, and 0.25 μm particle sizes. Each specimen surface was covered with nail varnish (Revlon Nail Enamel #270, New York, NY) to prevent demineralization except for a window measuring approximately 2.5 x 2.5 mm (Figure 1).

Artificial carious lesions approximately 140 μm deep were induced by exposing the surface to a demineralizing solution consisting of 0.05 M acetate buffer containing 2.2 mM calcium phosphate and adjusted to pH 5.0 for 66 hours, a demineralization treatment determined by prior kinetics studies.[7]

After artificial caries lesions were produced, the specimens (n = 3/group) were remineralized for a period of 2 weeks using restorative material to cover exposed 2.5x2.5mm window. The 5 groups included (n=3/group): Biocem, Biocem + pAsp (20% or 40% by weight), Fuji I GI or Fuji IX (Table 1). Each restorative was handled according to manufacturer instructions. Restorative material was allowed to be fully set prior to proceeding to next step.

Another group of the specimens were remineralized for a period of 6 weeks. The groups included (n=3/group): Biocem, Biocem + 20% pAsp and Biocem + 40% pAsp.

Simulated body fluid (SBF) to mimic *in vivo* pulpal fluid was prepared according to the composition shown by Kokubo et al.[11] Nail varnish from the bottom side (opposite

restorative material) of each sample was removed and etched for 60 seconds and rinsed with distilled water. Each sample was placed in a 50mL conical centrifuge tube with 40mL of SBF. Each group of specimens were incubated at 37 degrees Celsius while being continually rocked for a period of 2 or 6 weeks. The pH of SBF tested at the start of the remineralization cycle was 7.4.

After remineralization period was complete, samples were removed, gently rinsed with de-ionized water and dried. The blocks containing the lesions were embedded in room temperature curing epoxy (Epoxicure, Buehler, Ltd, Lake Bluff, IL). Each specimen was sectioned through the 2.5x2.5 mm window. The window was ground with SiC abrasive papers from 320 to 1200 grit, and then polished with aqueous diamond suspensions (Buehler, Lake Bluff, IL) of 1.0, and 0.25 μm particle sizes. (Figure 1).

The cross sections were imaged with reflective light microscopy to determine the amount of dentin collapsed after the remineralization cycle. The cross sections were imaged at 4x with an Olympus BH-2 microscope (Olympus America Inc., San Diego, CA). The lesion depth, the distance between the original intact dentin surface protected by the nail varnish protected and exposed treated dentin, was measured using OMAX ToupView (Touptek Photonics, Zhejiang, P.R.China). The shrinkage from junction of restorative surface to surface of lesion was measured in micrometers for each specimen, which in some cases was zero.

This data was compared to controls consisting of the non-remineralized lesion group (n = 3) and normal untreated dentin from the area protected by nail varnish during demineralization. Pairwise t-test was used with significance level was set as $P<0.05$.

Subsequently, select specimens were studied with AFM-based nanoindentation to evaluate the mechanical properties recovered. Indentations were performed perpendicular to the lesion using a Berkovich diamond testing probe (Hysitron Inc, Minneapolis, MN) with a (Nanoscope IIIa Controller, Bruker). Measurements were taken in parallel lines spaced by five micrometer (μm) and were executed by moving sagittally between the interface of dentin and cement to sound dentin, covering the length of approximately 140 μm.

Results:

This initial study tested the applicability of a commercial cement, Biocem, modified by the addition of pAsp to support remineralization of collagen and reinforcement of demineralized dentin. The modified cements, BC20 and BC40, were compared to commercial dental cements BC, F1 and F9. The addition of pAsp to BC did not affect the setting behavior of the cement. BC20 and BC40 set upon light-curing for 20 seconds. When exposed to SBF solutions the cements did not show visible signs of dissolution in the 2 week or 6 week of remineralization treatment. While BC, as a resin-modified glass ionomer cement, lacks largely any porosity, both BC20 and BC40 showed substantial amount of pores ranging from 1 to 20 μm in size (Figure 2c and 2d). Porosity was minimal in F1 and F9 (Figure 2e and 2f). Using light microscopy on

polished specimens, the integrity of the cement-dentin interface and the occurrence of shrinkage of the lesion after the remineralization treatment was analyzed. All commercial cements, BC, F1 and F9, showed a gap developing between the cement and the lesion which is due to a collapse of the demineralized portion of dentin, e.g. shrinkage. For the 2 week group, the size of the gap in BC and F1 was not statistically different from the shrinkage observed on demineralized dentin, but was somewhat reduced in F9 (Figure 2). Further reduction of shrinkage was observed when pAsp was added to the cement. However, that shrinkage appeared to be predominantly related to compression of the demineralized zone when the cement was placed and pushed down the demineralized matrix, which can be determined when comparing to the level of normal dentin in Figure 2. The interface between BC20 and BC40 to the underlying dentin was intact in most of these specimens and no gap formation was observed by light microscopy after dehydration of the samples (Figure 2c and 2d). To evaluate the improvement the cement-induced remineralization process may have had on the demineralized dentin. The shrinkage from junction of restorative surface to surface of lesion was measured in micrometers for each specimen and mean shrinkage was plotted (Figure 3). Then the measured shrinkage as a percentage value compared to the demineralized control sample was plotted (Figure 4). This illustrates that BC20, BC40 and F9 significantly reduced the shrinkage after the two weeks of remineralization treatment, with BC40 showing the highest recovery of tissue stability. The shrinkage was not reduced by Biocem only treatments, but was reduced by 54% ($P<0.01$) and 75% ($P<0.01$) with 20% or 40% pAsp added, respectively. Fuji I and Fuji IX reduced shrinkage by 24% ($P>0.05$) and 40% ($P<0.05$), respectively (Table 2).

A longer remineralization period of 6 weeks was completed for the BC, BC20, and BC40 groups. Porosities in the modified Biocem were visible and similar in size in both groups. The interface between BC20 and BC40 to the underlying dentin was intact in most of these specimens and however gap formation was observed by light microscopy after dehydration of the samples (Figure 5b and 5c). Again the shrinkage from junction of restorative surface to surface of lesion was measured in micrometers for each specimen and mean shrinkage was plotted (Figure 6). The shrinkage was not reduced by Biocem only or BC20 treatments, but BC40 reduced shrinkage by 31% ($P<0.02$) with 40% pAsp added (Table 3). This illustrates that addition of 40% by weight pAsp to Biocem significantly reduced the shrinkage after six weeks of remineralization treatment.

Shrinkage after 2 weeks v. 6 weeks of remineralization was compared (Figure 7). Shrinkage did not appear to be reduced with a longer remineralization period. Pairwise t-test between groups yielded a significant comparison for B40 group ($P<0.05$).

Nanoindentations were performed across the demineralized and remineralized lesions comparing the demineralized tissue to BC40 remineralization treatments of 2 weeks (Figure 8). Comparing the average elastic modulus values from the surface of the lesion into the demineralized dentin, a very soft outer zone to about 80 μm depth was observed which was associated with fully demineralized collagen (Burwell et al).[7] Moduli gradually increased, as residual mineral reinforced the collagen matrix at increasing

amounts between 80μm and 140μm depth when values of normal dentin (18 GPa) were reached (Figure 8). Restoring the lesion with BC40 and exposure to SBF solution for 2 weeks resulted in increased E-moduli values. The graded region shifted towards the outer zone, and normal modulus values were reached at about 90 μm depth. The outer zone showed a slow gradual increase from about 0.3 GPa to about 6 GPa at 80 μm depth indicating that the mineralization process was initiated.

Similar, in the 6 week treatment with BC 40, moduli gradually increased as residual mineral reinforced the collagen matrix at increasing amounts between 50μm and 140μm depth when values of normal dentin (18 GPa) were reached (Figure 9). The graded region shifted towards the outer zone, and normal modulus values were reached at about 50 μm depth. Restoring the lesion with BC40 and exposure to SBF solution for 2 weeks and 6 weeks resulted in increased E-moduli values.

Discussion:

This study tested the effect of a series of glass ionomer cements on their ability to functionally remineralize artificial lesions in dentin. Shrinkage measurements, which evaluate the amount of collapse of dentin matrix upon drying, were used as indicator for insufficient mineralization. Shrinkage was significantly reduced when pAsp was added to BC in 2 week remineralization experiments, indicating that mineral was reintroduced into collagen fibrils in the demineralized zone. At 6 weeks of remineralization, the BC40 group showed significant remineralization and reduction in the collapse of the collagen.

The BC20 group also had a reduction in shrinkage as compared to the demineralized control, however was not statistically significant.

These findings may indicate the mineral was deposited into the previously collapsed collagen. Then fibrils are being reinforced with this new mineral, which lead to a limitation in the further collapse of the artificial lesion. This indicates that when compared to the demineralized control group, there was a deposition of mineral beyond just on the surface of the lesions.

Nanoindentation before and after treatments of demineralized dentin with BC40 confirmed this finding as mechanical properties recovered across the lesion after 2 weeks of treatment and continued for at least 6 weeks. Restoring the lesion with BC40 and exposure to SBF solution for 2 weeks and 6 weeks resulted in increased E-moduli values. This suggests that apatite mineral is deposited in the intrafibrillar sites in collagen and is restoring the functionality of dentin.[5] This effect is expected to continue with time and to lead to further, possibly complete recovery of the dentin. To definitively determine the presence of the newly remineralized apatite in the cement-dentin interface, it is necessary to perform compositional analysis to identify the presence and relative molar ratios or relationship between calcium, phosphate, and hydroxyl components. TEM analysis in the near future may shed some light.

One limitation of study is length of remineralization was limited to 6 weeks, for further studies, longer periods of remineralization should be investigated. Additionally it would

be ideal to test natural caries lesions. The presence of bacteria, infected versus affected dentin may have an effect on the PILP process. In this experiment, the artificial lesions tested were about 140 μm, however natural lesion may well exceed this depth. The light microscope images of pAsp modified Biocem showed porosities in the dental cement. These porosities may have an effect on the properties of the material itself such as bond strength or compressive strength.

Moving forward, if the PILP process can be adapted into a clinical delivery system, it shows great promise for the future of minimally invasive dentistry. Looking forward, rather than modifying restorative material, the PILP process may be achieved via delivery of polyacids as a liner or base.

Conclusions:

This is the first study to show the benefits of pAsp addition to a commercial glass ionomer cement.

1. The release of PILP-components at the cement-dentin interface led to functional remineralization of collagen fibrils and initiated the repair of demineralized dentin
2. The use of PILP-releasing cements appears a suitable restorative application to translate the PILP-method into the clinic and allow for dentin remineralization in carious dentin and the conservation of natural tissue.

This study was supported by NIH/NIDCR grant RO1-DE016849 and UCSF Catalyst Award.

Table 1: Treatment Groups with sample abbreviations

1. Biocem Control - (BC)
2. Biocem + 20% pAsp - (BC 20)
3. Biocem + 40% pAsp - (BC 40)
4. Fuji I (GI) - (F1)
5. Fuji IX (GI) - (F9)
6. Demineralized dentin - (Demin control)

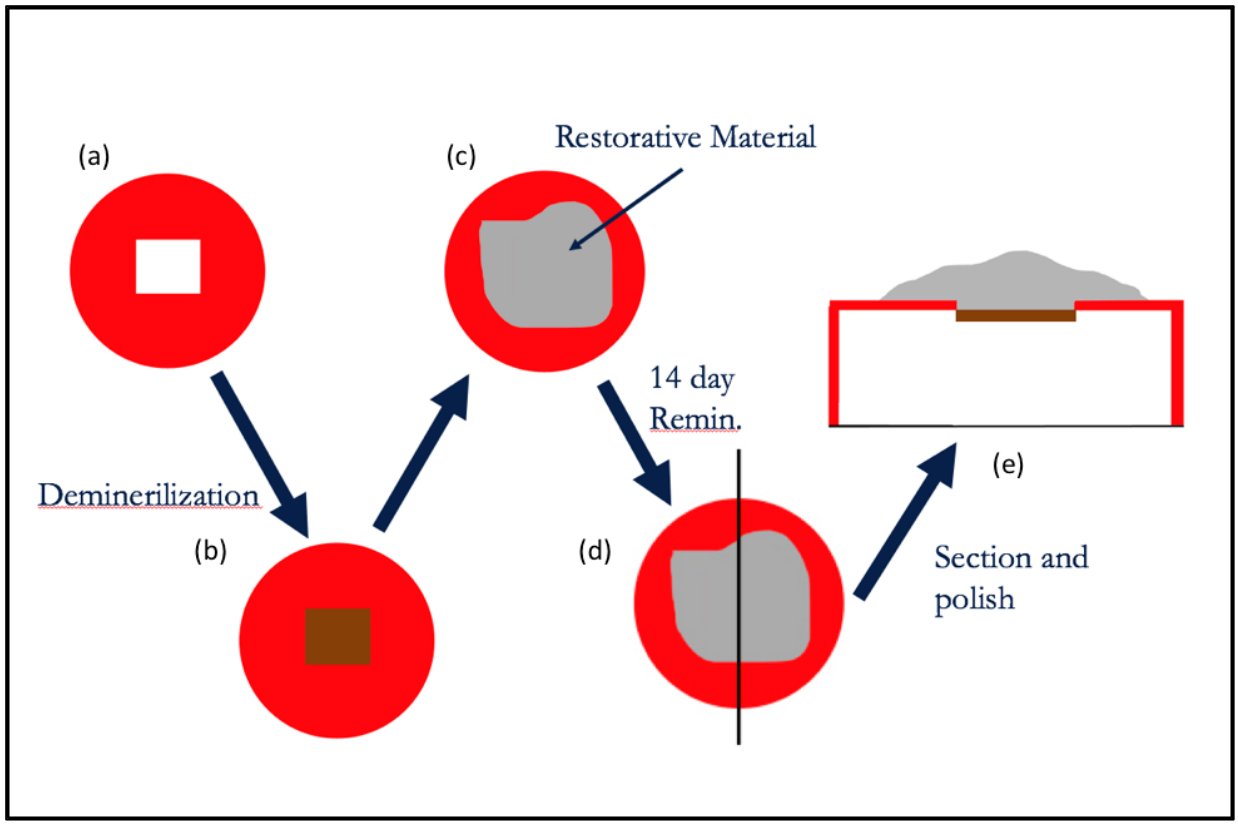

Figure 1: Specimen Preparation

(**a**) Dentin block covered in nail varnish with the exception of a 2.5x2.5mm window exposing healthy dentin. (**b**) Artificial carious lesions approximately 140 mm deep created by exposing the surface to a demineralizing solution consisting of 0.05 M acetate buffer containing 2.2 mM calcium phosphate and adjusted to pH 5.0 for 66 hours. (**c**) Specimen restored using one of treatment groups #1-5, for Demin group (#6) sample left untreated. (**d**) Specimen sectioned through 2.5x2.5 window (**e**) Sagittal view of specimen after sectioned, ready for optical microscopy.

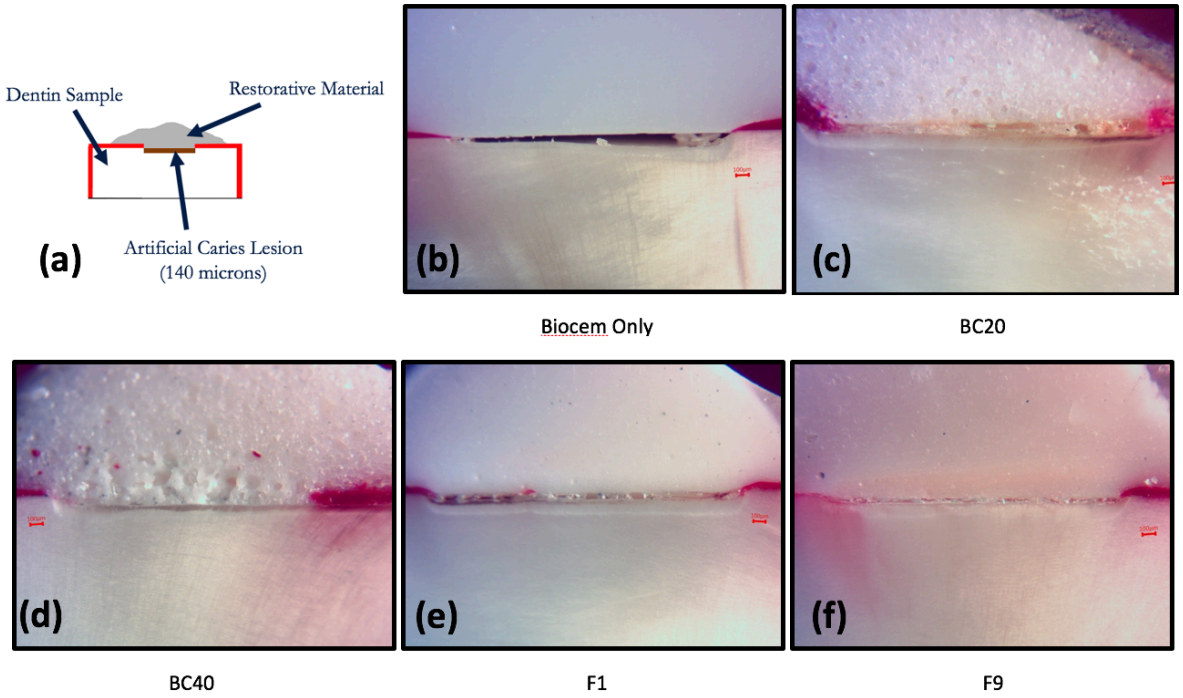

Figure 2: Optical microscopy (4x) of shrinkage at 2 week (14 days) of treatment

Shrinkage occurs upon dehydration of sample due to collapse collagen matrix in the demineralized dentin and formation of a gap. Shrinkage is measured as the distance (gap) from restorative material to surface of lesion. Shrinkage is reduced when lesion is functionally remineralized (FR). (**a**) Figure showing orientation of specimens: restorative material, artificial caries lesion, red nail varnish (**b**) Biocem only (**c**) Biocem+20% pAsp (**d**) Biocem+40% pAsp (**e**) Fuji I (**f**) Fuji IX

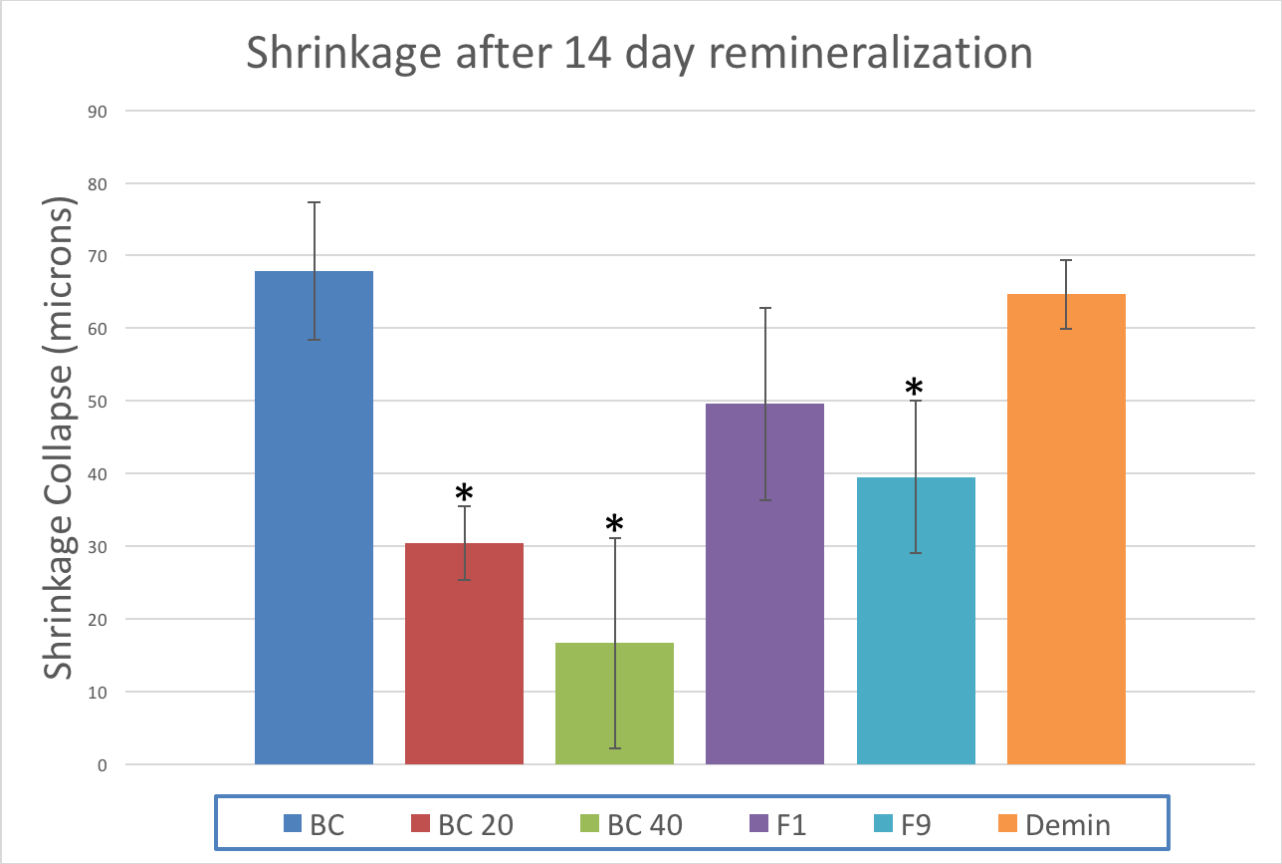

Figure 3: Shrinkage after 2 week (14 day) remineralization

Shrinkage Collapse (micrometers) and Standard deviation illustrated from Table 2. Statistical significance ($P<0.05$) from pairwise t-test compared to Demin control group show with (*).

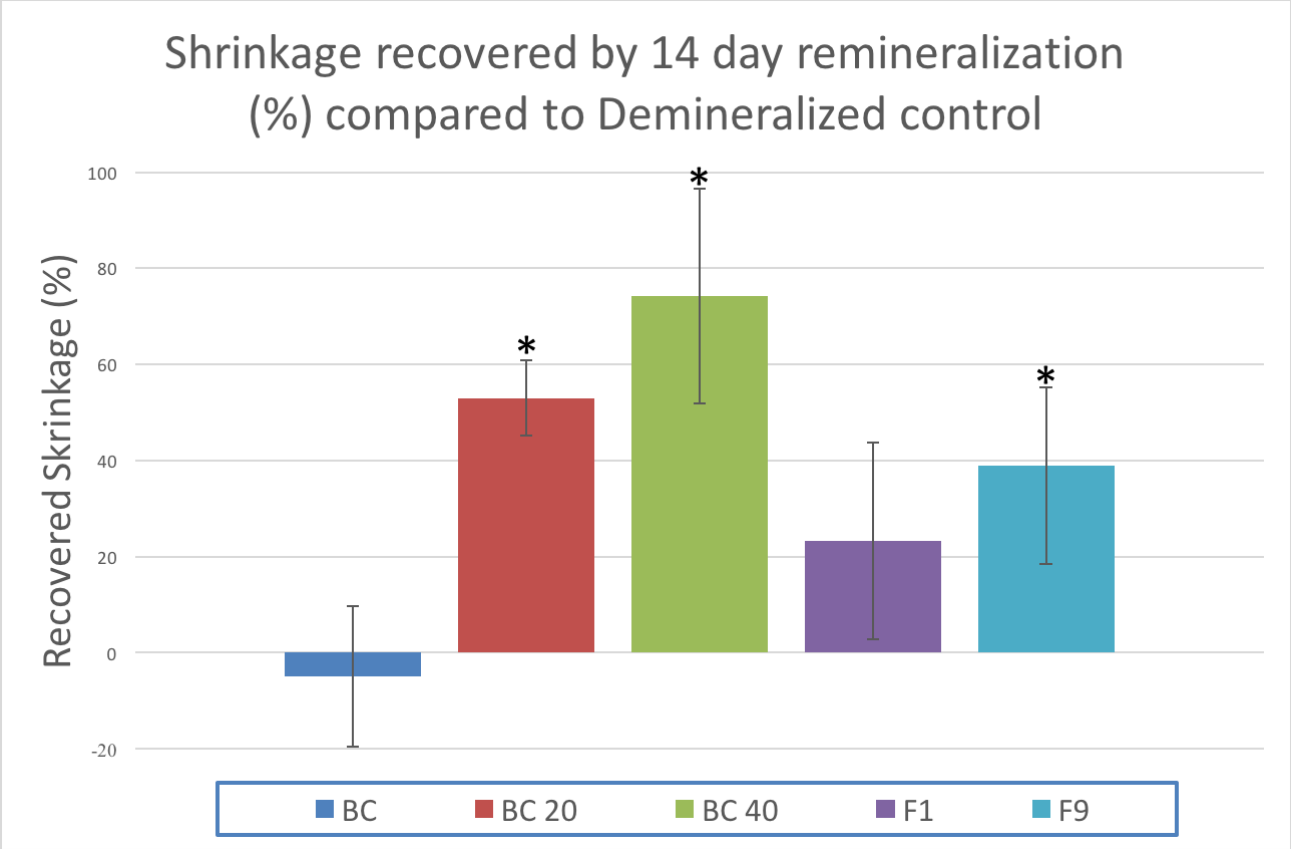

Figure 4: Shrinkage recovered by remineralization (%) compared to Demineralized control.

Graph illustrating the shrinkage recovered (%) when compared to Demin control group. This is the amount of shrinkage that was reduced by each group when compared to Demin control. Statistical significance ($P<0.05$) from pairwise t-test compared to Demin control group show with (*).

Table 2: Shrinkage after 2 week (14 day) remineralization (micrometers)

Group	BC	BC 20	BC 40	F1	F9	Demin Control
Average Shrinkage Collapse (n=3) (micrometers)	67.85	30.40	16.66	49.58	39.50	64.67
Standard Deviation	9.49	5.09	14.44	13.21	10.50	4.73
P-Value (pairwise to Demin control)	0.63	**0.001***	**0.006***	0.14	**0.02***	-

Average Shrinkage collapse and Standard deviation for groups shown in table.
P-Values from pairwise t-test comparison to Demin control group show for BC, B20, BC40, F1, F9 groups.
P<0.05 marked with (*).

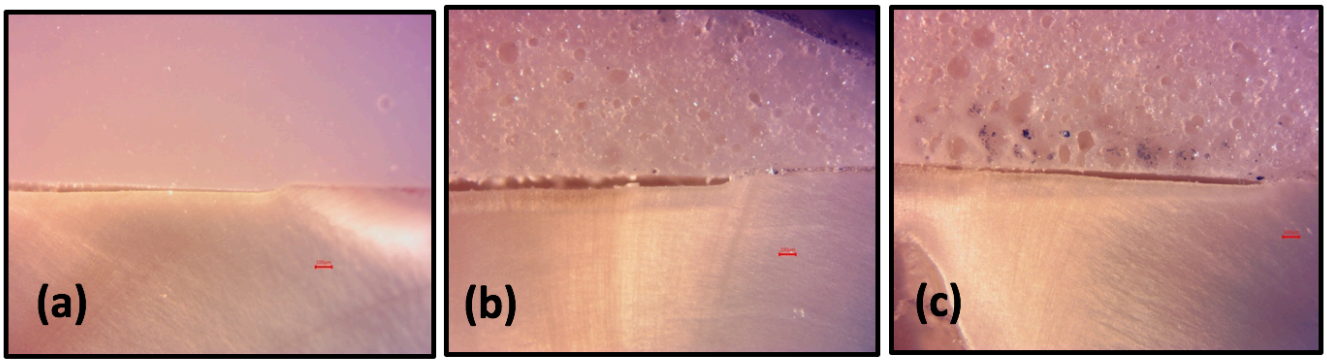

Figure 5: Optical microscopy (4x) of shrinkage at 6 weeks of treatment

Shrinkage occurs upon dehydration of sample due to collapse collagen matrix in the demineralized dentin and formation of a gap. Shrinkage is measured as the distance (gap) from restorative material to surface of lesion. Shrinkage is reduced when lesion is functionally remineralized (FR). (**a**) Biocem only (**c**) Biocem+20% pAsp (**c**) Biocem+40% pAsp

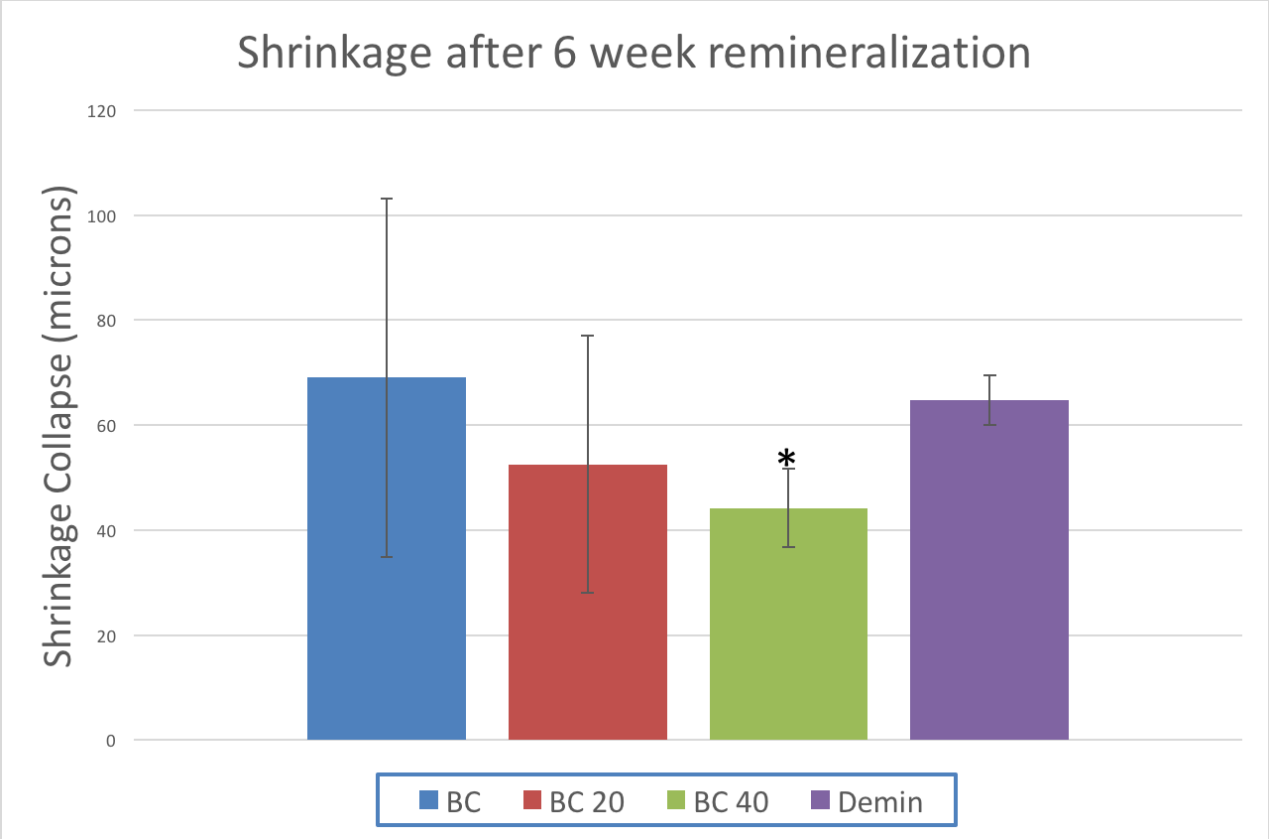

Figure 6: Shrinkage after 6 week remineralization

Shrinkage Collapse (micrometers) and Standard deviation illustrated from Table 3. Statistical significance ($P<0.05$) from pairwise t-test compared to Demin control group show with (*).

Table 3: Shrinkage after 6 week remineralization (micrometers)

Group	BC	BC 20	BC 40	Demin Control
Average Shrinkage Collapse (n=3) (microns)	68.99	52.52	44.19	64.67
Standard Deviation	34.18	24.53	7.42	4.73
P-Value (pairwise to Demin control)	0.83	0.44	**0.02***	-

Average Shrinkage collapse and Standard deviation for groups shown in table.
P-Values from pairwise t-test comparison to Demin control group show for BC, B20, BC40 groups.
P<0.05 marked with (*).

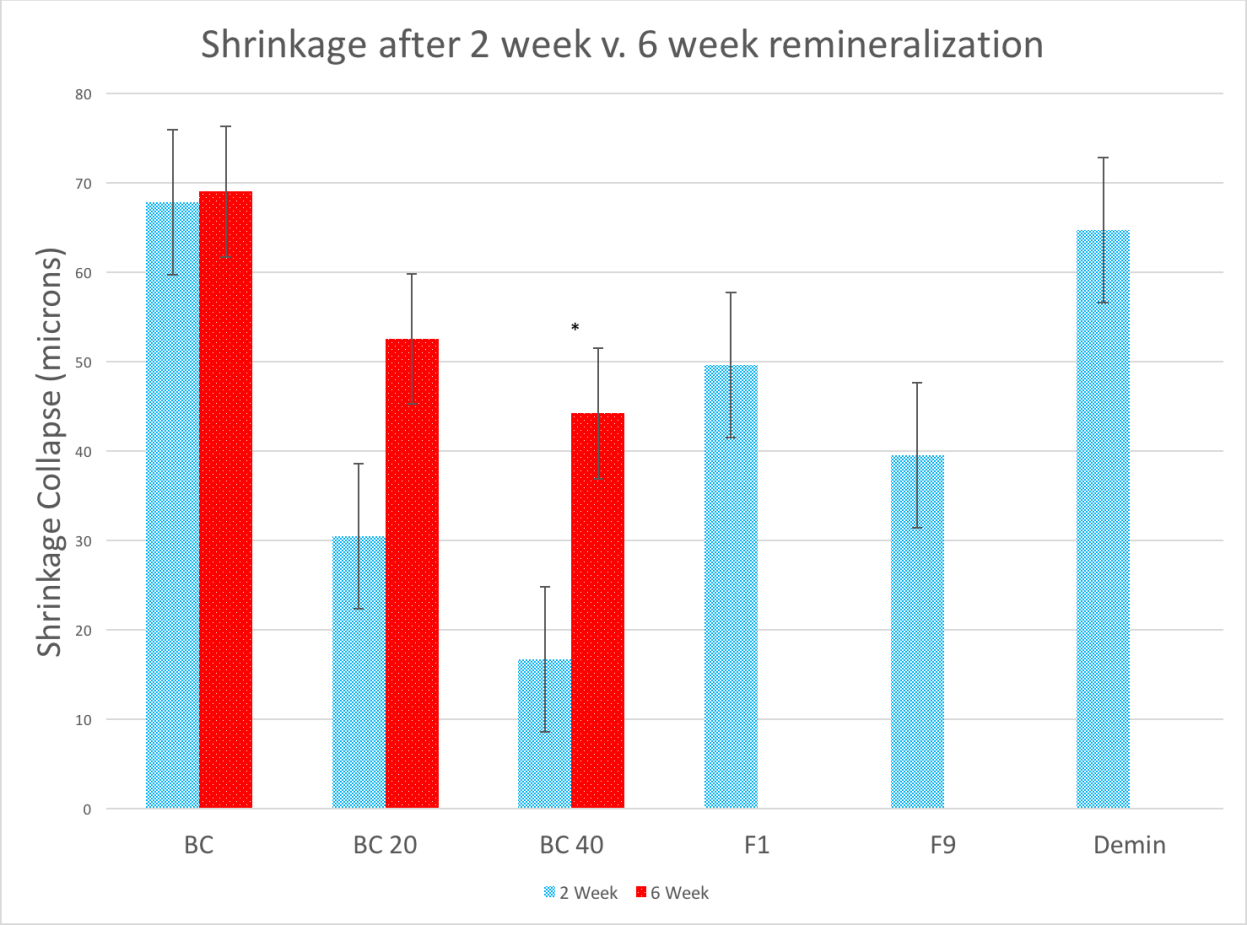

Figure 7: Shrinkage of 2 week versus 6 weeks remineralization

Shrinkage Collapse (micrometers) and Standard deviation illustrated from Table 2 and 3. Statistical significance ($P<0.05$) from pairwise t-test compared between two groups show with (*).

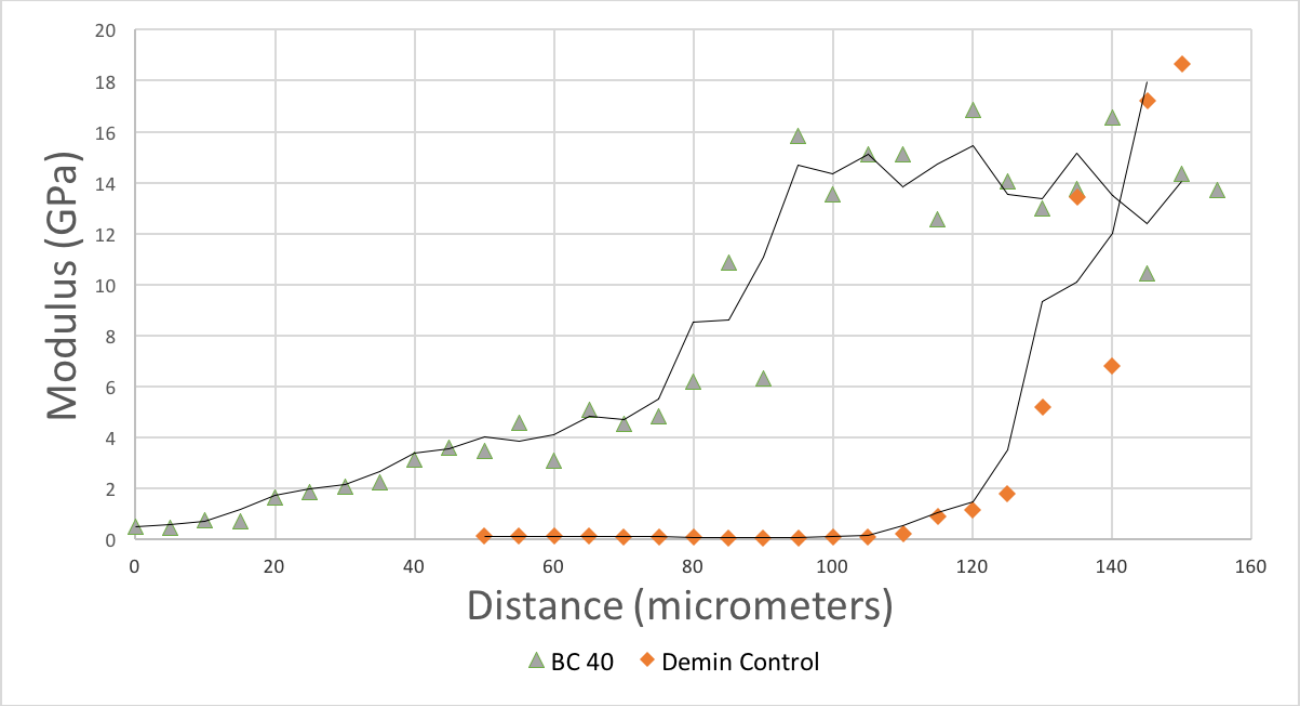

Figure 8: Elastic Modulus after 2 week remineralization

Elastic modulus profiles across artificial lesion from specimen surface towards normal dentin comparing demineralized dentin before and after BC40 application and remineralization for **2 Weeks** (14 days**)**.

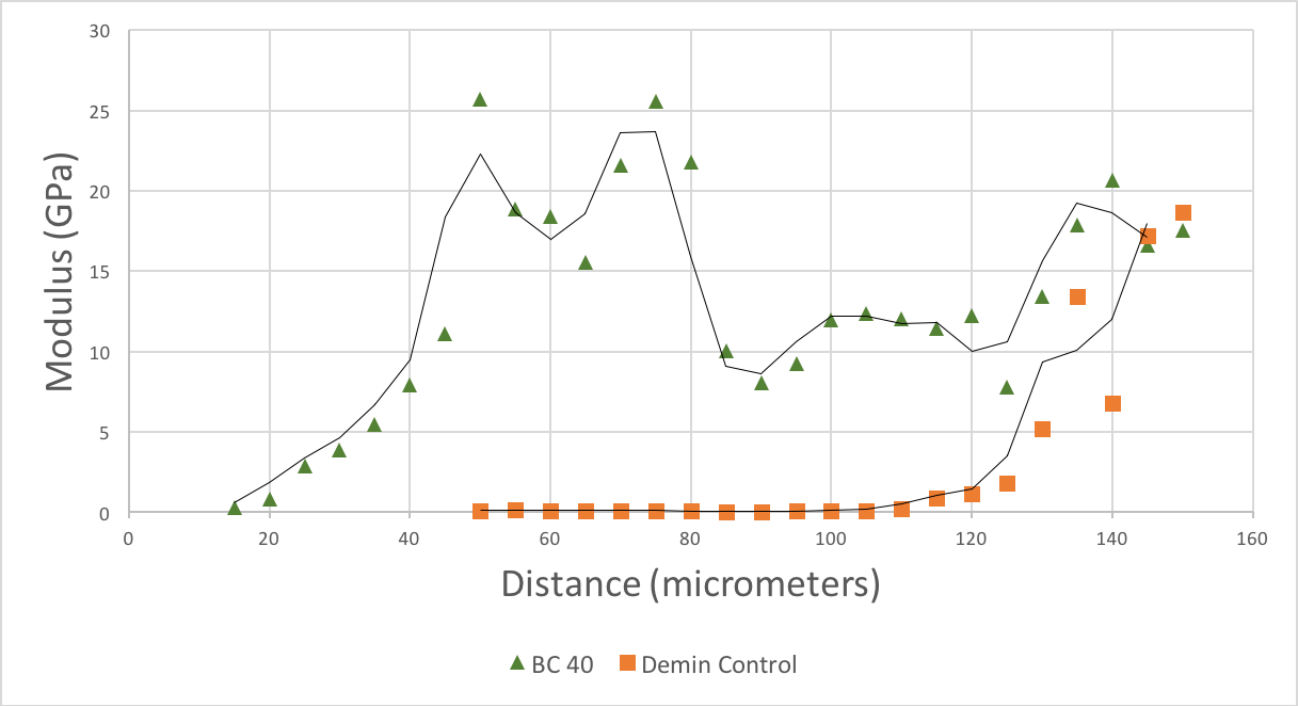

Figure 9: Elastic Modulus after 6 week remineralization

Elastic modulus profiles across artificial lesion from specimen surface towards normal dentin comparing demineralized dentin before and after BC40 application and remineralization for **6 weeks**.

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