Potential of Barnacle Cement in Dentistry

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Summary

The barnacle adheres tenaciously to underwater substrata by secreting cement. Lauded for its mechanical properties and water insolubility, barnacle cement was further examined for its potential in dental application. Its biocompatibility, speed of polymerization, aesthetic appeal and acid-resistance was investigated. It was observed to blend in with the tooth's natural color. Rate of barnacle cement polymerization, however, was found to be slower than existing adhesives and it was not acid-resistant. Thus, its applicability as a dental adhesive is limited. With further research on enhancing acid-tolerance and polymerization rate, barnacle cement could still be a potential dental adhesive.

(97 Words)

Abstract

The barnacle, a marine arthropod, adheres tenaciously to underwater substrata by secreting cement. Lauded by past researchers as a prospective dental adhesive due to its mechanical properties and water insolubility, barnacle cement was further examined in this study for its potential in dental applications. The biocompatibility, speed of polymerization, aesthetic appeal and acid-resistance of this cement was investigated. To study acid-resistance, tooth samples coated with barnacle cement were immersed in Coke and visual alterations of barnacle cement on tooth surfaces were observed at 24-hour intervals. The effect of Ca^{2+} ions and different electromagnetic radiation (UV and infrared radiation) on cement polymerization time was recorded based on visual and timed assessments of the cement polymerizing on teeth. To study the biocompatibility of barnacle cement, *Caenorhabditis elegans* was exposed to barnacle cement and the mortality rate was recorded after 30min, 1hr, 2hr and 3hr. Barnacle cement did not induce death of *C. elegans* and was observed to blend in with the tooth's natural color. The rate of barnacle cement polymerization, however, was found to be slower as compared to existing adhesives and it did not provide much protection to teeth from acidic conditions. Hence, given current understanding of barnacle cement, we concluded that the applicability of barnacle cement as a dental adhesive is limited. With further research on enhancing acid tolerance and rate of polymerization, barnacle cement could still be a potential dental adhesive.

Introduction

Today, dental sealants are placed on pits and fissures of the teeth to provide a physical barrier against cariogenic bacteria. Enamel and dentin surfaces, however, are wet in the mouth, inhomogeneous in composition, rough, covered with various types of debris, and have relatively low surface energy. These conditions obstruct adhesion and reduce the effectiveness of sealants in treating caries (Phillips, 1988). For example, salivary enzymatic hydrolysis of ester linkages in resin-based sealants poses a challenge to their durability as a sealant (Azarpazhooh & Main, 2008). In addition, in vivo studies have shown that certain dental adhesives (*Admira Bond*, *Gluma Comfort Bond*) have cytotoxic effects (Koulaouzidou, 2009).

For dental applications to be clinically successful, the adhesive must bond permanently, be biocompatible, be of high colour stability, and can be handled conveniently by dentists (Phillips, 1988). Also, the adhesive should be resistant to acids and able to withstand mechanical forces. Most importantly, a strong adhesive that is insensitive to water contamination would be convenient in dental practice (Bishara, 1975). One possible adhesive to be considered is the barnacle cement.

Barnacles permanently adhere to nearly any solid substrata in the marine environment using secreted cement. Barnacle cement is 90% protein and is able to resist both enzymatic and chemical degradation at surrounding temperatures (Khandeparker et al, 2006). Being insoluble, able to rapidly polymerize and adhere to a variety of substrates in aqueous environment, barnacle cement may be a potentially powerful dental adhesive. Studies have shown that barnacle cement compared favorably with other dental adhesives in terms of bonding strength. Its ability to polymerize underwater is also an advantage. When placed on a wet surface, barnacle cement was favored over other commercial dental adhesives such as zinc phosphate cement (Despain et al, 1973). Pull-off strength for barnacle cement on polystyrene surface is substantial, averaging 1.05×10^5 Nm^{-2} with a range from 0.14×10^5 Nm^{-2} to 2.79×10^5 Nm^{-2} (Dougherty, 1990). Also, the bond strength of barnacle cement on wet plastic surface has been measured to be 0.48×10^5 Nm^{-2} (Bowlin, 2008).

However, there are no studies regarding the application of barnacle cement as a dental adhesive and sealant. Past researches advocated the potential of barnacle cement in dentistry solely based on its mechanical properties and biochemical composition. Yet, other properties vital in dentistry such as acid resistance, biocompatibility, speed of polymerization and aesthetic appeal have not been studied in context. Therefore, our study aims to investigate these properties of barnacle cement and determine its applicability as an alternative dental adhesive and sealant.

Hypothesis - *Amphibalanus amphitrite* barnacle cement possesses the necessary properties to act as a dental adhesive and sealant.

Objective - To study the properties of barnacle cement - biocompatibility, acid resistance, speed of polymerization and aesthetic appeal so as to determine its applicability as an effective and novel alternative dental material.

Methods and Materials

I) Preparation of Barnacle Cement

Dyeing and Extraction of Barnacle Cement

To obtain a clear visualization of barnacle cement on the tooth surface, a simple but novel experiment was conducted. Thirty droplets of Cochineal Red (E120) food coloring were added to 175ml of seawater and *Amphibalanus amphitrite* barnacles were placed in dyed seawater for at least a day before cement extraction. The extraction method used was adapted from Dr. Gary Howard Dickinson (Dickinson et al. 2009). *Amphibalanus amphitrite* barnacles were rinsed with de-ionized water, dried with a Kimwipe, and then sat in air for 2 to 3 hours. A dissecting needle was used to pick gently at the periphery of the base plate while it was gently squeezed to stimulate the secretion of 1-2 μl drops of barnacle cement. The barnacle cement was then extracted using a micropipette.

II) Evaluation of Barnacle Cement in Dental Applications

A) Biocompatibility Test

A mixture of *Caenorhabditis elegans* was prepared using 4ml of PBS buffer to wash a culture of *C. elegans* stock. The mixture, containing *C. elegans*, *Escherichia coli* and PBS buffer, was transferred into a 15ml Falcon tube and centrifuged at 2000 rpm for 10 minutes to separate the *C. elegans* and *Escherichia coli*. 2ml of supernatant containing *E. coli* was removed and an additional 2ml of PBS buffer was added. The tube was vortexed to mix the solution well. 50μl of solution containing *C. elegans* was mixed with 5μl of barnacle cement and 10μl of Trypan blue dye in a 1.5ml Eppendorf tube. A control tube without barnacle cement was also prepared. The tubes were then incubated at 25°C for 30 minutes. After incubation, the mixture was extracted and placed in a 48-well microtiter plate (Iwaki). The number of *C. elegans* was counted under a stereomicroscope (Zeiss Stemi DV4) to determine mortality rate, with dead *C. elegans* stained blue by Trypan blue dye. The above procedure was repeated at different incubation times (1/2 hr, 1 hr, 2hrs, 3 hrs) with triplicates each.

B) Morphological Analysis

2 μl of barnacle cement was placed on tooth surfaces and the process of polymerization was observed under a stereomicroscope (Olympus SZ-CTV). The polymerized barnacle cement was then observed for its texture, color, and its general appearance on the tooth.

C) Rate of Polymerization Test

1. Determining Optimal Volume of Barnacle Cement

The volume of barnacle cement used affects the time required for it to polymerize. However, if too little was used, cavities in teeth might not be sealed properly. Hence, a balance between the amount of barnacle cement used and the time for polymerization had to be reached.

0.5μl of un-dyed barnacle cement extracted from *Amphibalanus amphitrite* barnacles was placed on a microscope slide and observed under a light microscope at room temperature. The process and time taken for polymerization of barnacle cement were observed and recorded. The above procedure was then repeated with different volumes of barnacle cement (1.0, 2.0, 3.0, 4.0μl) to determine the optimum volume of barnacle cement and its corresponding time taken for polymerization. 2.0μl was found to be the optimal volume.

2. Ensuring the Integrity of Dyed Barnacle Cement

2.0μl of dyed barnacle cement was extracted and placed on a microscope slide for observation. The time taken for protein structures to first appear (start of polymerization) and their saturation (completion of polymerization) were recorded and analyzed to ensure that the process and time of polymerization of the dyed barnacle cement did not vary significantly from the un-dyed barnacle cement.

3. Investigating Polymerization under Various Conditions

To simulate oral conditions, each tooth was coated with saliva at 37°C. An additional layer of 1% Ca^{2+} solution was added on the tooth surface. Barnacle cement was then applied and time taken for complete polymerization was recorded. The above steps were repeated under exposure to radiation [UV light (254nm) and infrared radiation (1000nm)] a distance of 0.3 m for 40s. A control for all factors without the addition of 1% Ca^{2+} solution was also carried out. Triplicates of each tooth under each set of conditions were observed.

D) Acid Resistance Test

1) Using acid-resistant red nail varnish, 4 windows were demarcated on each tooth, 2 on the crown surfaces and 2 on the root surfaces.

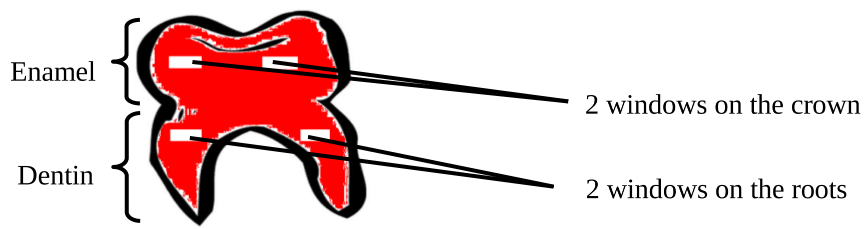


Fig 1. Diagram of demarcated tooth

3) Pictures of the windows were then taken using a stereomicroscope to act as baselines for comparison.

4) One of the 2 windows on the crown and the root was selected randomly and coated with barnacle cement.

5) The other window, coated with transparent, acid-resistant nail varnish, functioned as a positive control.

6) Using the stereomicroscope, pictures of the windows were taken again for observation and comparison.

7) Tooth samples were then adhered to microscope slides.

8) The slide was then placed on top of a beaker, with the tooth submerged in the solution, as shown in Fig 2.

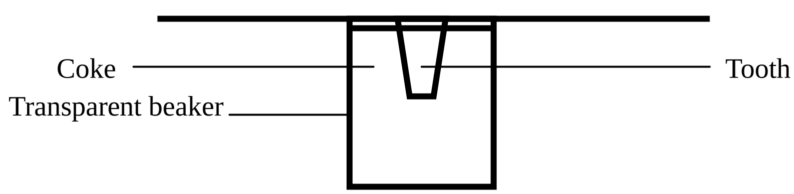


Fig 2. Schematic diagram of set up

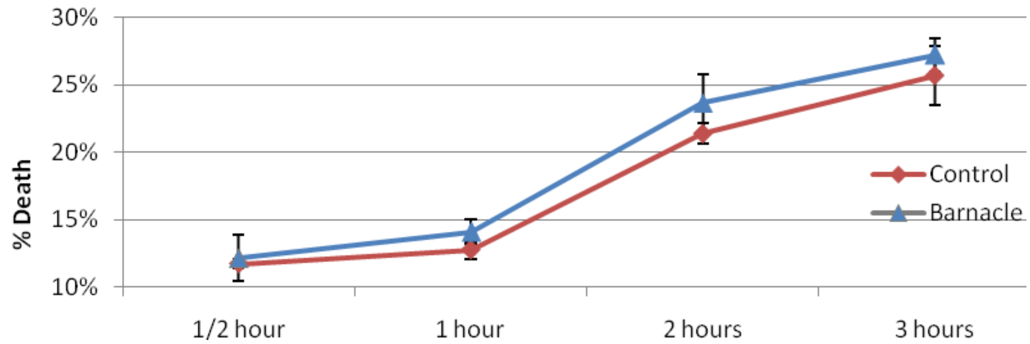
9) The pH of Coke was measured and each tooth sample was submerged in it for 72 hrs.

10) The Coke was changed and pH re-measured at 24-hour intervals.

11) The teeth were then gently rinsed and images of the windows were taken using the stereomicroscope.

<u>Results & Analysis</u>

A) Biocompatibility Test



p-value	0.698	0.107	0.164	0.357

Fig 3. Mortality of C. *elegans* against time exposed to barnacle cement (n=3)

Mortality of *C. elegans* was observed to increase over time in both groups: control and those exposed to barnacle cement. The difference between mortality of both groups is small (0.42-2.27%) and does not follow a clear trend across time, implying that the differences may have arisen from random errors. P-values obtained from T-test were significantly larger than 0.005, falling short of the 99.5% confidence level. This suggests that barnacle cement does not have a significant negative impact on the survival of *C. elegans*.

B) Morphological Analysis - Aesthetic Appeal of Barnacle Cement

Fig 4. Barnacle cement on tooth surface (n=3)

On the tooth surface, cement first appears as a clear fluid. As time proceeds, the cement appears to be less wet. As shown in figure 4, polymerized barnacle cement cannot be seen easily on tooth surfaces and matches the shade of the surrounding tooth. Therefore, barnacle cement has a good aesthetic appearance.

C) Rate of Polymerization Test

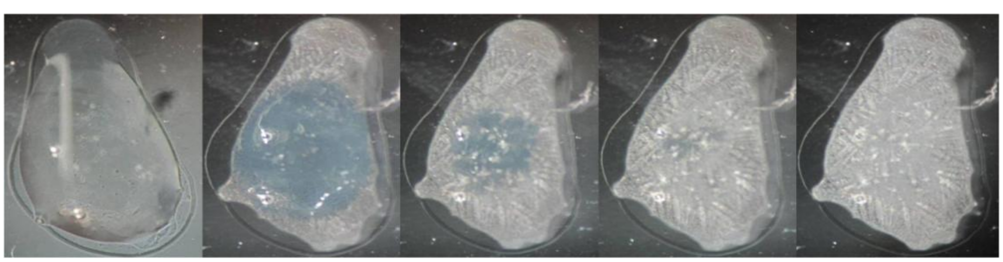


Fig 5. Polymerization of barnacle cement on glass slide (Under Stereomicroscope)

Initially, cement appears as a clear, non-viscous fluid. Polymerization of cement begins at the periphery of the fluid before further occurring towards the centre. Viscosity gradually increases before becoming an opaque and dry solid mass. Appearance of the hardened cement indicates end of polymerization (*ref. App I*)

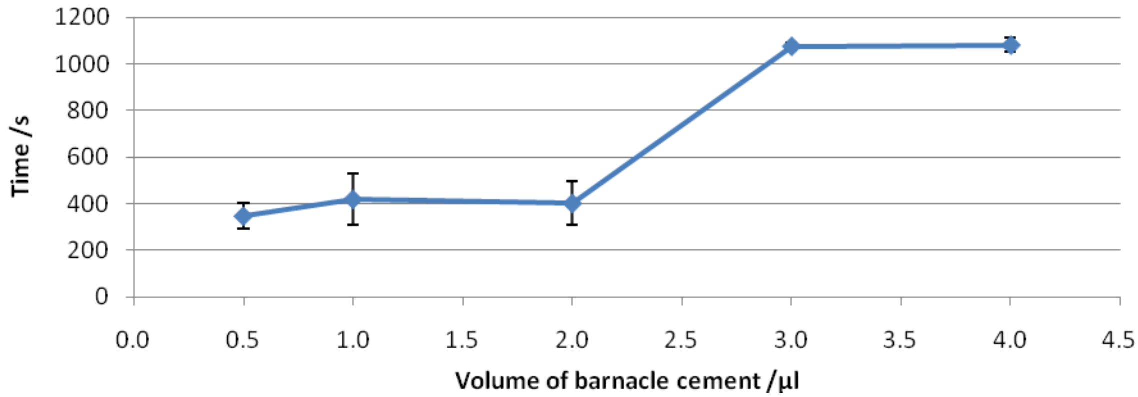


Fig 6. Time taken for barnacle cement to polymerize at varying volume of barnacle cement (n=3)

Time taken for polymerization increased with the volume of cement used. In the first 2µl of barnacle cement used, the time taken for polymerization is roughly constant. Above this threshold, the time of polymerization sharply increased. Hence, the optimum volume of barnacle cement used was determined to be nearest to 2µl.

		1	2	3	4	Avg
Dyed	Start /s	52	45	47	38	45.5
	Stop /s	275	290	309	303	294.25
Undyed	Start /s	54	53	55	51	53.25
	Stop /s	288	302	254	283	281.75

p-value = 0.723

Fig 7. Comparison of time taken for dyed and undyed barnacle cement to polymerize on glass slide (n=4)

The times taken for the first key-like crystallized protein structure to form and for the cement to be saturated with these were recorded. Our statistical analysis of the results via T-test indicates a p-value of 0.723, implying that the difference in time of polymerization between dyed and undyed cement is insignificant. We thus conclude that the addition of red dye to aid visualization will not significantly impact our results in following experiments.

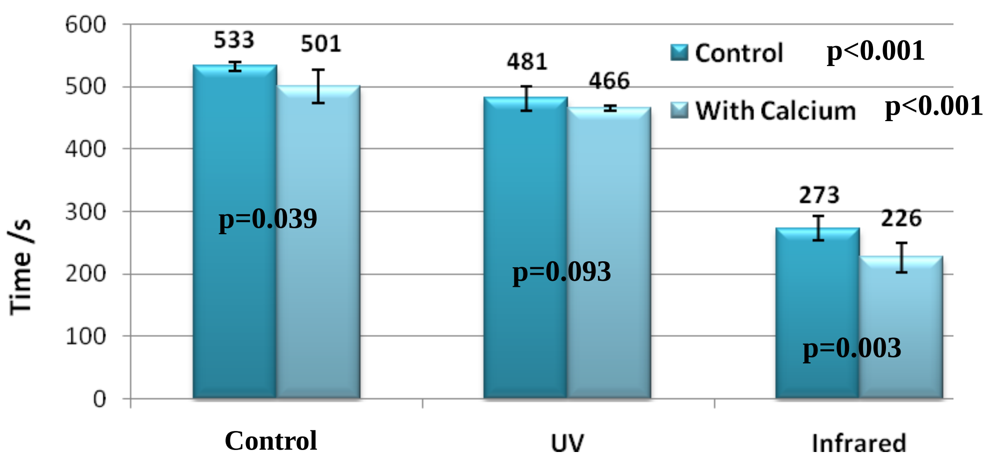


Fig 8. Comparison of time taken by barnacle cement to polymerize on teeth under exposure to calcium, UV and Infrared radiation (n=4)

T-test revealed that the enhancing effect of Ca^{2+} ions was significant only in the infrared group, achieving more than 99.5% confidence. Meanwhile, Anova Two-factor with Replication test among the different radiations suggested that both treatments have improved the time for polymerization significantly. The fastest rate of barnacle cement polymerization was achieved in presence of Ca^{2+} ions and infra radiation.

D) Acid Resistance Test

On Crown Surfaces

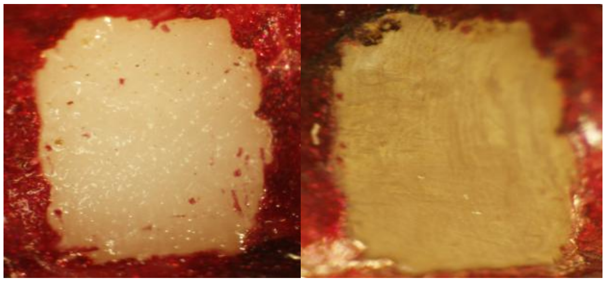


Fig 9A. Window on enamel coated with Barnacle Cement

Fig 9B. Window on enamel immersed in Coke (pH 3.3) for 72

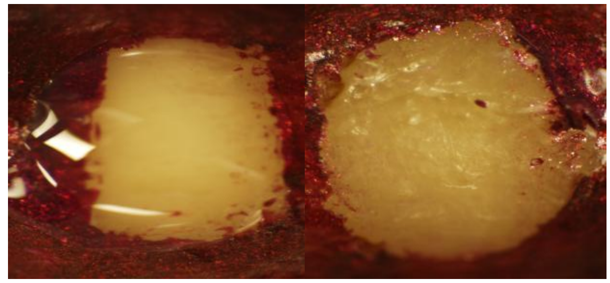


Fig 10A. Window on enamel coated with transparent, acid-resistant nail varnish

Fig 10B. Window on enamel immersed in Coke (pH 3.3) for 72 hrs

On Root Surfaces

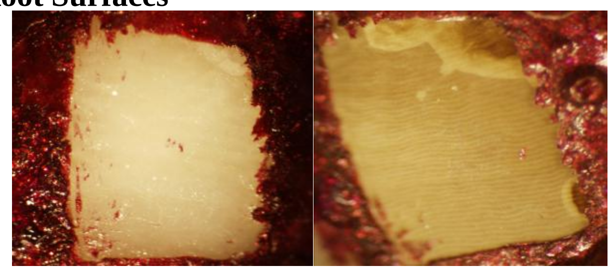


Fig 11A. Window on dentin coated with Barnacle Cement

Fig 11B. Window on dentin tooth immersed in Coke (pH 3.3) for 72 hrs

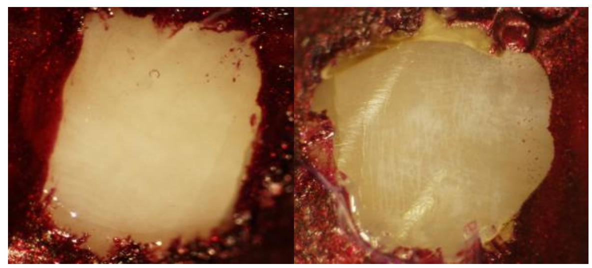


Fig 12A. Window on dentin coated with transparent, acid-resistant nail varnish

Fig 12B. Window on dentin immersed in Coke (pH 3.3) for 72 hrs

As time passed, the surfaces that were coated with a layer of barnacle cement and immersed in coke for 72 hours gradually stained brown and some degree of erosion occurred. However, the surfaces coated with a layer of transparent, acid-resistance nail varnish remained constant.

Discussion/Conclusion

Biocompatibility

We examined the toxicological effects of barnacle cement using *C. elegans*, a common model organism for neurobiology and toxicology studies. Our results showed that barnacle cement does not affect mortality of *C. elegans*, suggesting that barnacle cement exhibits no toxicity to C. *elegans*. This is the first study to show that barnacle cement has the potential, in terms of biocompatibility, as a novel material for medical applications. Barnacle cement can thus be considered over some existing adhesives which have cytotoxic effects. However, biocompatibility of dental materials is characterized by many parameters such as mucosal irritation, mutagenicity, carcinogenicity, etc (Hauman et al, 2003). Low deaths over a short timeframe do not necessarily imply that barnacle cement is completely safe to humans in the long run. To make a conclusive stand on the biocompatibility of barnacle cement, a clinical trial over a longer period of time may be needed.

Rate of barnacle cement polymerization

In dentistry, light curing sealants and adhesives are cured by exposure to radiant energies. In our tests, barnacle cement was exposed to UV and infrared radiations. However, the time taken for cement polymerization was not greatly reduced by UV exposure. Barnacle cement is largely composed of proteins (Kamino et al. 1996). Hence, the high energy of ionizing UV light, duration and proximity in which barnacle cement was exposed to might have denatured cement proteins. This might explain the negligible reduction in time taken for cement polymerization. Past research suggested that a high dose of UV radiation has had a destructive effect on proteins such as complement factor C4 (Artyukhov, 2007) and affected the stability of other structural proteins like those found in egg white (Liu, 2009). On the other hand, infrared photons contained less energy, and hence lesser extent of or no denaturation might have occurred, while additional energy was provided. Thus, infrared light speeds up polymerization time as it increases reaction rates and promotes drying. Still, the time required for complete polymerization remains slower than existing adhesives that take below 2 minutes. Our results indicated that Ca^{2+} ions increased the rate of cement polymerization, a phenomenon previously reported by Dougherty (1996) who showed that Ca^{2+} ions enhanced enzymatic activity in *Chthamalus fragilis* barnacle cement. With exception to the set-ups exposed by UV radiation, further statistical analysis showed significant differences in time taken resulting from the addition of Ca^{2+} ions. This might be also accounted to the destructive effects of UV radiation on cement proteins.

Acid Resistance Test

In our study, we observed that windows of both enamel and dentin, coated with a layer of barnacle cement, became stained and eroded after 72 hours of immersion in Coke. This indicated that barnacle cement was not effective as a protective layer. In a highly acidic environment (pH of Coke = 3.3), denaturation of the cement proteins might have occurred, resulting in its inability to prevent staining and acidic erosion by Coke. Another reason for its ineffectiveness might be over-exposure to such solution. Based on preliminary experimentations, however, such long exposure was necessary so that visible changes to the tooth surfaces can be observed. Thus, over-exposure to Coke could adversely affect the structure and hence function of the cement proteins, resulting in its ineffectiveness to adhere to tooth surfaces. Still, future studies can investigate if barnacle cement helps promote remineralization of teeth, instead of reducing demineralization.

Future Work

This study can be extended in the following ways:

1) To carry out human cell culture tests to validate the biocompatibility of *Amphibalanus amphitrite* cement
2) To investigate the adhesion strength of *Amphibalanus amphitrite* barnacle cement to tooth surfaces in vitro
3) To assess the effect of barnacle cement on remineralization of enamel and dentin

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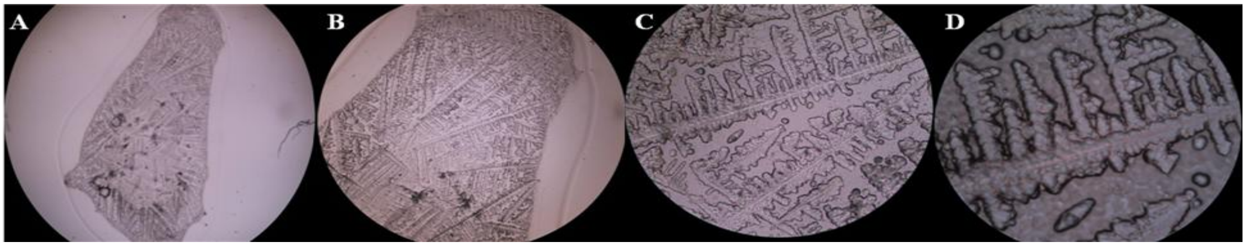
Appendix

I. Polymerized Barnacle Cement (Under Light microscope)



4X Magnification 10X Magnification 40X Magnification 100X Magnification

Fig 13. Polymerized barnacle cement on a glass slide (Under Light microscope)

Likewise, under the light microscope, key-like crystallized protein structures gradually appear as polymerization of cement occurs. When the cement droplet is completely covered with these key-like protein structures, it marks the end of polymerization and the cement has turned into an opaque solid.

II. Polymerization of red dyed barnacle cement on teeth surface

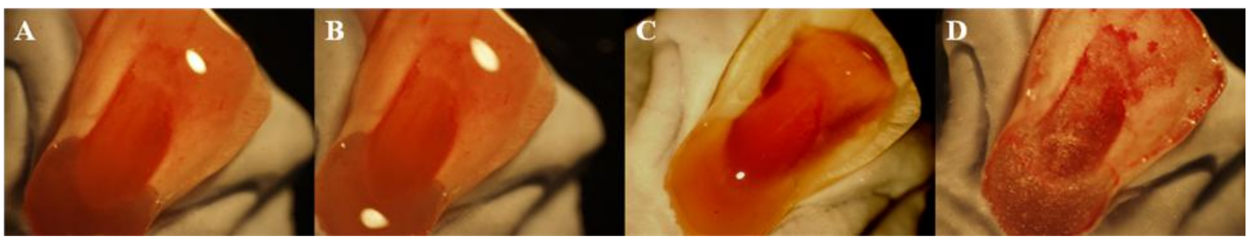


Fig 14. Polymerization of red dyed barnacle cement on teeth surface

Aesthetically, it is feasible to consider the barnacle cement in dental applications (*ref. Fig 5*). However, due to highly similar color between the cement and tooth surface, it is almost impossible to observe the end point of polymerization of barnacle cement. Hence, a simple but novel experiment was conducted in an attempt to solve the problem. By rearing the barnacles in red dyed seawater, the cement extracted from them was consequently red. Upon application of the red cement on teeth surface, it is clearer to observe the end point of polymerization (*ref. Fig 14D*). Thus, in further experiments concerning the speed of cement polymerization on teeth surface, the red dyed barnacle cement was used.