The Potential of Self-assembling Peptides for Enhancement of In Vitro Remineralisation of White Spot Lesions as Measured by Quantitative Laser Fluorescence

Luca Golland[a] / Patrick R. Schmidlin[b] / Marc Schätzle[c]

Purpose: To test the remineralisation potential of a single application of self-assembling peptides or acidic fluoride solution using quantitative light-induced fluorescence (QLF) in vitro.

Materials and Methods: Bovine enamel disks were prepared, and white spot lesions were created on one half of the disk with an acidic buffer solution. After demineralisation, disks were allocated into three groups of 11 specimens each. Group A served as a control group and received no treatment. Group B had a single application of fluoride, and group C was treated once with self-assembling peptides. All disks were embedded in a plastic mold (diameter 15 mm, height 9 mm) with an a-silicone, and remineralisation was initiated using a pH-cycling protocol for five days. Four experimental regions on each disk were measured using QLF prior to the start of the study (T_0), after demineralisation (T_1) and after the remineralisation process (T_2).

Results: After demineralisation, all areas showed a distinct loss of fluorescence, with no statistically significant difference between the groups (ΔF from -69.3 to -10.2). After remineralisation, samples of group B (treated with fluoride) showed a statistically significant fluorescence increase (ΔF from T_1 to T_2 15.2 ± 7.3) indicating remineralisation, whereas the samples of control group A and group C (treated with self-assembling peptides) showed no significant changes in ΔF of 1.1 ± 1.9 and 2.5 ± 1.9, respectively.

Conclusions: Application of self-assembling peptides on demineralised bovine enamel did not lead to increased fluorescence using QLF, indicating either lack of remineralisation or irregular crystals. Increased fluorescence using QLF indicated mineral gain following a single application of a highly concentrated fluoride.

Key words: peptides, quantitative light-induced fluorescence, self-assembly, white spot lesions

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Despite advances in contemporary orthodontics, insufficient oral hygiene remains a clinical problem and results in increased plaque accumulation and the subsequent development of demineralised areas around fixed orthodontic appliances. The prevalence of white spot lesions (WSLs) after debonding on at least one tooth surface varies greatly, ranging from 2% to 97%.[3,6,12,19] However, initially demineralised areas do not always reach a critical degree of visibility (above ICDAS code 0 and 1[21]), even though they are visually detectable after prolonged air drying or with sensitive methods such as quantitative light-induced fluorescence (QLF).[1,13] In addition, the visibility of many WSLs may decrease after debonding or even disappear due to improved oral hygiene by remineralisation.[3] Different treatment modalities have been proposed to handle WSLs.[5] In situ, a single application of highly concentrated fluoride has also been shown to increase the remineralisation process.[8] In incipient lesions, the remineralisation process seems to be dose and time related,[2] and remineralisation was shown to be significantly greater with a 1% amine fluoride liquid than with a 0.5% amine fluoride liquid. At both concentrations, mineral gain followed a linear relationship within the observation time.[2] In contrast, products containing low doses of fluoride, calcium, and phosphate in a bioactive form (casein phosphopeptide amorphous calcium phosphate) have been shown to enhance the biological remineralisation potential of the saliva.[5] A more cosmetic method to camouflage WSLs is bleaching of the adjacent enamel, microabrasion, or infiltrating the etched enamel with a low-viscosity resin.[18] However, infiltration with resin impedes any potential remineralisation. In recent years, 'smart' biomaterials have been introduced to regenerative dentistry aiming not only to pre-

[a] *Orthodontist, Clinic of Orthodontics and Pediatric Dentistry, Center of Dental Medicine, University of Zurich, Zurich, Switzerland. Experimental design, study setup, performed laboratory work, wrote and proofread the manuscript.*

[b] *Professor, Clinic of Preventive Dentistry, Periodontology and Cariology, Center of Dental Medicine, University of Zurich, Zurich, Switzerland. Experimental design, study setup, statistical analysis, proofread the manuscript.*

[c] *Orthodontist, Clinic of Orthodontics and Pediatric Dentistry, Center of Dental Medicine, University of Zurich, Zurich, Switzerland. Idea, proofread the manuscript.*

Correspondence: *Luca Golland, Bahnhofplatz 7, 7000 Chur, Switzerland. Tel: +41-79-734-9439; Email: ortho@golland.ch*

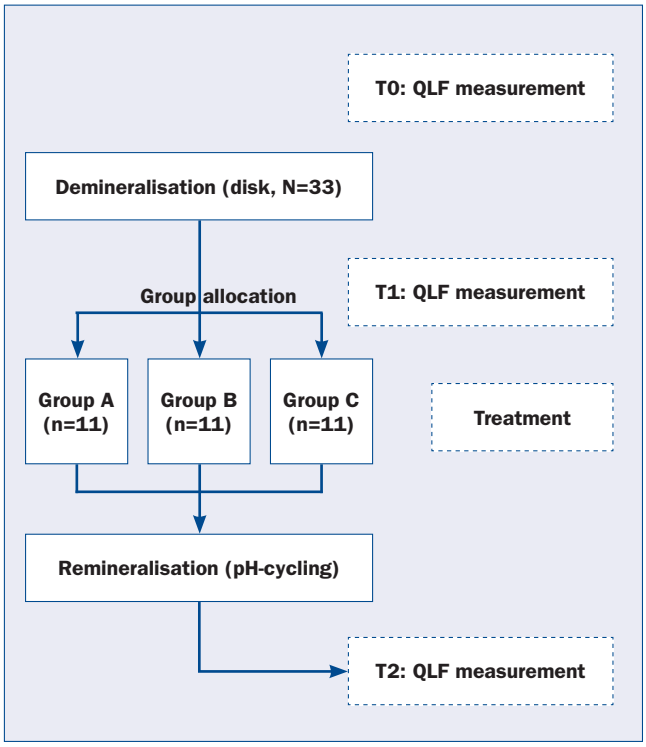

Fig 1 Experimental design.

vent further decay by influencing oral biofilms but also to repair enamel lesions.[14] Recent studies describe peptides that may undergo spontaneous self-assembly after infiltrating a carious lesion. These fibrillar networks are claimed to mimic 'natural histogenesis' and have been shown to introduce mineral deposition in enamel lesions.[16]

The use of QLF offers a non-invasive method to assess changes in the enamel mineral content.[24] This study therefore aimed to assess the remineralisation potential of a self-assembling peptide solution when treating artificially demineralised bovine enamel disks using QLF. A fluoride solution was used as a control. It was hypothesised that the application of self-assembling peptides (SAP) results in a comparable or even better remineralisation than the application of a highly concentrated fluoride treatment or untreated controls.

MATERIALS AND METHODS

Bovine Enamel Disk Preparation

Thirty-three bovine enamel disks with a diameter of 6.8 mm and a thickness of 1.5–1.6 mm were prepared from freshly extracted mandibular bovine second incisors using a diamond-coated trephine under constant water cooling, so that the enamel-dentin junction was in the middle of the disk.[11] The cylinders obtained were ground flat on both sides using 500-grit silicon carbide sand paper (FEPA P standard, Struers; Birmensdorf, Switzerland). A flowchart depicts the experimental design as described in detail below (Fig 1).

A notch was placed on the edge of the disk for orientation with respect to coating, disk alignment during specimen preparation, treatment and analysis using QLF measurements (for more details see below). The disks were then stored in the dark in tap water at a temperature of 4°C until the experiment started.[11]

Prior to the start of the study, all disks were placed in artificial saliva (1000 ml distilled water, 0.002 g ascorbic acid, 0.03 g glucose, 0.58 g NaCl, 0.17 g $CaCl_2$, 0.16 g NH_4Cl, 1.27 g KCl, 0.16 g NaSCN, 0.33 g KH_2PO_4, 2.7 g mucin, 0.34 g Na_2HPO_4, and 0.2 g urea at pH 6.4) under agitation for seven days to induce uniform baseline mineralisation at 37°C.[17] The saliva was changed every 3 days. All chemical products were obtained from VWR International (Dietikon, Switzerland), except for mucin (Bacto-Mucin Bacteriological, Difco Laboratories; Detroit, MI, USA).

Lesion Formation

The notch indicated a 12 o'clock position in the coronocervical direction. The right half of all 33 disks was covered with water-resistant adhesive tape (electrical insulating tape No. 288, 0.19 mm x 19 mm x 20 m, Nitto Europe; Duisburg, Germany) to avoid any exposure to the demineralisation or mineralisation agent.[23] Disks were then embedded in a plastic mold (diameter 15 mm, height 9 mm) with an amorphous (a) silicone (Coltène President light body, Coltène/Whaledent; Altstätten, Switzerland) to protect the dentin of the disk and were then placed for five days in 30 ml of Buske's demineralisation solution (5 L distilled water, 2.205 g $CaCl_2$ + 2 g H_2O, 2.041 g KH_2PO_4, 10 ml MHDP-solution [100 ml distilled water with 0.0528 g methylenediphosphoric acid], 14.3 ml CH_3COOH and 10 M KOH to titrate the solution at pH 4.95) under agitation at 37°C,[9] which was changed after 2.5 days. All chemical products were produced by VWR International.

Group Allocation, Treatment and Remineralisation

After demineralisation, the a-silicone and the tape were removed, and the disks were rinsed for 30 s with tap water. Baseline demineralisation was measured using a QLF device (Inspektor Research Systems; Amsterdam, The Netherlands) and samples were stratified to equally distribute those with higher and lower QLF differences into three groups of 11 samples each (details of the QLF evaluation are described below). The coronal half of the sample was again covered with water-resistant adhesive tape (Nitto electrical insulating tape, No. 288) to avoid any exposure to test or remineralisation solution after treatment. These protected areas served as controls (Fig 2A).

Group A (n = 11) served as a control group and received no treatment. Before the agent was applied in groups B and C, the specimens were dried with a paper tissue. Group B (n = 11) received a single application of a highly concentrated amine fluoride agent (elmex fluid, GABA International; Münchenstein, Switzerland; 10,000 ppm amine fluoride olaflur and dectaflur, pH 3.9) for 5 min. Afterwards, disks were rinsed for 30 s with tap water. Group C specimens (n = 11) received an application of 25 µl of a 10 mg/ml monomeric

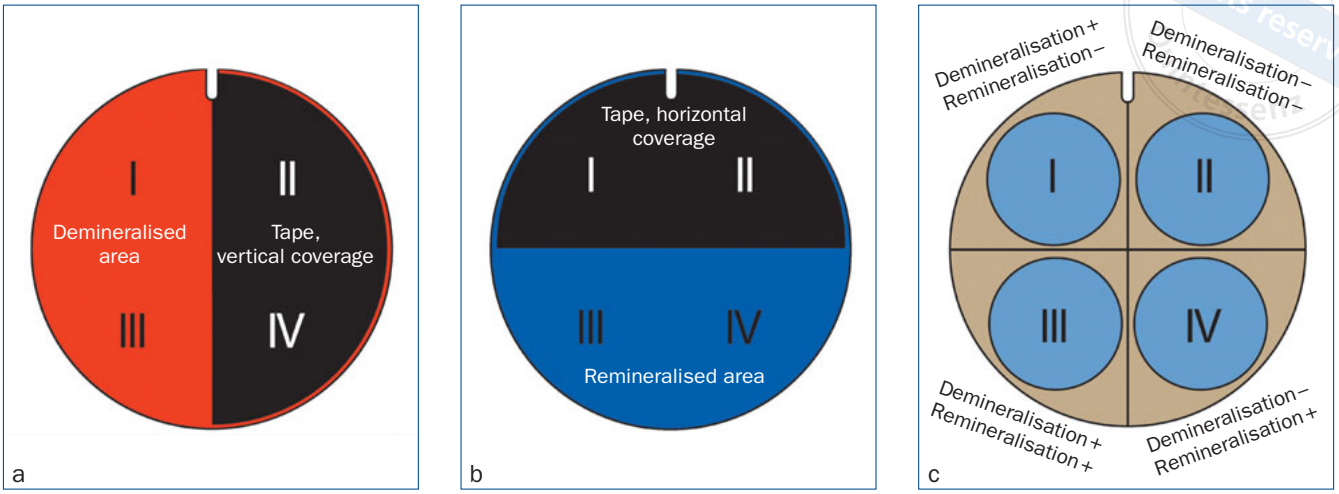

Fig 2 a: Situation during demineralisation, tape coverage of areas II and IV. b: Situation during demineralisation, tape coverage of areas I and II. c: Measurement patch allocation (areas I, II, III and IV).

P11-4 solution titrated to pH 8.4 ± 0.4 using a 20 mM tris-buffer. The solution was a prototype of the now commercially available product CURODONT Repair (Credentis; Windisch, Switzerland). The solution was left for 5 min on the disk at room temperature to allow soaking into the enamel surface. Afterwards, disks were rinsed for 2 min with tap water.

After treatment, disks were re-embedded in a plastic mold (diameter 15 mm, height 9 mm) with an a-silicone (Coltène President light body) to protect the dentin side of the disk, and remineralisation was initiated using a modified pH-cycling protocol[22] for five days using the re- and demineralisation solutions as described above.[9,17] Stock solutions were changed every 24 h. During the day (16 h), all specimens of one group were placed in 80 ml of heated (37°C), agitated artificial saliva for remineralisation. Three times during this 16-h period, each group was exposed to 150 ml of the demineralisation solution for 20 min. Before and after exposure to the demineralisation agent, the disks were rinsed for 30 s with tap water. During the night, all specimens of one group were stored in 80 ml artificial saliva at 37°C for 8 h.

In Vitro Quantitative Light-induced Fluorescence (QLF)

After treatment and remineralisation, the tape was removed again and QLF images were taken at all four regions (areas I to IV), which were defined with freely moveable optical patches that overlaid the pre- and post-intervention image. The patches had a standardised area of 3.1 mm^2 and were placed centrally in each quarter of the disks (Fig 2C). The edges of the disks were not included because of the potential fluorescence edge loss in this region. QLF measurements were conducted before demineralisation (T_0), after demineralisation (T_1) and after five days of remineralisation and pH cycling (T_2).

QLF images were taken with a special camera (Sony CCD Camera DXC-LS1P, Sony; Tokyo, Japan) equipped with a 12-mm focal lens and mounted with a fixed focal distance of 33.35 mm from the specimen surface. Images recorded by the video camera were obtained in real time by a personal computer and instantly compared with a previously recorded reference image of the same area within each specimen. To precisely position a disk on the stage, the live image was lined up with the red outline of the reference image using QLF-Patient software (version 30.0.026, Inspektor Research Systems). When optimum specimen positioning was achieved, a definitive image was stored in bitmap format.[11]

Pre- and post-intervention images were compared by subtraction analysis using the software Substract 1.1.0.4 (Inspektor Research Systems). The outcome measure was ΔF, defined as the percent change from the pre- to post-intervention image in fluorescent radiance in each image pixel averaged over the analysis area.[11]

Statistical Analysis

All of the data were coded in Microsoft Excel for MAC 2011, version 14.0.0 (Microsoft; Redmond, WA, USA) and analysed by StatView, version 5 (Abacus Concepts; Berkley, CA, USA).

To assess the differences between the treatment groups at the respective areas, ANOVA followed by Scheffé's post-hoc test was used to determine significant differences between the groups. In all tests, $p < 0.05$ was considered statistically significant.

RESULTS

After Demineralisation

After demineralisation (T_0 to T_1), areas showed a distinct loss of laser fluorescence, with minimum and maximum values in ΔF ranging from -69.3 to -10.2. Area I (demineralisation only) showed a mean reduction in ΔF of -44.4 ± 18.6 in group A, -38.6 ± 31.8 in group B and

Table 1 Substrate preparation (demineralisation and protection) effect after demineralisation without treatment and subsequent remineralisation (difference T_0-T_1)

Area	Substrate	Group A	Group B	Group C
	Non-remineralised*			
I	Demineralised	-44.4 ± 18.6^A	-38.6 ± 31.8^A	-49.7 ± 12.8^A
II	Sound enamel	0.2 ± 3.7^A	-2.0 ± 5.7^A	-2.4 ± 4.1^A
	Remineralised*			
III	Demineralised	-46.9 ± 15.4^A	-48.8 ± 16.0^A	-43.5 ± 21.7^A
IV	Sound enamel	-0.7 ± 3.5^A	-2.1 ± 2.3^A	-2.2 ± 4.1^A

*The mineralisation terminology refers to the respective areas and treatments only. Identical superscript capitals in rows represent values which did not significantly differ from each other (Scheffé's test).

Table 2 Treatment effects expressed as the calculated differences between the measured QLF values (ΔF) after demineralisation and treatment/remineralisation (difference T_1-T_2)

		Group A	Group B	Group C
			Treatment	
Area	**Substrate**	No	No	No
	Non-remineralised			
I	Demineralised	-1.1 ± 2.1^A	0.9 ± 2.2^A	0.4 ± 3.1^A
II	Sound enamel	-3.3 ± 1.9^A	-2.9 ± 1.0^A	-1.8 ± 2.5^A
			Treatment	
	Substrate	None	Fluoride	SAP
	Remineralised			
III	Demineralised	1.1 ± 1.9^A	15.2 ± 7.3^B	2.5 ± 1.9^A
IV	Sound enamel	-3.1 ± 1.9^A	-2.7 ± 1.3^A	-3.0 ± 1.9^A

Positive values indicate a remineralising potential. Identical superscript capitals in rows represent values which did not significantly differ from each other (Scheffé's test).

-49.7 ± 12.8 in group C, with no statistically significant difference between the groups. Additionally, area III (demineralisation and remineralisation) showed no significant difference in the mean reduction of ΔF between group A (-46.9 ± 15.4), group B (-48.8 ± 16.0) and group C (-43.5 ± 21.7), irrespective of whether a treatment was performed or not (Table 1).

The initially non-demineralised areas II and IV displayed only minute changes in the ΔF values from T_0 to T_1 with minimum and maximum values ranging from -17.1 to +10.1. The mean change of ΔF in area II was 0.2 ± 3.7 in group A, -2.0 ± 5.7 in group B and -2.4 ± 4.1 in group C, with no statistically significant difference between the groups. Area IV showed ΔF of -0.7 ± 3.5 in group A, of -2.1 ± 2.3 in group B, and of -2.2 ± 4.1 in group C. Again, no statistically significant difference between the groups could be detected (Table 1).

After Treatment and Remineralisation

In the protected control areas I and II, only minute changes could be detected in all groups from T_1 to T_2, ranging from -7.1 to 4.6. The mean ΔF in area I in groups A, B and C was -1.1 ± 2.1, 0.9 ± 2.2 and 0.4 ± 3.1, respectively, with no statistically significant difference between the groups. In area II, the mean ΔF in group A was -3.3 ± 1.9, in group B -2.9 ± 1.0 and in group C -1.8 ± 2.5, with no statistically significant difference between the groups (Table 2).

With regard to the different treatments, samples of group B in area III (treated with a highly concentrated fluoride) showed a significant mineral gain (ΔF from T_1 to T_2 15.2 ± 7.3). Area III in groups A (untreated control) and C (treated with self-assembling peptides) showed no significant ΔF of 1.1 ± 1.9 and 2.5 ± 1.9, respectively, but did show positive values (Table 2).

Values obtained in the non-demineralised area IV showed no statistically significant different between the three groups. All groups showed a slight loss of fluorescence: group A -3.1 ± 1.9, group B -2.7 ± 1.3 and group C -3.0 ± 1.9.

DISCUSSION

This in vitro study aimed to test the remineralisation potential of a self-assembling peptide (SAP) compared with a single application of highly concentrated fluoride using quantitative light-induced fluorescence. Our study could not confirm any remineralisation after application of SAP as hypothesised. Only the application of fluoride was able to show some mineral gain, which was statistically significant as confirmed by QLF as a surrogate parameter. In situ, a single application of highly concentrated fluoride was shown to increase remineralisation.[8] In incipient lesions, the remineralisation process seems to be dose and time related,[2] and the remineralisation was shown to be significantly higher with a 1% amine fluoride fluid than with a 0.5% amine fluoride fluid. At both concentrations, mineral gain followed a linear relationship within the observation time.[2]

Casein phosphopeptide amorphous calcium phosphate (CPP-ACP) supports the biological remineralisation process and the mineral gain after orthodontically induced WSLs.[7] This was reflected in reduced fluorescence and reduced area assessed by QLF. However, the improvement was not superior to the regression of WSLs using a daily toothpaste program.[7] For this reason, CPP-ACP was not tested in this study.

Although scaffold-forming SAPs are available for daily use in the dental clinic to treat WSL, no clinical trial has proven the effectiveness of the material. In vitro studies showed a significantly increased mineral gain after a single application of a P11-4 monomeric solution for 30 min and pH cycling for five days.[16] It is questionable whether a 30-min infiltration time was feasible in an in vivo setup. The tested solution was a prototype of a now commercially available P11-4 monomeric solution. The clinical guidelines of this product (CURODONT Repair, Credentis) recommend an infiltration time from only 3 to 5 min. The treatment includes etching the affected area and area surrounding the enamel surface with 35% phosphoric acid for 20 s to allow SAP to infiltrate. This approach is also used to reopen inactive subsurface WSLs to treat the area with resin infiltration.[18] In the present study, the bovine disk surface was free of pellicle and plaque, and due to preparation, was relatively rough. These conditions should allow good infiltration of the peptide solution within 5 min without surface etching. By protecting half of the disk during demineralisation and application of the solutions, one area in every quarter of the disk was preserved after every step to obtain a reference area in each disk for intra-sample comparison. The results showed solution leakage during the observation period.

Most clinical QLF studies have focused on smooth-surface caries associated with natural buccal lesions resulting from poor oral hygiene or those associated with orthodontic banding.[10] This method may be used for quantitative monitoring of mineral changes in lesions on surfaces that can be visually inspected within a time period of only a few months. Therefore, evaluation using this technique makes sense in view of the clinical applicability and the indication; optical changes are also assessed. It has been shown that demineralisation surrounding orthodontic brackets can be reproducibly quantified using computerised image analysis from photos converted to digital images and QLF, and that the two techniques show good agreement with respect to quantifying the area of demineralisation.[4] However, no direct conclusions regarding mineral deposition can be made, because samples underwent neither hardness nor radiographic evaluation (e.g. Transverse Microradiography [TMR]). These destructive methods would not have been possible in this complex caries model, in which a longitudinal evaluation was envisaged.[15]

Factors such as hydration status, lesion shape and localisation, influence of plaque accumulation or staining, as well as the influence of ambient light were minimised in this laboratory evaluation by employing standardised preparation processes and measurements.[10]

Interestingly, the lower visibility of a white spot lesion may also be achieved by etching the affected enamel and reopening the surface for an undisturbed remineralisation with saliva, as shown previously[1] in a study which assessed WSLs with laser fluorescence over a 12-month period after debonding. The mineral gain and decrease in the demineralised area followed a time trend in which the fluorescence regain was pronounced during the first months and then continued at a slower rate. Therefore, the use of remineralisation enhancing methods is still warranted in these cases, but no reliable material has been identified and thoroughly studied to date.

Limitations

This study has certain limitations. For instance, due to the rough surface of the disks, the hardness could not be tested. Further, Kirkham et al[16] detected a net mineral gain in artificial WSLs after applying a SAP and remineralising in a pH-cycling model for five days. QLF is an acceptable method to assess changes in the enamel mineral content. The mineralisation time of five days might have been too short in the present study to detect a mineral gain, although the greatest change in mineral content was at the beginning of the mineralisation period. A possible long-term effect of enhanced mineral gain after the application of SAP must be tested in further studies.

Further assessments (e.g. TMR) must be performed to understand how the remineralisation of WSLs after applying SAP occurred and if the QLF method is a valid tool to detect mineral gain after treatment with SAP.

CONCLUSIONS

- Mineral gain in demineralised enamel was enhanced by even a single application of a highly concentrated fluoride.
- Newly developed self-assembling peptides for dental application did not result in significant remineralisation after five days in demineralised enamel compared with artificial saliva.

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