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Investigating the addition of collagen and its integrin binding sequence (RGD) to glass polyalkenoate; in terms of material and cellular properties to explore a more biocompatible method of root caries restoration

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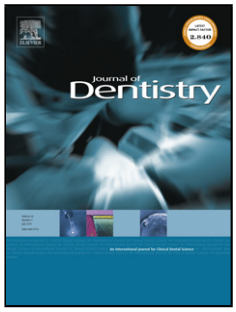

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INVESTIGATING THE ADDITION OF COLLAGEN AND ITS INTEGRIN BINDING SEQUENCE (RGD) TO GLASS POLYALKENOATE; IN TERMS OF MATERIAL AND CELLULAR PROPERTIES TO EXPLORE A MORE BIOCOMPATIBLE METHOD OF ROOT CARIES RESTORATION.

Short Title: INVESTIGATING THE ADDITION OF COLLAGEN AND ITS INTEGRIN BINDING SEQUENCE (RGD) TO GLASS POLYALKENOATE .

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ABSTRACT

Placement of a restoration to treat root caries disrupts many tissues. There is scope for the restorative material to interact with these to augment reductions in micro leakage afforded by an adhesive restorative material. **Objectives –** 1) To investigate the effects of incorporating bioactive molecules into a glass polyalkenoate (GPA) 2) To quantify the changes in physical properties of the material. **Methods -** Biocompatibility of the GPA cement (Chemfil Superior, Dentsply De Trey, Konstanz, Germany) in unmodified and modified forms was ascertained using cell culture techniques. The optimum concentration of bioactive components required to promote cell attachment was determined indirectly by quantification and localisation of the fibroblast marker vimentin. The properties of surface hardness, compressive strength and adhesive bond strength were also determined prior to and following addition of the bio-additives: collagen type I and a pentapeptide containing Arg-Gly-Asp (RGD). **Results -** Addition of Type I Collagen (100 µg/ml) and RGD (5 mg/ml) to ChemFil Superior had no statistically significant effect upon the compressive strength and bond strength to bovine enamel but significantly ($P < 0.05$) increased the materials shore hardness. The addition of RGD to ChemFil Superior increased most the expression of vimentin, indicating that the cells had become more fibroblastic. This may be indicative of increased synthesis of extracellular matrix macromolecules with the potential to foster adhesion of the modified glass polyalkenoate to distracted gingival tissues. **Conclusions** -The results suggest that addition of bioactive molecules to GPA cement for subgingival restorations has potential clinical applications.

Keywords: glass polyalkenoate, cells, adhesion, biopolymer

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Clinical significance - It is possible to envisage that the additions, as described in this paper, could foster the attachment of displaced gingival tissues to GPA restorative materials placed subgingivally where root caries has been treated. This would offer potential to form a seal around the restoration by the attached gingival tissues avoiding a periodontal pocket and depriving residual cariogenic bacteria of a nutrient supply. Further investigation of the effects upon other similar materials of such additions is warranted.

INTRODUCTION

Root surface caries has been defined as " a soft irregular shaped lesion either (a) totally confined to the root surface or (b) involving the undermining of enamel at the cemento-enamel junction clinically indicating that the lesion initiated on the root surface" (1). Root surface caries usually occurs supragingivally at or close to (within 2 mm) the cemento-enamel junction (CEJ) (2). Experts generally agree that root caries can occur anywhere on the root surface occlusal to the gingival margins, but there are contradictory views about root lesions involving the cemento-enamel junction (CEJ). These relate to the classification of such lesions for some think they should be classed as root surface caries extending onto the crown or indeed as coronal caries extending onto the root or even both. This however is more a measurement issue than a diagnostic one (3).

The occurrence and location of root surface caries is usually associated with age and gingival recession. This is consistent with the idea that root caries occurs in a location close to the crest of the gingiva, where dental plaque accumulates. Root surface caries most commonly occurs on the proximal surfaces followed by the facial surfaces of the tooth (2).

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The prevalence of root surface caries in the general population increases with age (4). This increase in prevalence is related to the longer retention of teeth in older people, than in previous generations. Also, root surfaces at this age become exposed due to gingival recession putting the root surface at greater risk (5). The occurrences of root surface caries can be prevented using a variety of preventive methods e.g. water fluoridation and use of fluoridated dentifrices. Maximum efficiency of prevention could be achieved if high-risk individuals were identified earlier and appropriate preventive methods instituted (6).

The management of root surface caries should start by preventive and remineralisation therapies that will help inhibit or eliminate the lesion before further damage to dental tissues occurs. Restorative treatment is indicated where there is excessive distraction of the tooth tissues by active root surface caries (7). It is generally accepted that root surface caries can be prevented or arrested by plaque removal, diet modification and topical fluoride application (8).

When the active caries root lesion progresses, it causes destruction of the root tissues and so restorative treatment is indicated to remove the caries and replace the destroyed tissues. Sometimes such lesions extend subgingivally and the clinician is faced with many difficulties in removing the caries. These include impaired visibility, limited access, limited moisture control, pulpal proximity and the nature of the dentinal tissues themselves (7).

Many different restorative materials may be used to restore the root surface. Glass polyalkenoate (Conventional/Resin modified) cements are considered to be the materials of choice for restorative treatment of most root surface caries lesions (7). This is because such materials provide good adhesion to the hard tissues of the tooth and have anti-cariogenic effects due to sustained fluoride release. Resin composite materials, although possessing

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aesthetic qualities, undergo polymerisation shrinkage and have no or limited fluoride release (7). As these materials are resin based, they are intolerant of moisture. In the past dental amalgam was used, but its use is to be phased down (9), on environmental grounds, and its poor aesthetics do not endear it to an increasingly demanding public (10).

Placement of a restoration for a root caries lesion involves the disruption of many tissues namely the attached gingiva, periodontal ligament, enamel, dentine and cementum. Scope therefore exists for surface interactions of the restorative material with the cell populations of both the attached gingiva and periodontal ligament. The main component of the extracellular matrix of the periodontal ligament and gingiva is comprised of Type I collagen and its bioactive motif (RGD) (11). This offers the potential for cellular attachment to these tissues to augment the reductions in restoration microleakage afforded by the adhesive bonding of glass polyalkenoates to the hard tissues of the tooth.

This work sought to identify bioactive additions, potentially suitable for chairside incorporation at the time of mixing into glass poyalkenoate cement, to foster cellular attachment to subgingival restorations of root caries lesions. Although not tested in this study, the purpose of these additions was to provide a tissue seal for such restorations with a view to depriving residual cariogenic bacteria of their nutrient supply. As such additions could potentially affect the physical properties of the materials, those thought most likely to contribute to clinical success were determined for the material tested in both unmodified and modified form.

Materials and methods

Preliminary work, utilising cell culture and viability assays, identified that the glass polyalkenoate (GPA) Chemfil Superior was sufficiently biocompatible to advance this work. The compositional details of this material are summarised in Table I.

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The methods used in this study are best described under the subheadings of

(a) Determination of optimal concentration of bioactive additions to promote cellular adhesion.

(b) Effects of bioactive additions on the cements properties

Unless otherwise stated GraphPad PRISM software (version 5.0, GraphPad Software Inc, San Diego, California, USA) was used for all statistical analysis. Statistical significance was signified at $P < 0.05$.

(A) Determination of optimal concentration of bioactive additions to promote cellular adhesion.

The effects of two different bioactive additions [1- Type I Collagen (Prepared in house (12)) and 2- Gly-Arg-Gly-Asp-Ser (RGD) (SIGMA-ALDRICH, St. Louis, MO, USA)] to ChemFil Superior GPA at two different concentrations were investigated.

In summary the additions investigated were;

- Type I collagen was added to the mixing water of the GPA in two different weight ratios, 10 µg/ml and 100 µg/ml, to form aqueous solutions of 0.1% and 0.01% type I collagen.
- RGD was added to the GPA mixing water at 1 mg/ml and 5 mg/ml.

These concentrations were chosen with reference to previously conducted cell attachment studies (13).

All bioactive additives were incorporated into the material and mixed according to the manufacturers' instructions at a powder: liquid ratio of 1: 1.

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To make the necessary material specimens for this aspect of the investigation, Polytetrafluoroethylene (PTFE) disc shaped moulds, with an inner diameter of 12 mm and thickness of 2 mm were used. Prior to use, they were washed thoroughly with water and detergent (Lipsol®, Scilabware, Stoke-on-Trent, UK) for 5 minutes, and then sterilised by exposure to ultraviolet light for 24 hours. All subsequent preparations of the glass polyalkenoate cement, in any form, were carried out under sterile conditions using sterilised instruments. To achieve this scoops, droppers and plastic spatulas provided by the manufacturers were rinsed thoroughly in 70% (v/v) ethanol and left to dry under ultraviolet light for 24 hours before use. All specimens of the unmodified glass GPA cement were mixed according to the manufacturers' instructions and condensed using plastic instruments into the PTFE mould. To achieve smooth specimen upper and lower surfaces, the mould, containing unset GPA cement, was sandwiched between two PTFE plates before being compressed between two metal plates. After setting the disks were removed from the mould by gentle hand pressure, following unscrewing of the metal plates. To ensure sterility, this work was undertaken under sterile conditions under a microbiological safety cabinet. The set discs were glued to the centre of the dishes using superglue (SHERAMEGA 200, Espohsrabe 53, Lemforde, Germany). Once all the specimens had adhered, the dishes were labelled and placed beneath a microbiological hood under UV light overnight (lid removed) for sterilisation. The specimens were washed over the next 24 hours with Hanks balanced salt solution then left in serum free medium (SF-MEM) overnight prior to the biological testing.

Cell culture and Viability scores - Normal oral mucosa fibroblast cells (MM1), Source – Dr M Macluskey University of Dundee (14) were cultured in 90 mm dishes with 6 ml growth medium each. These were incubated at 37 ºC in a CO_2 incubator with 5% CO_2 until confluent. For all subsequent experiments, cells from these were seeded in 60 mm dishes around and over the material specimens at a concentration of 5 x 10^5 cells/dish for 21 days at 37 ºC and

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5% CO_2. Control dishes contained cells and medium only. The medium was changed every 48 hours. The cells were monitored under the light microscope (Olympus IX70-S8F2, Olympus, South-End-On-Sea, UK) Photographs were taken of the cells close (adjacent to the attached material) to and remote (at the perimeter of the dish) from the specimens every 3 days. Ten examiners, experienced in cell biology, were asked to rank cell viability using a visual observation method as detailed in Table II. The effects of the material and additives on the cells were compared to that of the control using the scoring values as indicators of cell viability seen over all observation times. This was undertaken by conducting a non-parametric one-way ANOVA (Kruskal-Wallis test) test of the data with localisation of significant differences using Dunn's multiple comparison test. A note of the most common ranking scores for each experimental group was also made.

Cell viability testing (MTT Assay) - The MTT Assay was performed to determine the optimum concentration for each bio-additive to improve biocompatibility of the glass ionomer cement and promote cellular adhesion. The MTT assay is a colorimetric assay for measuring cellular viability (15, 16).

Disks made from ChemFil Superior, without and with additives, were placed inside the wells of sterile plastic 48 well plates. These were then sterilised overnight under UV light. Specimens were then washed over the next 24 hours with Hanks balanced salt solution, then left in SF-MEM overnight.

MM1 cells were seeded in two 48-well culture plates (50,000 cells per well) at a concentration of 5×10^4 cells/ml for 24 hours and 72 hours at 37 ºC and 5% CO_2. This concentration had previously been found to be optimal in pilot work for the purpose of the experiment.

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After 24 hours of incubation, cell attachment, morphology and confluence was checked on both plates and 250 µL of fresh growth medium was added to each well of the second plate, which was then returned to the incubator for another 48 hours at 37 ℃ and 5% CO_2. The medium was removed from the wells of the first plate by aspiration and the wells were then washed twice with SF-MEM. 250 µL MTT solution was added to each well and the plate was then incubated at 37 ºC, 5 % CO_2 for 3 hours. The MTT solution was then removed from the wells by gently tapping the contents against paper tissues. Subsequently, 250 µL of DMSO was added to each well and the plate was placed on an orbital shaker for 20 minutes at room temperature. Optical density of the wells was then measured using a Fluorostar Optima plate reader (FLUOstar Optima, BMG Labtech, Aylesbury, Bucks, UK) at a wavelength of 540 nm.

The same procedure was repeated for the second plate after 72 hours of incubation at 37 ºC, 5% CO_2.

The Absorbance values obtained for each well represent the amount of MTT reduction, which is proportional to the number of viable cells. In order to assess the percentage of viable cells present in each well, the absorbance values were related to those of the control. This was achieved by setting the mean absorbance of the control to 100%. The percentage of viable cells was calculated using the equation:

$$Percentage\ of\ viable\ cells = \frac{\text{Absorbance value}}{\text{Mean Absorbance of the control}}\ \text{X}\ 100\ \%$$

The effects of the material modification state on the cells were compared to each other using the percent viability values as indicators of cell numbers. An unpaired t test was used to analyse the data to determine the effect of material state on cell viability.

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Examination for the expression of Vimentin – The expression of vimentin gives a measure of the fibroblastic activity level of the cells used in this work. A number of techniques were used to assess this namely: Immunocytochemistry and Protein Biochemistry.

Immunocytochemistry (ICC) to localise and quantify Vimentin expression - The objective was to label MM1 fixed *in situ* with primary antibody against vimentin and visualise this by labelling with a fluorescent secondary antibody. The protocol used was the standard laboratory procedure as described in Islam *et al* (14) with vimentin (R28 rabbit monoclonal antibody # 3932S, Cell Signalling Technology) and fluorescent secondary antibody (anti-Rabbit IgG conjugated with Alexa Fluor 488 (#4412, Cell Signaling Technology).

Protein biochemistry – This involved the analysis of lysates of MM1 cells cultured alongside the glass polyalkenoate with and without additions of biopolymers.

Cell lysis – The normal oral mucosa fibroblast cell line (MM1) was cultured along with glass polyalkenoate and bio-modified glass polyalkenoate cements at an initial density of 0.5×10^6 cells per 60 mm dish. After 3 weeks in culture, the medium was aspirated from the dishes and the cells were washed 3 times using phosphate-buffered saline (PBS). Total cell protein was then harvested using a RIPA (Radio immunoprecipitation Buffer) cell lysis buffer (14) containing protease inhibitors (# 04693132001, Roche Diagnostics, Burgess Hill, UK). 500 µl of lysis buffer was added to each dish followed by incubation on ice for 10 minutes; finally, each dish was scraped and the lysates were then collected in Eppendorf tubes and stored at -20°C.

SDS PAGE (polyacrylamide gel electrophoresis) - Frozen lysates were thawed and then spun at 13000 rpm for 5 minutes. Samples were combined with equal volume of

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Laemmli loading buffer (BioRad, Hemel Hempstead, Hertfordshire, UK) and were heated at 95 °C for 5 minutes, prior to loading onto the Any KD gel (Bio Rad). 5 μL cell lysate was loaded per well, and 3 μL of Magicmark XP (Invitrogen Ltd, Paisely UK) was also loaded onto the gel, for molecular weight estimation. Gels were run at a constant voltage of 110 -150 volts in TGS running buffer (BioRad) until the dye front reached the bottom of the gel. The gel was then removed from the cassette and placed into transfer buffer.

Western blotting - Proteins were transferred from the gel to nitrocellulose membrane using a semi-dry blotter (TransBlot Semi-Dry Transfer Cell, BioRad, Hemel Hempstead, Hertfordshire, UK) and Towbin (17) transfer buffer for 42 minutes at 15 V. After blotting, the membranes were blocked in 1% milk TBST for 10 minutes and then exposed to a 1:2000 dilution of the anti-vimentin antibody (Cell Signalling #3932, Leiden, The Netherlands) overnight. The blot was then washed 3 times with TBS-T for 20 minutes for 20 minutes each wash. The membrane was then incubated with a 1:2000 dilution of the secondary antibody (Cell Signaling Anti-rabbit IgG – HRP labelled no. 7074) for an hour. Membranes were then washed again with TBST as previously described and then incubated with SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific, Life Technologies Ltd., Paisley, UK). Finally, the chemiluminescence was detected and documented by using a BioRad gel doc system. Protein expression was quantified using ImageLab software (BioRad 4.0.1. build 6).

In order to assess the percentage of Vimentin expression by cells cultured with materials, the band densities, following normalisation for gel loading, were related to those of the control (MM1 + ChemFil superior). This was achieved by setting the density of the control to 100%.

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$$Percentage\ of\ vimentin\ expressed\ by\ cells = \frac{Band\ density\ of\ each\ material}{Band\ density\ of\ the\ control} \times 100\%$$

(B) Effects of bioactive additions on the cements properties

For this aspect of the work Table III summarises the types of specimens fabricated and both the dimensions and material of the moulds used. It also details the number of specimens fabricated for each state of the material tested and where used the mould release agent.

In all cases the mixed cement was applied into the well of the mould using a plastic spatula, with packing action, to slight excess. A cellulose matrix strip was then applied to the exposed surface and pressure applied to the material through a flat glass slab on which was placed a 5 Kg weight for 5 minutes. After 30 minutes had elapsed the specimen was then removed from the mould. If upon visual inspection no defects were found, the specimen was accepted for storage and testing. All specimens were then stored in distilled water at 37 °C for one week prior to testing.

All baseline specimens were proportioned and mixed according to the manufacturer's instructions. The bioactive additions; (a) collagen Type I and (b) RGD, were added as described below. Such additions were identified from the cellular work of this study as offering greatest potential to foster cellular interaction. The material was dispensed according to the manufacturer's instructions at a powder: liquid ratio of 1:1. The additions investigated for this part of the work were type I collagen 100 µg/ml and RGD 5 mg/ml.

In addition to the above, 15 bond strength specimens, per cement variant, were prepared from longitudinally sectioned bovine molar teeth mounted in circular epoxy resin blocks (Bonda

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Clear Casting Resin, Bondaglass Vost Ltd., Kent, UK) with their buccal/palatal surfaces upper most. The exposed surfaces were rendered flat flush with the surrounding epoxy resin using a PM5 precision lapping and polishing machine (Logitech, Glasgow, Scotland) and a slurry of calcined aluminium oxide powder with a particle size of 9 μm ((Logitech, Glasgow, Scotland), for subsequent cement application. Prior to application of the cement, all prepared bovine samples were stored at 37 °C in distilled water for one month in an endeavour to ensure uniform specimen hydration. Thereafter, the specimens were removed from storage, blotted surface dry and a circular washer (5 mm diameter x 1.5 mm deep) was placed upon the exposed tooth surface. Through this, the mixed glass polyalkenoate cement was applied, using a flat plastic instrument and once clinically set, the washer was removed. The completed specimen was then stored in distilled water at 37 °C for one week prior to testing.

An Instron Universal testing machine (Model 4469, Instron Ltd., and High Wycombe, UK) was used to perform all tests unless stated otherwise.

Surface hardness - A type D Shore Durometer (Shore Instrument and manufacturing Co, Jamaica, New York, USA) was used to measure this property. Prior to its use, its calibration was checked against its supplied calibrator and each sample was subjected to one indentation, yielding a shore hardness value. For each state material hardness values were expressed as a mean and standard deviation of five samples, measured once each.

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Compressive strength - was determined at a cross head speed of 1 mm min^{-1}. Prior to testing the length and diameter of each specimen was measured using a micrometer and the flat ends of the specimens were covered with a circular disc of wet filter paper (Whatman No.1, Whatman International Ltd., Maidstone, UK) as recommended by Baig (18) 6 mm in diameter. Compressive strength was calculated using the formula:

$4P/\pi d^2$

where P was the load to failure (N) and d the specimen diameter (mm).The results for each material and state were expressed as a mean and standard deviation. This data was subject to analysis of variance with *post hoc* testing using the Tukey comparison of means test.

Adhesive shear bond strength to bovine enamel – A previously described jig (19) mounted upon the load cell of the Instron Universal Testing Machine was used to determine the shear bond strengths of the GPA cement to bovine tooth substance in both manufactured and modified form. For each combination of materials a total of 15 specimens were tested to failure. The blade of the assembly was applied, as close as possible, to the cement/tooth interface at a crosshead speed of 0.5 mm min^{-1}.

The shear bond strength was calculated using the formula:

$$\text{Shear bond strength (MPa)} = \frac{\text{Force at Failure (N)}}{\text{Bonded Area (mm}^2\text{)}}$$

This data was subject to analysis of variance with *post hoc* testing using the Tukey comparison of means test.

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RESULTS

Determination of optimal concentration of bioactive additions to promote cellular adhesion – Table IV, for all states of Chemfil Superior evaluated in this work, gives the most common cell viability rating, its range and lowest and highest values.

In relation to the additions of type I collagen to ChemFil superior the pooled rankings of cell viabilities of the cells close and away from ChemFil superior glass ionomer cement, modified by the addition of two different concentrations of type I collagen (0.01%, 0.1%) and control, at all the observed time points demonstrated very highly statistically significant differences ($P < 0.0001$) between the observed cell viabilities. For each proximity of observation these were localised using a Dunn's multiple comparison test whose outcome is summarised in table V.

In relation to the additions of RGD to ChemFil superior the pooled rankings of cell viabilities of the cells close and away from ChemFil superior glass ionomer cement, modified by the addition of two different concentrations of RGD (1 mg/ml, 5 mg/ml) and control, at all the observed time points demonstrated very highly statistically significant differences ($P < 0.0001$) between the observed cell viabilities. For each proximity of observation these were localised using a Dunn's multiple comparison test, whose outcome is summarised in table VI.

Figure 1 summarises the relative effects of the additions of Type I collagen and RGD to Chemfil superior upon cell viability, after 24 and 72 hours, compared to the cement with no additions. After 24 hours, cells exposed to modified ChemFil Superior with 0.1 % Type I collagen and 0.01% Type I collagen showed lower viability than the control, around 89.9% and 71.24% respectively. After 72 hours, the average percentages of the viability of the cells had markedly increased for both materials i.e. (0.01 % Type I collagen + ChemFil superior 146.7 % and 0.1 %Type I collagen +ChemFil superior 156.62 %). Statistical analysis (one-

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way ANOVA) showed significant effects between the groups of different Type I collagen concentrations ($P < 0.05$) on cell viability. Follow up comparison by Tukey's multiple comparison test showed no significant differences between the different concentrations of the Type I collagen.

It is thus evident that the highest cell viability was associated with ChemFil Superior modified with 0.1% Type I Collagen.

After 24 hours, cells exposed to bio- modified ChemFil Superior (1 mg/ml RGD and 5 mg/ml RGD) showed lower viability than the unmodified ChemFil Superior, around 76.22 % for 1mg/ml RGD and for ChemFil superior 93.85 % for 5 mg/ml RGD. After 72 hours the oral mucosa fibroblast cells associated with 5 mg/ml RGD + ChemFil Superior showed greater viability then the cells associated with the control, by more than 50%.

Statistical analysis (one-way ANOVA) showed highly significant effects between the groups of different RGD concentration ($P<0.001$) on cell viability. Follow up comparison by Tukey's multiple comparison test showed no significant differences between the different concentrations of the RGD

It is thus evident that the highest cell viability was associated with ChemFil Superior modified with 5mg/ml RGD.

Vimentin expression Immunocytochemistry (ICC) demonstrated that in all cases the oral mucosa fibroblast cells expressed vimentin as revealed by the level of immunofluorescence staining. Cells cultured around ChemFil Superior with 0.1% type collagen added showed the highest level of vimentin expression (Figure 2). Western blotting of the oral mucosal

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fibroblast cells, cultured with either Chemfil Superior (negative control) or the different glass polyalkenoate additives, showed that although all cells used in this experiment expressed vimentin the level of expression varied depending upon the materials present. Oral mucosa fibroblast cells cultured with modified ChemFil superior (GIC) with bio-additives specimens expressed more vimentin compared to the cells cultured with unmodified ChemFil superior (GIC). The percentage of Vimentin expression is summarised in Figure 3. It is clear that this is highest for the cells cultured with ChemFil superior modified with RGD (36% more than the control GPA).

Determination of the physical properties of the glass polyalkenoate cements at baseline and following bioactive additions - Table VII summarises the observed mean values and their standard deviations for all properties determined for Chemfil Superior with and without the addition of RGD and Type 1 collagen. For each property analysis of variance with post testing by the Tukey comparison of means test demonstrated no statistically significant ($P > 0.05$) differences following the addition of RGD and Type 1 collagen upon compressive strength and observed adhesive bond strength to bovine enamel. Both additions however improved significantly ($P < 0.05$) the shore hardness of the set material.

Discussion

This laboratory work sought to investigate the potential to modify existing GPA's to promote cellular adhesion to improve the treatment of root caries. In order to establish if modification had an adverse effect upon material properties, baseline property values were determined for the unmodified materials using recognised laboratory testing techniques.

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This discussion focusses upon the laboratory methods used in this study and thereafter the effects of the biological additions upon them.

It should be noted that in preparing the specimens for testing, a clinically realistic mixing regime was adopted. This was because it was envisaged that the material, and any additions to it, would be placed immediately into the root surface cavity; and thus contrary to the manufacturer's instructions would be exposed to blood and moisture. It was to part simulate this, that no water impervious coatings were applied to the specimens prior to storage and this may well account for the low property values observed when compared to other papers where similar materials were protected from moisture until setting was complete.

The work carried out here used a material presented in powder and liquid format for hand mixing. This presentation was favoured over encapsulated materials for it readily permitted modification of the mix by the inclusion of the biological additives tested. The authors are however aware that dispensation of the cement's components by the manufacturer supplied scoop and dropper bottle potentially introduces both variations in quantities and physical properties (20, 21), but this was not observed in the present work perhaps as proportioning and mixing was undertaken by a single operator.

The shore hardness test was selected over the more usually performed Vickers hardness test for it did not require the specimen surface to be highly polished in order to visualise the diamond indent. At the time of planning the study it was not known how the incorporation of additives would affect polishability of the samples and it seemed prudent therefore to adopt a relative ease of penetration test, such as afforded by the Shore tester, to enable a hardness assessment to be made. A downside of this approach is that the results presented here cannot be compared to other literature values for no other workers have employed this test for GPA's. It does however permit inter specimen comparison within the study.

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Compressive strength testing of specimens was undertaken using an Instron universal testing machine at 1mm min^{-1}. This test in the manner conducted has been demonstrated to be a discriminatory mechanical testing performance indicator for hand-mixed GPA's compared to three – point flexure strength, biaxial flexure strength and Hertzian indentation (18). It was for this reason that this was the sole strength test undertaken.

In this work an assessment of the bond of the glass ionomer to tooth substance was made. Self-adhesion is considered important to the proposed application of the modified materials as it will provide a marginal cavity seal and thus, if durable prevent the ingress of bacteria and development of recurrent caries. Its determination is however, controversial in respect of testing method and tooth substrate used. This study utilised macro testing (one tooth per test) compared to micro testing (sections of the same tooth used in multiple tests). In a critical review of bond strength test methods Armstrong *et al*., (22) supported the continuation of macro testing. In terms of geometry and technique of testing it is acknowledged, as reviewed by Van Noort *et al*., (23) that the results of such testing are affected by the specimen geometry, loading configuration and material stiffness. In the present work, the first two of these were controlled by standardisation of the methods, but it is acknowledged that the material stiffness could have been affected by the additions. Due to the relatively easier collection of bovine teeth as compared to human teeth, the former tissue was used for testing. Some have indicated that the bond strengths to bovine enamel are in the range of 21% to 44% less than to human enamel (24). Given also that such a test is static and does not reflect environmental interactions and multiple loading cycles (25) its findings are at best only confirmatory of adhesion.

Materials used for the cell assays were washed in HBSS for 24 hours prior to use, during which time the pH increased from 2.2 to 6.6 as at low pH, the cells would die (26). The washed materials were analysed using a number of techniques to analyse cell viability

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including microscope observation and MTT assay. In order to investigate a number of materials quickly and easily we used a simple observational technique, similar to work by Caughman et al (27). This involved observing cells close to and away from the materials. Cells growing on the materials could not be seen. After initial experiments, work focused on the MMT assay to check the ability of cells to attach and grow on the materials, together with immunocytochemistry and Western blotting to investigate the expression of vimentin. Vimentin is a cytoskeletal protein from the intermediate filament family of proteins, with a molecular weight of 57 kD, and is used primarily as a fibroblast marker (28). This study indicated that the MM1 cells appear to become more fibroblastic in character, in response to GPA with added Type I collagen and RGD.

With the exception of shore hardness the biological additions made no significant difference compared to the baseline values for ChemFil Superior in unaltered state. Although no improvements in bond strength were observed the results, accepting the limitations of the test, conformed that despite the additions the material maintained adhesive capability. Perhaps this is not surprising as the additions do not have an obvious mechanism for degrading or improving this bond.

GPA adheres to tooth substance by the formation of chemical linkages with the calcium of the tooth substance. They cannot roughen the surface of the tooth in order to promote micromechanical attachment as they are not of sufficiently low pH.

It has been observed by others that the pH of a setting conventional glass ionomer (ChemFil) changes with time, commencing around 2.2 and increasing to 6.2 after 1440 minutes (26). This range of pH values is conducive for the collagen Type I addition to form molecular aggregates, fibrils and ultimately fibres (29). Previously the formation of 70 nm collagen granules upon glass ionomers has been observed in SEM and AFM studies (30). These have

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the potential to facilitate cellular attachment to the glass ionomer and also the fibrils have the potential to bring about fibre reinforcement of the glass ionomer providing they make attachment to the material. Some indirect evidence for this is provided by the improvement in shore hardness, when Type I collagen was added, but further work is required to test this hypothesis.

In relation to the improvements in surface hardness seen when RGD was added to the conventional glass ionomer, an exact mechanism has not been found in this work. It is however possible that the amino acids (arginine, glycine, and aspartic acid) in some way become involved in the setting reaction. They have the capacity to crosslink by ionic bridges to the calcium and aluminium of the glass particles and to bond with the material's particles and also by dipole dipole interaction and/or hydrogen bonding with the material's parent acid. Further work would be required to determine if this was the case. An appreciation however, of their molecular structure indicates this is possible. Others have demonstrated that RGD can promote adhesion and osteoblast activation in relation to a pure calcium phosphate cement (31). It is worth noting that in this application the biological scaffold Chitosan improved the calcium phosphate's properties by a fibre reinforcing mechanism (31). It therefore does not seem as remote a possibility as the first thought suggests.

In conclusion the addition of RGD and Type I collagen to ChemFil Superior increased the expression of vimentin as determined by immunocytochemistry and Western blotting indicating that the cells have become more fibroblastic. There is thus the potential that such additions could promote cellular attachment to restorations of glass ionomer cement to improve the marginal seal in subgingival restorations placed to treat root caries without affecting detrimentally the materials compressive strength and adhesive capability.

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Figure 1: The relative effects of additions of Type I collagen and RGD to Chemfil superior upon cell viability, after 24 and 72 hours, compared to the cement with no additions (100 %).

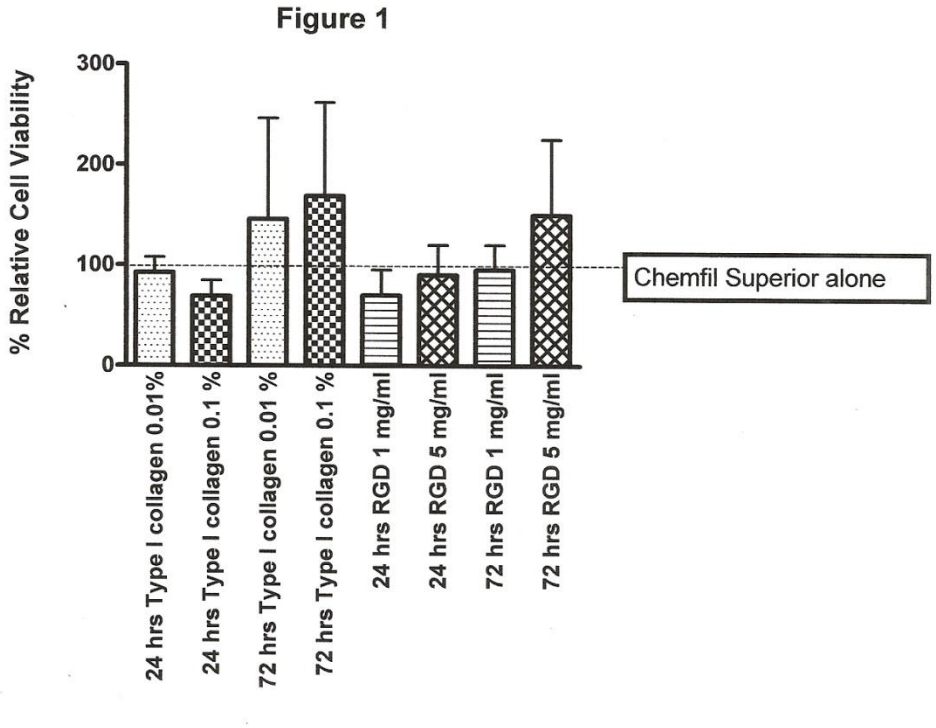

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Figure 2*: Oral mucosa fibroblast cultured in presence of the unmodified and bio-modified glass ionomer cement (ChemFil superior) and the same cultures fluorescently labelled for Vimentin. ChemFil superior + 0.1 % type collagen showed the highest level of Vimentin expression.*

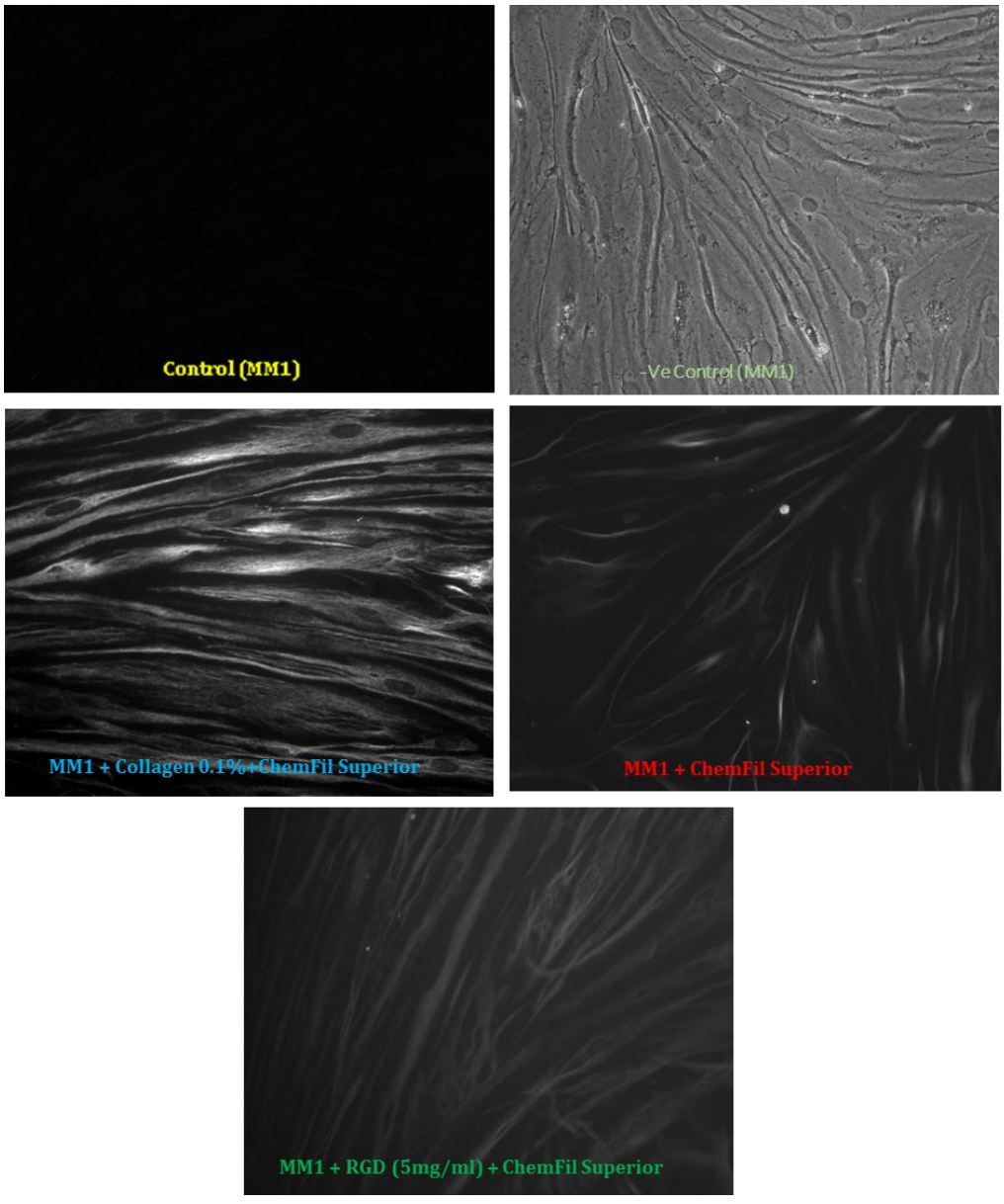

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Figure 3: The percentage of Vimentin expressed by oral mucosa fibroblast cells (MM1) cultured with Bio-modified and unmodified ChemFil superior (GIC) where ChemFil superior represents 100%.

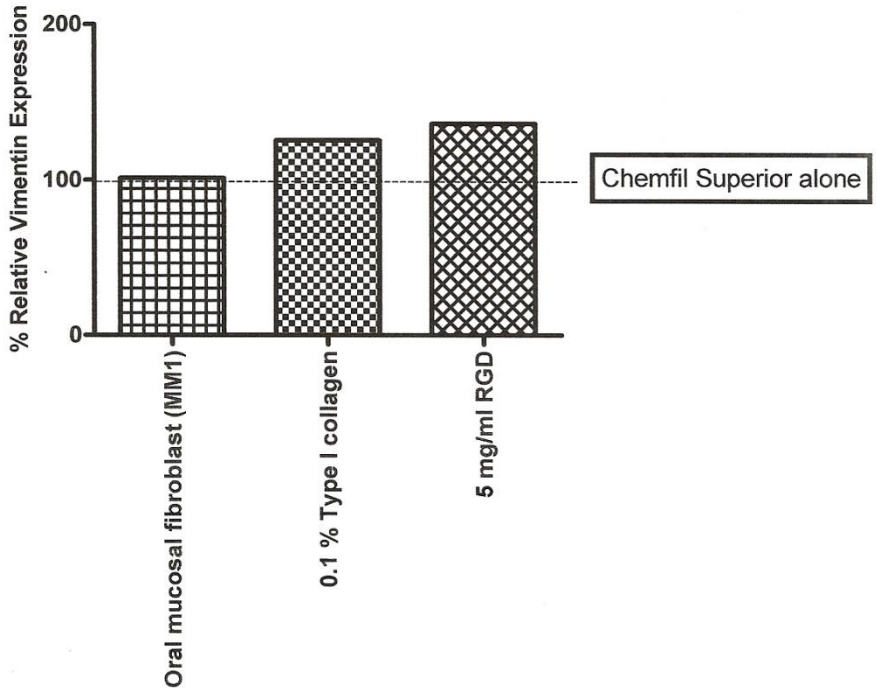

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Table I Details of the glass polyalkenoate cement studied

	ChemFil Superior	
Manufacturer	DENTSPLY D_ET_{REY} GmbH 78467 Konstanz GERMANY	
Composition	**Powder (1g)**	-Aluminium-sodium-calcium-fluoro-phosphoro-silicate (18:9:8:16:3:46) 0.84g -Polyacrylic acid (MW 30000-45000) 0.15g
	Liquid	Distilled/deionized water
Colour	L 2	
Batch number	1110001332	

Data derived from manufactur's material safety data sheet.

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Table II: Visual Observation Scoring Table for Cell Viability.

VIABILITY SCORE	**BRIEF DESCRIPTION**
1	Normal cell morphology and cell density
2	Altered cell morphology and/or small gaps between cells
3	Altered cell morphology and/or large gaps between cells
4	Few (or no) visible cells

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Table III: Summary of specimens made and mould used.

Test	n	Mould Material	Specimen Dimensions (mm)	Release Agent
Surface hardness	5	Silicone Rubber	2 thick x 12 diameter	No
Compressive strength	20	Split Stainless Steel	6 long x 4 diameter	Petroleum jelly

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Table IV: Cell culture viability scores for control and Chemfil Superior specimens in unmodified and modified forms close and away from specimen.

Specimen	Most Common Score	Range	Lowest Score	Highest Score
Control (Close)	1	2	1	3
Control (Away)	1	1	1	2
Chemfil (Close)	3	3	1	4
Chemfil (Away)	2	2	2	4
Chemfil & 0.1% Collagen (Close)	2	2	1	3
Chemfil & 0.1% Collagen (Away)	2	2	1	3
Chemfil & 0.01% Collagen (Close)	3	3	1	4
Chemfil & 0.01% Collagen (Away)	4	3	1	4
Chemfil & RGD 1mg/ml (Close)	3	3	1	3
Chemfil & RGD 1mg/ml (Away)	2	3	1	4
Chemfil & RGD 5 mg/ml (Close)	2	2	1	3
Chemfil & RGD 5 mg/ml (Away)	2	3	1	4

Key: 1 = Normal cell morphology and cell density, 2 = Altered cell morphology and/or small gaps between cells, 3 = Altered cell morphology and/or large gaps between cells, 4 = Few (or no) visible cells.

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Table V: Dunn's Multiple comparison test of rankings of cell viabilities cells close to and away from unmodified and modified, by the addition of different concentration of type I collagen (0.01%, 0.1%), ChemFil superior glass ionomer cement.

Versus	0.1% ChemFil Superior + Collagen type I (Close)	ChemFil Superior + 0.01% Collagen type I (Close)
ChemFil Superior (Close)	***	***
ChemFil Superior + 0.1% Collagen type I (Close)	—	***
ChemFil Superior (Away)	**NS**	*
ChemFil Superior + 0.1% type I Collagen (Away)	—	***
	0.1% ChemFil Superior + Collagen type I (Away)	ChemFil Superior + 0.01% Collagen type I (Away)

Key; **NS** = not significant, * = $p < 0.05$, **= $p < 0.01$, *** = $p < 0.0001$,
Note: No comparison between the results close and away from the specimen is made.

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Table VI: Dunn's multiple comparison test of rankings of cell viabilities cells close to and away from unmodified and modified, by the addition of different concentrations of RGD (1 mg/ml, 5 mg/ml) to ChemFil superior glass ionomer cement.

Versus	ChemFil Superior + RGD 1mg/ml (Close)	ChemFil Superior + RGD 5ml/ml (Close)
ChemFil Superior (Close)	**NS**	***
ChemFil Superior +RGD 5mg/ml (Close)	***	___
ChemFil Superior (Away)	NS	**
ChemFil Superior +RGD 5mg/ml (Away)	*	____
	ChemFil Superior + RGD 1mg/ml (Away)	ChemFil Superior + RGD 5ml/ml (Away)

Key; **NS** = not significant, * = $p < 0.05$, **= $p < 0.01$, *** = $p < 0.0001$,

Note: No comparison between the results close and away from the specimen is made.

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Table VII: Summary of mean property values, Standard Deviations and number of specimens tested of Chemfil Superior with and without addition of RGD and Type 1 collagen.

Property/Material State	**Chemfil Superior**	**Chemfil Superior plus RGD**	**Chemfil Superior plus Type 1 collagen**
Shore Surface Hardness	36.0 (7.4) n = 5	50.5 (9.7) n = 5	54.7 (8.8) n = 5
Compressive strength (MPa)	63.36 (18.97) n = 20	62.34 (24.72) n = 20	75.57 (23.28) n = 20