1. Introduction

Bioactive glasses (BAGs) were first used in dentistry for the treatment of bony defects [1]. This material was further developed as a commercial dentifrice to treat teeth with dentine hypersensitivity and enamel demineralisation [2]. The reduction of dentine hypersensitivity occurs by occluding exposed dentinal tubules with apatite formation using a calcium-sodium-phospho-silicate glass. Remineralisation of small enamel defects caused by acid erosion or caries is enhanced through the release of calcium and phosphate ions [3, 4]. More recent research has focused on incorporating BAGs in dental composites and adhesives [5] to prevent and/or treat demineralisation of teeth through beneficial long term ion release and their acid neutralising characteristics [6, 7]. Depending upon the glass composition, restorative materials containing BAG can release locally, and more continually, a range of therapeutic ions such as Ca^{2+}, Sr^{2+}, PO_4^{3-} and F^- at the site of demand, rather than less specifically through toothpaste or remineralising gel.

The mechanism of enamel demineralisation and remineralisation has been extensively studied over several decades. Hydroxyapatite crystals in enamel are in quasi dynamic equilibrium with the aqueous phases of saliva and plaque fluids [8]. The amount of dissolution of enamel is directly related to both the pH and the concentration of calcium and phosphate ions in the solution [2, 9] . On the other hand the rate of apatite formation of the BAG has been shown to increase dramatically upon increasing the phosphate content, whilst maintaining the network connectivity (NC) at a low value [10, 11]. Another factor that increases the rate of apatite formation is the addition of a small amount of fluoride, whilst again maintaining the NC [11-14]. The composition of the immersion solution can also affect the apatite formation process depending on the presence or absence of ions necessary for apatite crystal formation

and on the pH of the solution. In our previous study [15], we manufactured and tested a novel fluoride containing BAG resin adhesive for its potential on ion release and acid neutralising effect. It was found that F^{-}, Ca^{+2}, PO_4^{-3} were released substantially over a six-month period and the release was higher and faster in acidic media at pH4 compared to pH7. The BAG resin was also shown to have a long term neutralising effect [15].

Very few studies exist evaluating the ability of the BAG adhesives or composites to form apatite, particularly with regards to the effect of the immersion media on apatite formation. The aim of the present study was to investigate the potential of the previously reported BAG resin adhesive [15] to form apatite in three different solutions: Artificial Saliva at pH4 (AS4), and pH7 (AS7), and Tris buffer (TB) at pH7.35, simulating the extreme range of conditions present in the mouth. The characterisation of the apatite would also be investigated.

2. Materials and methods:

The detailed composition of the novel BAG resin adhesive was described in the previous study [15]. The BAG was composed of 35.25% SiO_2, 6% Na_2O, 43% CaO, 5.75% P_2O_5, and 10% CaF_2 and was prepared via the melt quench technique. The resin was composed of 42.25% BisEMA, 55% TEGDMA, 0.25% DMAEM, 0.5% camphorquinone and 2% 4-Meta.

2.1. Preparation of the BAG-resin disks:

Ninety disks were prepared using Teflon moulds measuring 10mm in diameter and 1.2mm in thickness. The BAG-resin:weight ratio was 80:20%. The disks were divided into three groups (n=30) to be immersed individually in three types of solutions. Each

disk was immersed in a 15 ml polypropylene centrifuge tube (Fisher Scientific UK Ltd, Leicestershire, UK) containing 10 ml of the solution.

2.2. Preparation of the immersion media:

2.2.1. Tris buffer (TB)

The preparation of TB solution was by dissolving 15.09g tris-(hydroxymethyl)aminomethane (Sigma–Aldrich) in 800 ml deionized water, adding 44.2 ml 1M hydrochloric acid (Sigma–Aldrich) and overnight heating to 37 °C. The pH was adjusted to 7.3 the next day, using 1M hydrochloric acid and a pH meter (Oakton Instruments, Nijkerk, the Netherlands). Deionized water was then added to make up a total volume of 2.0 litres. The solution was kept at 37 °C [11].

2.2.2. Artificial saliva

Artificial saliva (demineralising and remineralising buffers) were prepared according to Ten Cate et al [16]:

1- Demineralising buffer (AS4): 2.0 litres AS4 was prepared by dissolving 0.4411g of $CaCl_2.2H_2O$, 0.245g KH_2PO_4 (all Sigma–Aldrich) in 800ml of deionized water, and adding 5.72ml acetic acid (Sigma–Aldrich). The pH was adjusted to 4, by adding 0.5M KOH (Sigma–Aldrich), and deionized water was then added to make up a total volume of 2.0. The solution was stored in a fridge at 2°C.

2- Remineralising buffer (AS7): 2.0 litres AS7 was prepared by dissolving 0.4411g $CaCl_2.2H_2O$, 0.245g KH_2PO_4, 9.532 Hepes and 19.386g KCL (all Sigma–Aldrich) in 800ml of deionized water. The pH was adjusted to 7, by adding 0.5M

KOH (Sigma–Aldrich) and deionized water was then added to make up a total volume of 2.0 litres. The solution was stored in a fridge at 2°C.

2.4. Investigations for apatite formation:

For the characterisation, the samples were investigated by the following techniques:

(1) Attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) using a Spectrum GX (Perkin-Elmer, Waltham, MA, USA) - The disk was pressed against the ATIR-FTIR lens to attain maximum signal intensity. Data collected from 1800 to 500 cm^{-1} in absorbance mode.

(2) X-ray diffraction (XRD) using an X'pert Pro Diffractometer (Panalytical, Netherlands) with Cu-Kα alpha radiation. The samples were analysed using a 2θ range of 3-70°, with a step size of 0.03 and a step time of 200 seconds.

(3) Scanning electron microscope (SEM) - The immersed disk was halved and one half was imbedded in a cold cure acrylic resin in a Teflon mould of 10.2mm diameter and 5mm depth. The embedded disk was polished at the fracture surface using a sequence of silicon carbide grinding papers (P300, P1000 and P4000 respectively) in a Kent 4 Automatic Lapping and Polishing Unit (Kemet International Ltd, Maidestone UK). The samples were attached to SEM stubs and carbon coated to minimize charging and improve the imaging resolution.

2.4.1. Bioactivity of BAG - 24 hour study:

In order to investigate the bioactivity and its potential of the BAG, an initial 24hr study was carried out. A sample of the BAG powder (75mg) was immersed in 50 ml of TB in a 250 ml polypropylene container and one BAG-resin disk was immersed in 10 ml of TB in a 15 ml polypropylene centrifuge tube. Both containers were stored in an

incubator (KS 4000i control, IKA) at 37°C with a 60 rpm agitation. After 24 hours, the BAG powder was collected by passing the solution through a filter paper, and dried overnight at 37°C. The disk was removed, washed using deionised water and dried. The samples were investigated using ATR-FTIR and XRD.

2.4.2. BAG-resin disks – long term study:

For the long term study, the disks, after immersion in the solutions, were investigated at 10 time points. At each time point (6, 12 and 24h, 3, 7, 14, 30, 60, 90 and 180 days), 3 disks were removed from each of the solutions, washed with deionised water and dried. Each disk was then investigated by ATR-FTIR, XRD and SEM. The rest of the disks were washed in deionised water and reimmersed in a fresh solution.

3. Results:

3.1. Characterisation of the BAG bioactivity in Tris buffer:

The XRD pattern and ATR-FTIR spectra for BAG powder before and after immersion are shown as a reference in Figs 1 and 2, respectively. It can be observed that the BAG powder changed from an amorphous pattern (Figs 1a and 2a) to form apatite, similar to that of HAP after 24h of TB immersion (Figs 1b and 2b). For the BAG-resin disk before immersion (Fig 3b), the ATR-FTIR spectra have a broad band between 1200-800 cm^{-3} representing the amorphous BAG (Fig 3a) and peaks at 1724cm^{-1} representing the resin [17] (Fig 3d). After 24h immersion in TB, new peaks at 560, 600 and 1030 cm^{-1} were found in the BAG-resin spectra (Figs 3c), similar to that for the BAG powder after immersion (Fig 2b). On the other hand, the resin component of the spectrum at 1724cm^{-1} did not show any change.

3.2. Bioactivities of BAG-resin disks after long term immersion

After long term immersion, 6h-6 months, the change of the ATR-FTIR spectra and XRD pattern with time for the BAG-resin in TB was similar to that in AS4 (Figs 4a and b, and Figs 5a and b, respectively), but the ATR-FTIR bands around 560, 600 and 1030 cm^{-1} are more defined and the XRD peaks at 25.8° and 31.8° 2θ are sharper for AS4. It was also noted that the bands at 1724cm^{-1} diminished after 3 months and furthermore disappeared after 6 months of AS4 immersion. After immersion in AS7, the 1724cm^{-1} peak in the ATR-FTIR spectra was lost and the bands around 560, 600 and 1030 cm^{-1} were formed at 6h. There was little change of the spectra after 6h (Fig 4c) In the corresponding XRD, the patterns were similar to that of HAP from 6h to 3 months. At 6 months, the peak 25.8° became very intense (Fig 5c).

The SEM images of the disks show that the BAG particles had reacted in the surface layer of the BAG-resin disk after both TB and AS4 immersion (Figs 6a and b). For AS7, a very thin reacted layer was found in the BAG-resin disk but a distinct fairly thick precipitated layer was formed on the surface of the disk (Figure 6c). A precipitated layer was also seen on the AS4 disk at 6 months.

4. Discussion:

4.1. Characterisation of the bioactivity of BAG:

The 24h experiment shows that BAG is bioactive, forming apatite when it is immersed in a neutral solution (TB) which contains no Ca or PO_4 ions. The XRD patterns were compared to the reference patterns of hydroxyapatite (JCPDS 09-432), fluorapatite, FAP (15-876), carbonated hydroxyapatite (JCPD 19-272) and carbonated fluorapatite (JCPDS 31-267). However, due to the overlap of these diffraction patterns, they are

indistinguishable and will be referred to as HAP pattern. It was shown by the XRD that the BAG was mainly amorphous (with a halo centred around 30° 2θ) before immersion (Fig 1a). There was a very small amount (<5%) of the BAG revealing a crystalline structure as indicated by the small peaks at 31.8° and 33.9° 2θ. However, the amount was so small that it was not detected by the ATR-FTIR technique which spectra showed wide bands of the non-bridging oxygen at 1012-935 cm^{-1} (Fig 2a). Following 24 hrs TB immersion, the XRD pattern showed peaks emerging at 25.8°, 28.2° and 31.8°2θ, and small peaks at 39° 2θ and 46-49° 2θ corresponding to apatite (Fig 1b). The ATR-FTIR spectra (Fig 2b) are characterised by the disappearance of the non-bridging oxygen vibrations, changes in the Si-O stretches at 770cm^{-1}, 935cm^{-1} and 1012 cm^{-1}, emergence of a peak at 1030 cm^{-1} and the split peaks of the P-O vibrations at 560 and 600 cm^{-1}. As these spectra pattern are similar to those for HAP, it indicates that the BAG powder bioactively changes from an amorphous structure to form crystalline HAP apatite [10, 11, 18, 19].

4.2. Apatite formation of BAG-resin in 3 different media:

The long term abilities of the BAG-resin to form apatite were investigated by 3 techniques. It is noted that the infra-red rays of the ATR-FTIR only scan the surface and only provide information on the surface of the sample; whilst the X-rays in XRD penetrate through the surface layer and react with atoms along their pathway, thus providing information at a deeper layer (~50 μm) including the sub-surface (Fig 7). Hence SEM was used to ascertain what layer the spectra represented.

ATR-FTIR showed that apatite formed in TB (Fig 4a) as early as six hours, but the intensity of apatite did not change significantly upon longer immersion, while in AS4 (Fig 4b), apatite formed after 6 hours of immersion and the intensity increased

throughout the immersion period. However, the XRD patterns reveal an increase in intensity upon immersion in both solutions (Fig 5a and b). This would suggest that in AS4, the particles at the surface of the disk did not react completely because of the rapid diffusion of the solution to react with the deeper layer to form apatite incrementally. On the other hand, in the TB, the BAG particles on the surface were degraded before the solution moved deeper to react with more particles. This interpretation is supported by the SEM images which reveal the layer of reactive BAG-resin was thinner after TB, than that after AS4 immersions (Fig 6a and b respectively). This might also explain the presence of diffraction peaks of the non-reacted glass particles in the TB samples. In AS7 ATR-FTIR spectra, the resin peak at 1724cm^{-1} disappeared after 6h (Fig 4c). As the SEM image shows the very thin BAG-resin reactive layer and a distinct precipitated layer on the surface (Fig 6c). This precipitated layer could be regarded as a *de novo* layer formed from the components (Ca and PO_4) in the AS7 solution and from the BAG-resin disk. This observation is also noted on the BAG-resin disk after 6 months of AS4 immersion.

The potential of the BAG adhesive to form apatite which was demonstrated by this FTIR and XRD study is consistent with the pattern of ion release presented by the authors previously [15]. Despite the BAG used was deliberately designed with low soda content to minimise loss of the mechanical properties of the adhesive upon water storage, there was a significant cumulative release of Ca^{+2} and PO_4^{-3} indicated the ability of the material to deliver these ions especially when required during acidic attack. The PO_4^{-3} concentration was subsequently started to level off especially in TB and AS7 solution suggesting that the released phosphate was consumed in apatite formation which is consistent with the apatite formation potential found with the three solutions.

4.2.1. BAG-resin in TB:

The ATR-FTIR spectra show that apatite started to form on the surface of the disk as early as 6h after TB immersion (Fig 4a), evidenced by the emergence of the split bands at 560 and 600cm^{-1} (vibration of the P-O bond) and the Si-O-Si band around 1030cm^{-1}, but the intensity of these bands did not change significantly upon longer immersion until after 3 months. However, the XRD pattern show further increase in intensity of the diffraction peaks at 25.8° and 31.8° 2θ from 7 days (Fig 5a) suggesting a difference in depth of detection between the two techniques. In addition, the halo found around 18-25° 2θ indicates that there was a degradation of the BAG to form amorphous silica gel on the surface layer. In the SEM image (Fig 6a), no distinctive layer of precipitation was found. Therefore, it can be concluded that the reaction of the BAG-resin in TB is a surface phenomenon, affecting only a few microns on the surface of the disk.

4.2.2. BAG-resin in AS4:

The ATR-FTIR spectra also show apatite started to form after AS4 immersion by the emergence of very shallow peaks at 560 and 600cm^{-1} at 6h onwards (Fig 4b). The Si-O-Si band around 1030cm^{-1} also has similar pattern of increase intensity with time. The XRD peaks show more crystalline apatite were formed after AS4 immersion than TB, evidenced from the sharper and more intense peak at 25.8° and 31.8° 2θ (Fig 5b). As these sharper peaks are also found earlier after AS4 than TB immersion, it confirms that BAG dissolves faster in acidic than in a neutral media [20]. This also indicates that the rapid and high releases of ions from BAG in acidic solution, as reported in our previous study [15], do not only have a neutralising effect, but are also used to form

thicker apatite layer on the surface of the disk. This can be confirmed by the disappearance of the resin peak at 1724cm^{-1} in the ATR-FTIR spectra at 6 months time point, and the thin layer of deposit in SEM image (Fig 6b). In addition, the high release of F ion from the BAG-resin in acidic condition [15] might enhance the formation of apatite.

4.2.3. BAG adhesive disks in AS7:

Although both the ATR-FTIR and XRD show apatite formation on the disk surface after AS7 immersion (Figs 4c and 5c), a very thin surface reactive layer was observed in the SEM image (Fig 6c). A layer of precipitate appeared as a chalky white layer, on the disk surface. In addition, the resin peak at 1724cm^{-1} in the ATR-FTIR spectra was not found at all the time points from 6h of AS7 immersion. These features indicate that the apatite was precipitated using the Ca and PO_4 ions from the AS7 solution. It is also noted that the relative intensity in the XRD peaks at 25.8° (002 plane) to 31.8° (121 plane) after AS7 immersion, is about 20 times that of HAP. This indicates that the apatite crystals are more orientated, similar to that of human enamel when they are formed in a neutral solution saturated with Ca and PO_4 ions. Also, this preferential orientation increased with time as shown by the increase of the relative intensities (Fig 8). This preferential orientation of apatite crystals formed on BAG was found previously by Rehman *et al*. [21] on solid disks of Bioglass® immersed in simulated body fluid. However, they found a reduction of preferential orientation of these crystals with time and the orientation was much less marked compared to the present study. The variance in the findings is probably attributed to the difference in the composition of the BAG used. In the present study the glass has a lower Na and higher Ca content

and it also contains fluoride. The higher Ca concentration on the disk surface might affect the concentration gradient and lead to a more preferential crystal orientation. In addition, the fluoride content could favour more needle like crystal formation by promoting growth in the c- direction.

The relative intensity of the apatite formed was observed to increase as a function of time which was confirmed by the ATR-FTIR results. Fig 9 shows the band at 600cm^{-1} for apatite normalised to the intensity of the band at 1724cm^{-1} corresponding to the resin. The data indicates that apatite is forming in AS7 preferentially on the surface and the apatite that formed is becoming thicker with time. In the case of immersion in AS4, the apatite layer is only formed on the surface after 6 months which supports the SEM images.

4.2.4 Clinical application:

The current study shows that when the BAG-resin is in a neutral solution, there is little reactivity except that it may promote orientated apatite formation if the solution is saturated with Ca and PO_4 ions, probably enhanced by its F release. This is likened to the resting phase of saliva and the precipitation of apatite may form a protective layer on enamel against future attack. Hence, the BAG-resin does not react until it is challenged in an acidic environment (e.g. acid produced by bacterial plaque) to release neutralising ions and F. As these ions also promote apatite formation, the BAG-resin does not only have a preventive effect through acid neutralisation, but also importantly a reparative effect on demineralised enamel. Because of the high F content of the BAG, FAP instead of HAP may be formed. In the present study, it is not certain which type of apatite is formed due to the indistinguishable peaks of HAP and FAP in the FTIR spectra and XRD patterns. However, the water ligation band at 630 cm^{-1} in the FTIR associated with the O-H of hydroxyapatite [22] was not present, which could be due to structural disorder in the apatite lattice or be a result of a fluorapatite forming. Therefore, further study using ^{19}F magic angle spinning-nuclear magnetic resonance (MAS-NMR) spectroscopy is planned to establish if a fluorapatite forms. If FAP is formed, it will give an additional benefit as FAP is more stable and resistant to acid challenge than HAP.

Finally, as many studies have investigated the addition of bioactive components such as amorphous calcium phosphate and hydroxyapatite to dental composites to reduce the potential for demineralisation and caries [23-25], few of them have studied the effect of incorporating BAG as the active component [5, 26, 27]. It was found that the BAG composites neutralise acid pHs, release beneficial ions and have a potential demineralisation prevention effect, which is in agreement with the results of the current and the previously presented studies [15]. However, the current study provides additional information regarding the possible impact of the pH and the saturation of the immersion solution on apatite formation and demonstrated some correlations between the detected ion concentrations and the tendency to form apatite.

Conclusions:

This novel BAG-resin has the potential to be developed into an orthodontic adhesive due to its long term protective and repairable effect to form apatite.

References:

[1] Hench LL, Splinter RJ, Allen W, Greenlee T. Bonding mechanisms at the interface of ceramic prosthetic materials. Journal of biomedical materials research 1971;5:117-41.
[2] Hill R. An alternative view of the degradation of bioglass. Journal of Materials Science Letters 1996;15:1122-5.

[3] Burwell A, Litkowski L, Greenspan D. Calcium sodium phosphosilicate (NovaMin®): remineralization potential. Advances in dental research 2009;21:35-9.
[4] Featherstone J. Remineralization, the natural caries repair process—the need for new approaches. Advances in dental research 2009;21:4-7.
[5] Brown ML, Davis HB, Tufekci E, Crowe JJ, Covell DA, Mitchell JC. Ion release from a novel orthodontic resin bonding agent for the reduction and/or prevention of white spot lesions: an in vitro study. The Angle orthodontist 2011;81:1014-20.
[6] Yang S-Y, Piao Y-Z, Kim S-M, Lee Y-K, Kim K-N, Kim K-M. Acid neutralizing, mechanical and physical properties of pit and fissure sealants containing melt-derived 45S5 bioactive glass. Dent Mater 2013;29:1228-35.
[7] Davis HB, Gwinner F, Mitchell JC, Ferracane JL. Ion release from, and fluoride recharge of a composite with a fluoride-containing bioactive glass. Dent Mater 2014;30:1187-94.
[8] Sollböhmer O, May K-P, Anders M. Force microscopical investigation of human teeth in liquids. Thin Solid Films 1995;264:176-83.
[9] Dorozhkin SV. Surface Reactions of Apatite Dissolution. Journal of colloid and interface science 1997;191:489-97.
[10] O'Donnell M, Watts S, Hill R, Law R. The effect of phosphate content on the bioactivity of soda-lime-phosphosilicate glasses. Journal of Materials Science: Materials in Medicine 2009;20:1611-8.
[11] Mneimne M, Hill RG, Bushby AJ, Brauer DS. High phosphate content significantly increases apatite formation of fluoride-containing bioactive glasses. Acta biomaterialia 2011;7:1827-34.
[12] Brauer DS, Mneimne M, Hill RG. Fluoride-containing bioactive glasses: Fluoride loss during melting and ion release in tris buffer solution. Journal of Non-Crystalline Solids 2011;357:3328-33.
[13] Gentleman E, Stevens MM, Hill RG, Brauer DS. Surface properties and ion release from fluoride-containing bioactive glasses promote osteoblast differentiation and mineralization in vitro. Acta biomaterialia 2013;9:5771-9.
[14] Shah FA, Brauer DS, Desai N, Hill RG, Hing KA. Fluoride-containing bioactive glasses and Bioglass® 45S5 form apatite in low pH cell culture medium. Materials Letters 2014;119:96-9.
[15] Al-Eesa N, Wong F, Johal A, Hill R. Fluoride containing bioactive glass composite for orthodontic adhesives–ion release properties. Dent Mater 2017;33:1324-9.
[16] Ten Cate J, Exterkate R, Buijs M. The relative efficacy of fluoride toothpastes assessed with pH cycling. Caries research 2006;40:136-41.
[17] Ho S-M, Young AM. Synthesis, polymerisation and degradation of poly (lactide-co-propylene glycol) dimethacrylate adhesives. European polymer journal 2006;42:1775-85.
[18] Kim CY, Clark AE, Hench LL. Early stages of calcium-phosphate layer formation in bioglasses. Journal of Non-Crystalline Solids 1989;113:195-202.
[19] Brauer DS, Karpukhina N, O'Donnell MD, Law RV, Hill RG. Fluoride-containing bioactive glasses: effect of glass design and structure on degradation, pH and apatite formation in simulated body fluid. Acta biomaterialia 2010;6:3275-82.
[20] Bingel L, Groh D, Karpukhina N, Brauer DS. Influence of dissolution medium pH on ion release and apatite formation of Bioglass® 45S5. Materials Letters 2015;143:279-82.
[21] Rehman I, Knowles J, Bonfield W. Analysis of in vitro reaction layers formed on Bioglass® using thin-film X-ray diffraction and ATR-FTIR microspectroscopy. Journal of Biomedical Materials Research Part A 1998;41:162-6.
[22] Moshaverinia A, Ansari S, Movasaghi Z, Billington RW, Darr JA, Rehman IU. Modification of conventional glass-ionomer cements with N-vinylpyrrolidone containing polyacids, nano-hydroxy and fluoroapatite to improve mechanical properties. Dent Mater 2008;24:1381-90.
[23] Santos C, Luklinska Z, Clarke R, Davy K. Hydroxyapatite as a filler for dental composite materials: mechanical properties and in vitro bioactivity of composites. Journal of Materials Science: Materials in Medicine 2001;12:565-73.
[24] Skrtic D, Antonucci JM, Eanes E, Eidelman N. Dental composites based on hybrid and surface-modified amorphous calcium phosphates. Biomaterials 2004;25:1141-50.

[25] Liu F, Jiang X, Zhang Q, Zhu M. Strong and bioactive dental resin composite containing poly (Bis-GMA) grafted hydroxyapatite whiskers and silica nanoparticles. Composites Science and Technology 2014;101:86-93.
[26] Manfred L, Covell DA, Crowe JJ, Tufekci E, Mitchell JC. A novel biomimetic orthodontic bonding agent helps prevent white spot lesions adjacent to brackets. The Angle orthodontist 2012;83:97-103.
[27] Yang S-Y, Kim S-H, Choi S-Y, Kim K-M. Acid Neutralizing Ability and Shear Bond Strength Using Orthodontic Adhesives Containing Three Different Types of Bioactive Glass. Materials 2016;9:125.