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# Estimated depth of apatite and collagen degradation in human dentine by sequential exposure to sodium hypochlorite and EDTA: a quantitative FTIR study

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**Abstract**

**Aim** To characterise chemical degradation of the principal constituents of dentine after exposure to NaOCl and EDTA using Infrared Spectroscopy (ATR-FTIR).

**Methodology** Ground dentine particles, from extracted permanent human molars, were passed through sieves of 38 to1000 μm to provide six size ranges. Portions (250 mg) of each size range were reacted with 5mL of 2.5% NaOCl for 2-10 minutes; or 17% EDTA for 5-1440 minutes. Powders larger than 75μm were also sequentially exposed to NaOCl/EDTA/NaOCl each for 10 minutes. All experiments were repeated 5 times. Reacted and unreacted powders were washed and dried. Particles larger than 75μm were then reground. FTIR spectra of unground and reground reacted particles enabled assessment of particle surface versus bulk chemistry, respectively, plus estimation of reaction depth. Changes in the ratio of the 1640 $cm^{-1}$ collagen: 1010 $cm^{-1}$ phosphate peak height or its inverse were obtained. These were used to estimate surface and bulk fraction reacted and thus depth to which collagen or phosphate were reduced following immersion in NaOCl or EDTA, respectively. The data was analysed descriptively.

**Results** Surface collagen fraction declined by ~40% within 2 minutes of NaOCl exposure, and plateaued at ~60% between 6–10 minutes. Bulk spectra showed average depth of collagen loss at 10 minutes was 16±13 μm. Ten minute EDTA exposure caused ~60 % loss of surface phosphate. Average depth of phosphate loss was 19±12 μm and 89±43 μm after 10 and 1440 minutes EDTA immersion, respectively. Sequential NaOCl/EDTA immersion, yielded a 62±28 μm thick phosphate-depleted surface. Sequential NaOCl/EDTA/NaOCl treatment resulted in approximately 85 μm of collagen loss.

**Conclusions** Data revealed the sequential depletion of collagen by NaOCl and apatite by EDTA in dentine, simultaneously exposing the other moeity. Alternate exposure to NaOCl and EDTA therefore enhances the depth of erosion.

## Introduction

Root canal treatment subjects dentine to mechanical stresses and chemical exposure to control the resident microbiota. The procedure leads to profound changes in the physical (Niu *et al.* 2002), mechanical (Rajasingham *et al.* 2010), and chemical (Pascon *et al.* 2012) properties of the dentine. NaOCl acts predominantly on the organic component of dentine, exhibiting little or no apparent effect on the mineral content (Pascon *et al.* 2012). EDTA chelates calcium ions in hydroxyapatite crystals and facilitates demineralisation of dentine (von der Fehr & Östby 1963), which extends to approximately 20-50 μm into the root dentine (von der Fehr & Östby 1963, Fraser 1974, Verdelis *et al.* 1999).

Sequential, repeated 30-minute irrigation steps with 5.25% NaOCl increased tooth surface strain in cyclically loaded premolars but this tended to plateau after two irrigation steps (Sim *et al.* 2001). It was hypothesized that remaining mineral posed a barrier to deeper NaOCl penetration. Abolition of the strain plateau effect by alternate use of 5% NaOCl and 17% EDTA appeared to confirm this (Rajasingham *et al.* 2010). It was hypothesised that NaOCl and EDTA depleted the organic and mineral components, respectively, allowing a greater overall penetration of both.

Studies exploring the effect of chemical irrigants on mechanical properties of dentine are conclusive in showing their negative effect on tooth resilience, elastic modulus, and flexural strength. These changes are almost certainly due to the altered chemical composition of dentine. The nature and extent of the chemical change is the focus of the present study.

## Materials and Methods

### *Collection and storage of teeth*

This study was approved by the EDI/EDH Joint Research & Ethics Committee, University College London Hospitals NHS Trust (Study reference: 03/E016). Fifty, redundant freshly extracted, human, intact, non-carious, third molars were obtained with informed consent from the Eastman Dental Hospital. They were stored in 4 wt% formal-saline at room

temperature (n = 10); or saline immediately and then frozen at -2 °C (n = 40) until sample preparation, when they were left at room temperature to thaw.

A sample size of five per experiment exceeded the minimum of three, estimated using Power analysis (STAT version 13, Statcorp, Collage City, TX, USA) based on 0.9 power, 0.05 α, and a difference in 50 wt% of collagen or phosphate reacted with 2.5 wt% NaOCl or 17 wt% EDTA, respectively, compared to baseline.

***Sample preparation***

Coronal dentine samples were obtained after sectioning teeth at the cemento-enamel junction, removal of pulp tissue, and the enamel. The dentine samples in each storage-medium were separately pooled and ground to a powder using pestle and mortar. The powder from each pool was thoroughly mixed (Retsch, Haan, Germany) and sieved (Endecotts Ltd, London, UK) to give fractions of known particle size ranges; the sieves used were 38, 75, 106, 150, 500 and 1000 μm, providing fractions between these sizes.

***Baseline FTIR analysis***

The unreacted dentine samples from each storage-medium were allowed to dry in a vacuum oven (Townsen & Mercer, Altrincham, UK) at 40 °C for 48 h and then analysed using a diamond ATR-FTIR (Perkin Elmer series 2000, Beaconsfield, UK) (resolution 8 $cm^{-1}$, 4 scans, a wavenumber range 4000-500 $cm^{-1}$) to obtain baseline data. Spectra of the particle surface (up to approximately 1 μm in depth) were obtained by applying pressure with the ATR bridge on a dentine sample placed on the FTIR diamond. Samples with particles larger than 106 μm were then reground and a further “reground” (bulk) FTIR spectrum obtained to improve the spectrum absorbance level and generate an average spectrum representing the bulk chemistry of the particles.

***Exposure of dentine particle samples to test solution(s)***

Following confirmation that storage conditions imparted no observable effect on dentine FTIR spectra, particles of the same size range (<38, 38-75, 75-106, 106-150, 150-500, and 500-1000 μm) were pooled for further experiments.

The smaller dentine particles (up to 75-106 μm) were used to investigate the effects on *surface* collagen or phosphate by NaOCl or EDTA, respectively; while larger particles (106-150μm or larger) were used to study the effects on bulk collagen or phosphate. The effect of NaOCl was tested after exposure for 2 to 10 minutes, while the effect of EDTA was tested after: 10, 60, or 1440 minute exposures.

For each particle size range, two 250 mg samples of dentine particles were placed in 10 mL stirred pots on a multiple plate stirrer, 5 mL of either 2.5 wt% NaOCl, or 17 wt% EDTA was then added. This was equivalent to 1:0.5 dentine:solid NaOCl, or 1:3.4 dentine: EDTA, by weight. At each designated time interval, 0.5 mL of the suspension, containing an even distribution of particles, was removed. The reaction within a removed aliquot was immediately quenched with 8 mL of distilled water. After particle sedimentation, the clear supernatant was removed and replaced with more water. This washing, sedimentation, supernatant removal procedure was repeated 3 times. The final dentine sediment was allowed to dry in a vacuum oven. The average particle surface FTIR spectra from four repeated scans for each sample were obtained at each time point as described above. Samples with large particles were reground and re-analysed using FTIR to gain average bulk composition. Each experiment time point was repeated five times.

For the sequential reactions with NaOCl (2.5 wt%) and EDTA (17 wt%) solution, particles of 75-106 μm or larger were used. For each size range, fifteen samples of 250 mg were first individually reacted with 5 mL of NaOCl (2.5 wt%) for 10 minutes, washed and dried. Ten of these samples were subsequently reacted with 5 mL of EDTA (17 wt%) solution. Assuming dentine mass loss was relatively small after reaction with NaOCl, this was equivalent to 1:3.4 dentine:EDTA by weight. The EDTA reaction was quenched at 10 minutes and the resultant dentine particles rewashed and dried. Five of these samples were further reacted with 5 mL

of NaOCl (2.5 wt%) for 10 minutes and again samples washed and dried. All the reacted, washed and dried samples were *reground* and analysed by FTIR to gain average *bulk* composition.

A control experiment used distilled water.

***Assignment of FTIR collagen, phosphate and carbonate peaks to dentine spectra***

The average spectrum of each set of four repeated scans, and absorbance for each averaged spectrum at specific wavenumbers were obtained using Spectrum® software (Perkin Elmer series 2000). FTIR peaks were assigned for the main components of dentine and compared with existing data from the literature (Muyonga *et al.* 2004) and previous pilot studies (Tomson 2004). The collagen and phosphate peak heights at 1640 $cm^{-1}$ and 1010 $cm^{-1}$ were obtained by subtracting background absorbance at 1730 $cm^{-1}$ and 1180 $cm^{-1}$, respectively.

***Data analysis***

The fraction of collagen or phosphate reacted (F) was calculated using equation 1:

$$F = 1-(R_t/R_0) \qquad \textit{Equation 1}$$

To calculate collagen fraction reacted, R was taken as the height of the collagen peak above background divided by the phosphate peak height. For the fraction of phosphate reacted, R was taken as the inverse of this ratio. The subscripts t and 0 indicate use of spectra at time, t, or zero (i.e unreacted), respectively. The data from unreacted sample spectra were used as the baseline when assessing the effect of a single solution. When assessing the effect of sequential reaction to NaOCl and EDTA, spectra obtained at the end of the previous immersion step were used as baseline for the following reaction. The mean and confidence intervals of the fractions of collagen or phosphate reacted in five repeated experiments were calculated.

The depth of reaction was estimated based on the assumption that the bulk fraction of reacted collagen or phosphate, F, could be equated with the volume fraction of a fully

collagen- or phosphate-depleted shell of thickness, L around a spherical particle of original radius r. From equations for the volumes of a shell, it can then be shown that:

$$L= (1-(1-F)^{1/3})r \qquad \textit{Equation 2}$$

r is taken as equal to half the average of the two sieve sizes used to fractionate the particles within each range. The data of reacted and reground particles from all particle size ranges were pooled to calculate the mean and confidence intervals of the depth of effect. When assessing the depth of sequential reaction to NaOCl and EDTA, F was calculated using spectra obtained from untreated samples as baseline. The estimated total depth of collagen loss was the summation of depth of each reaction, accounting for the thickness of collagen exposed following apatite dissolution by EDTA and subsequently dissolved by NaOCl.

## Results

### *Baseline spectra*

There was an inverse relationship between particle size and FTIR spectrum strength, with <38 μm particle size on average giving the strongest spectra. This is a consequence of smaller particles making better contact with the ATR FTIR diamond. The unreacted baseline FTIR collagen / phosphate peak heights were less particle-size-dependent and more reproducible. The storage method had no obvious effect on the FTIR spectral peak absorbance positions or their relative peak intensities.

### *Effect of exposure to 2.5% NaOCl solution alone*

Examples of average spectra for the dried 75-106 μm particles reacted with NaOCl are shown in Figure 1. The height of 1640 $cm^{-1}$ collagen peak decreased by ~ 40% within the first two minutes of reaction and declined slowly thereafter. The peak height for the $PO_4^{-3}$ (at 1010 $cm^{1}$) remained relatively stable.

On average, the fractions of surface or bulk collagen reacted increased, while the variation amongst samples with different size ranges decreased, over time (Figure 2). For the unground smaller particles, there was no systematic effect of varying size on the fractions of surface collagen reacted. Conversely, fractional collagen loss obtained with reground,

originally larger particles, decreased with increasing fractionated particle size. The estimated mean depth of reaction after 10 minute exposure was 16±13 μm.

***Effect of 17% EDTA exposure alone***

Average FTIR spectra obtained for 75-106 μm particles, reacted with EDTA upto 60 minutes (without regrinding) (Figure 3) revealed a decrease in the phosphate (1010 $cm^{-1}$) and carbonate (871 $cm^{-1}$) peaks over time and a concomitant increase in the collagen peaks (1640 $cm^{-1}$, 1530 $cm^{-1}$, and 1240 $cm^{-1}$). The peak at 1410 $cm^{-1}$ (collagen and carbonate) remained relatively stable. These observations suggest more collagen was exposed and makes contact with the FTIR diamond upon carbonated hydroxyapatite dissolution. Similar spectral changes were observed irrespective of particle sizes or regrinding.

The data suggest a continuing deepening of phosphate loss over the twenty-four hours of EDTA exposure (Figure 4). Between 10 and 60 minutes, average *surface* phosphate reaction estimated for the three smaller particle size ranges increases over time. *Bulk* reaction for particles of <500 μm were comparable at these times but <0.2 for the largest particle size range. By twenty-four hours, bulk reaction for all particle size ranges was above 0.75. The estimated mean depth of reaction was: 19±12μm by 10 minutes; 27±13 μm by 60 minutes, and 89±43 μm by 24 h.

***Effect of sequential exposure to NaOCl, EDTA and NaOCl***

The results suggest that with the 75-106 μm particles, more than 60 % of the collagen was lost after a single NaOCl treatment, while cores of particles greater than 500 μm were barely affected (Figure 5). Subsequent treatment with EDTA resulted in 57±3 % (500-1000 μm) to 86±3 % (75-106 μm) phosphate reacted; with an inverse relationship with the particle size. This corresponded with dissolution of the 16±13 μm of apatite exposed following collagen loss in the initial NaOCl treatment plus a further 62±28 μm of apatite dissolution in deeper layers.

Further treatment with NaOCl resulted in similar fractional bulk collagen loss in smaller particles when compared with the first treatment. The largest particles (500-1000 μm) exhibited 59±3 % bulk loss of collagen. This corresponded with loss of 62±28 μm of collagen exposed following apatite dissolution by EDTA plus a further 7±4 μm of collagen loss in the deeper layers. The approximate overall extent of collagen loss following sequential exposure to NaOCl, EDTA and NaOCl was 85 μm.

Prior to reaction, collagen/phosphate peak ratios for ground particles were 0.23±0.01 for 75-106 μm; 0.26±0.01 for 106-150 μm; 0.26±0.02 for 150-500 μm; 0.26±0.02 for 500-1000 μm. The corresponding collagen/phosphate peak ratios after the full treatment regime were: 0.17±0.02; 0.20±0.01; 0.24±0.01, respectively.

**Discussion**

This study developed a consistent FTIR method for determination of changes in dentine collagen to apatite ratio and depth of reaction with NaOCl and / or EDTA. The problem of heterogeneity of dentine (Kinney *et al.* 2003) was overcome by using mortar-crushed dentine particles from many teeth. Storage of human dentine *disks* up to fourteen days in various media (distilled, purified and filtered water, phosphate buffered saline, ethanol, or buffered formalin) affected the dentine surface phosphate to collagen peak intensities because of surface mineral dissolution and/or deposition (Strawn *et al.* 1996). Use of dentine within 14 days of tooth extraction, and crushing it into particles before experimentation would reduce the effect of surface changes and consequent FTIR spectra due to storage. The dentine particles were oven-dried before FTIR analysis to minimise the confounding effect of water (1640 $cm^{-1}$). FTIR spectra before and after reacted particle regrinding informed both surface reaction rate and reaction depth.

The extracted teeth were stored in two different storage media prior to experimentation in order to verify their potential confounding effects. Storage of human dentine *disks* up to fourteen days in various solutions (distilled water, purified and filtered water, phosphate buffered saline, ethanol, or buffered formalin) has been found to have no effects on FTIR

peak positions of collagen (1630, 1538, 1445, 1395 $cm^{-1}$) or mineral (1030 $cm^{-1}$) (Strawn *et al.* 1996). Storage in different media was however shown to affect the dentine surface phosphate to collagen peak intensities (Strawn *et al.* 1996). These changes were attributed to pH variations leading to surface mineral dissolution and/or deposition (Strawn *et al*. 1996, Habelitz *et al.* 2002). Use of dentine within 14 days from extraction in addition to crushing the dentine into particles immediately before experimentation would have reduced the effect of any surface changes brought about by storage. This would explain the absence of any observed effect of storage condition (4% formal-saline at room temperature *versus* saline at -2$^{o}$C) on the FTIR spectra of dentine particles.

The volume of solution and duration of reaction tested in this study may seem incongruent with clinical practice but this was a proof of concept and methodology development study. The decision on the dentine: NaOCl/EDTA ratios was based on volume mass ratio required for complete reaction between the agent and target chemical molecules to occur. The surface area per unit volume mass for particles of 100 micron radius (0.01 cm) is 600 $cm^2$. A canal surface area of 0.35 $cm^2$ equates to 0.00058 g of 100 micron radius dentine particles (0.35 $cm^2$ / 600). The mass of 7 $mm^3$ of 2.5 w% NaOCl is 0.007 mL*2.5 w% = 0.0175 g. Therefore, the mass ratio of dentine: NaOCl equates to 1:30 (0.00058 g dentine: 0.0175 g NaOCl) in a clinical scenario based on an average preparation size of ISO 30/0.06. The relative mass of NaOCl (1:0.5) used in the present study is, therefore, in fact much lower than would be in a clinical scenario. The ratio used was, however, deemed sufficient for the purpose of the present study.

The mass ratio of 1:0.5 for dentine: NaOCl, corresponds with a collagen:NaOCl ratio of approximately 0.2:0.5. Since the average amino acid molecular weight in collagen is greater than that of NaOCl, there should be a molar excess of NaOCl relative to the amino acid groups in collagen. Reaction of the collagen would be incomplete if the hypochlorite did not penetrate into dentine within the exposure time.

FTIR indicated a reduction of the collagen peak at 1640 $cm^{-1}$ within the first two minute exposure to 2.5 wt% NaOCl, consistent with (Oyarzun *et al.* 2002), who used indirect

immunofluorescence and dentine exposed to 5 wt% NaOCl. The present findings of collagen peak reduction coupled with carbonate (1410 $cm^{-1}$) and the phosphate (1010 $cm^{-1}$) peaks remaining unchanged after ten minutes exposure, confirmed previous findings (Driscoll *et al.* 2002, Hu *et al.* 2010, Pascon *et al.* 2012).

NaOCl reaction with dentine may degrade collagen, rendering it water-soluble and removable by the washing. Surface hydroxyapatites could subsequently be released but may either re-precipitate as new water insoluble particles or back onto the dentine surface due to their limited water solubility. A combination of such processes may explain the decrease in surface collagen:hydroxyapatite ratio.

Smear layers (0.5-2 μm) form on dentine surfaces prepared by abrasion (Pashley 1992) and are composed of disorganised collagen and mineral (Wang & Spencer 2002). The calculated depth of reaction (16±13 μm) with hypochlorite over 10 minutes suggests removal of the smear layer plus substantial levels of underlying collagen. If the hypochlorite had penetrated dentinal tubules, then the level of collagen disruption might have been expected to occur at much greater depths. The apparent lack of bulk reaction for the largest particles could be a consequence of rapid apatite re-precipitation blocking dentine tubules or neutralisation of hypochlorite by the presence of the hydroxyapatite. This could either prevent hypochlorite reaction with deeper collagen and/or prevent deeper damaged collagen from being washed away. The protective role of hydroxyapatite on organic matrix stability has been hypothesised (Oyarzun *et al.* 2002).

The duration of clinical use of EDTA during root canal treatment varies according to clinical situation and degree of canal calcification. In addition, irrigation with 20 mL of 17% EDTA for 10 min has recently been recommended for pulp regenerative procedure to promote cell survival due to its ability to: (1) demineralize superficial dentine; (2) expose dentine organic matrix; (3) improve dental pulp stem cells (DPSCs) adhesion; and (4) release dentine endogenous growth factors (Diogenes *et al.* 2014). Clinically, any residual EDTA may remain active and react with the mineral component of the root dentine until all chelators had formed complexes with calcium (Patterson 1963). Furthermore, EDTA containing lubricants

have been found to possess antibacterial properties against *Enterococcus faecalis* and *Pseudomonas aeruginosa* over a 24 h incubation period (Wong *et al*. 2014). Therefore, the potential inclusion of such agents in inter-appointment medicaments may be suggested and investigation of their effects on dentine over a 24 h period of exposure is justified.

Exposure of dentine particles to 17 % EDTA was associated with reduction of the phosphate (1010 $cm^{-1}$) relative to the collagen (1640 $cm^{-1}$) peak intensity within five minutes. The observed relative reduction of phosphate peak (1010 $cm^{-1}$) affected a large proportion of particles up to 500 μm, within the first ten minutes, an effect that continued up to 24 h.

When regrinding the larger particles, they were observed to be soft and rubbery, and to flatten on compression with a spatula or by the FTIR diamond probe, especially when exposed for 24 h. The present findings are consistent with the previous reports (Zhang *et al.* 2010, Pascon *et al.* 2012), regardless of the methods used for measuring mineral content and duration of exposure. Investigation of the efficacy of smear layer removal had concluded that EDTA decalcification was not time-dependent (Machado-Silveiro *et al.* 2004). The mineral loss over 15 minutes was attributed to removal of loosely bound smear layers without any effect on subsurface dentine (Dogan & Qalt 2001). The present findings differ, revealing that the depth of reaction increased with duration of exposure and beyond any possible smear layer. The previous notion of EDTA exhaustion through reactive depletion (Patterson 1963) may not be justified if a reasonable volume of high concentration EDTA was used (Nikiforuk & Sreebny 1953). The effect of EDTA in the present study was more extensive than indicated by previous micro-radiogram data after 24 h exposure in teeth (von der Fehr & Östby 1963); where root canals exposed to 15% EDTAC (containing Cetavlon, a cationic surfactant), exhibited a demineralised zone extending 50 μm into dentine after 24-48 h. Exposure beyond 24 h had not increased the demineralisation zone. The difference in outcomes may be attributed to: (1) different environments (canal surface would have a lower volume:surface ratio); (2) supplementation of EDTA with a cationic surfactant; and (3) a less sensitive method for measuring mineral loss (von der Fehr & Östby 1963).

Present findings confirm that NaOCl and EDTA cause chemical changes in dentine and that alternating exposure promotes alternating loss of organic and inorganic material, enhancing their erosive effect (Calt & Serper 2002). Pre-treatment with NaOCl enables the EDTA to penetrate deeper into dentine; a second treatment with NaOCl resulted in further loss of collagen and the phosphate: collagen ratio returned to that of untreated dentine for all particle sizes. At this stage, only cores of dentine particles remained that both EDTA and NaOCl failed to reach.

The estimations of depth of reaction are prone to systematic errors due to the assumptions made and should therefore be interpreted with caution. The estimated mean depth of reaction with NaOCl for 10 minutes (overall estimate = 16±13 μm) may be as low as 9±7 μm based on the minimal size or as high as 23±20 μm based on the maximum size of the particles within each size range. Similarly, the estimated mean depths of reaction with EDTA may range between: 11±5 μm & 27±20 μm by 10 minutes (overall estimate = 19±12μm); 16±4 μm & 38±23 μm by 60 minutes (overall estimate = 27±13 μm); and 56±29 μm & 112±60 μm by 24 h (overall estimate = 89±43 μm). The limits of errors were within 10 μm for all cases except those exposed to EDTA for 24 h, where the limits of errors were within 30 μm.

As predicted (Rajasingham *et al.* 2010), penetration into dentine by NaOCl is limited by the apatite but the precise *in situ* extent has been elusive. Their explanation for the abolition of the plateauing effect of NaOCl through removal of the protective hydroxyapatite by EDTA was confirmed in this study. A chemical explanation for the mechanical findings in previous studies (Sim *et al.* 2001, Rajasingham *et al.* 2010) was proposed and provides some indication of the depth of effect. The precise depth of effect *in situ*, however, remains to be determined.

**Conclusions**

Sodium hypochlorite reduced the collagen content of dentine rapidly within the first four minutes of reaction, leading to a plateauing effect thereafter. Conversely, EDTA continuously reduced the phosphate content of dentine over twenty-four hours and exposed collagen in the process. The depth of hypochlorite reaction was 16±13 μm after 10 minutes. The depth of EDTA reaction increased with duration of exposure (19±12 μm by 10 minutes, 27±13 μm by 60 minutes, and 89±43 μm by 24 hours) and by pre-treatment with NaOCl (62±28 μm by 10 minutes). NaOCl/EDTA/NaOCl treatment resulted in an estimated further 62±28 μm plus 7±4 μm thick collagen-depleted surface compared to the 16±13 μm depletion by initial NaOCl treatment, alone.

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**Figure legends**

**Figure 1** Average spectra (n = 5) of 75-106 μm dentine particles following reaction with NaOCl for 2, 4, 6, 8, or 10 minutes

**Figure 2** The mean and 95 % confidence intervals of fraction of surface (for particle size ranges <38 μm, 38-76 μm, 75-106 μm) or bulk (for particle size ranges 106-150 μm, 150-500 μm, 500-1000 μm) collagen reacted following exposure for various times to 2.5 wt% NaOCl. For the largest particles fractions are all close to zero.

**Figure 3** Average spectra (n = 5) of 75-106 μm dentine particles following reaction with 17 wt% EDTA for 5, 10 or 60 minutes (without regrinding)

**Figure 4** The mean and 95 % confidence intervals of fraction of surface (for particle size ranges <38 μm, 38-76 μm, 75-106 μm) or bulk (for particle size ranges 106-150 μm, 150-500 μm, 500-1000 μm) phosphate reacted following exposure for various times to 17 wt% EDTA. (*Particles smaller than 106 μm were only tested up to 60 minutes; particles larger than 106 μm were only tested after 10 minute exposure*)

**Figure 5** The mean and 95 % confidence intervals of fraction of bulk collagen reacted with NaOCl or bulk phosphate reacted with EDTA following each step of sequential exposure to the agents and regrinding of particles.

**Figure 1. Average spectra (n = 5) of 75-106 µm dentine particles following reaction with NaOCl for 2, 4, 6, 8, or 10 minutes**

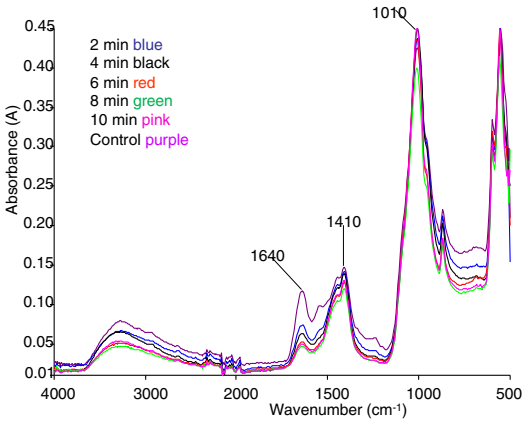


**Figure 2. The mean and 95 % confidence intervals of fraction of surface (for particle size ranges <38 µm, 38-76 µm, 75-106 µm) or bulk (for particle size ranges 106-150 µm, 150-500 µm, 500-1000 µm) collagen reacted following exposure for various times to 2.5 wt% NaOCl. For the largest particles fractions are all close to zero**

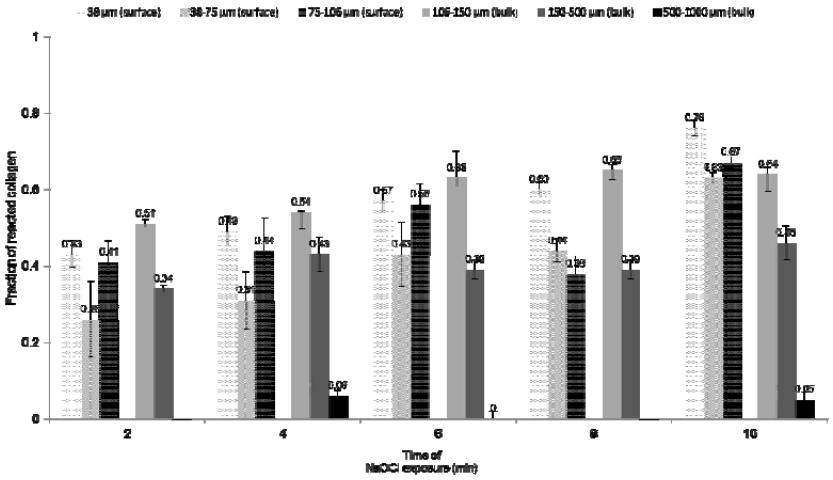

**Figure 3. Average spectra (n = 5) of 75-106 µm dentine particles following reaction with 17 wt% EDTA for 5, 10 or 60 minutes (without regrinding)**

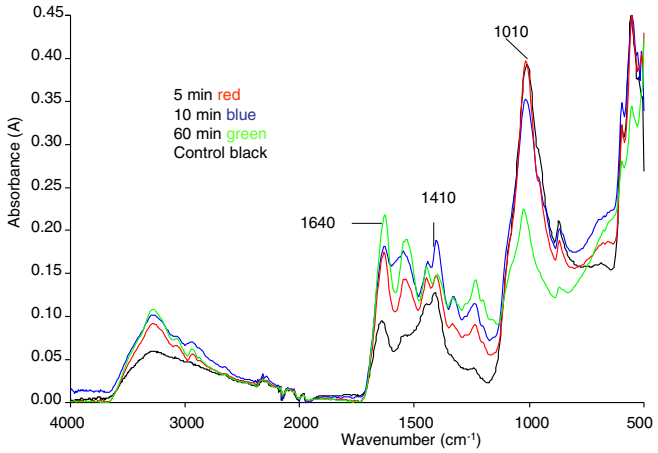


**Figure 4. The mean and 95 % confidence intervals of fraction of surface (for particle size ranges <38 µm, 38-76 µm, 75-106 µm) or bulk (for particle size ranges 106-150 µm, 150-500 µm, 500-1000 µm) phosphate reacted following exposure for various times to 17 wt% EDTA. (*Particles smaller than 106 µm were only tested up to 60 minutes; particles larger than 106 µm were only tested after 10 minute exposure*)**

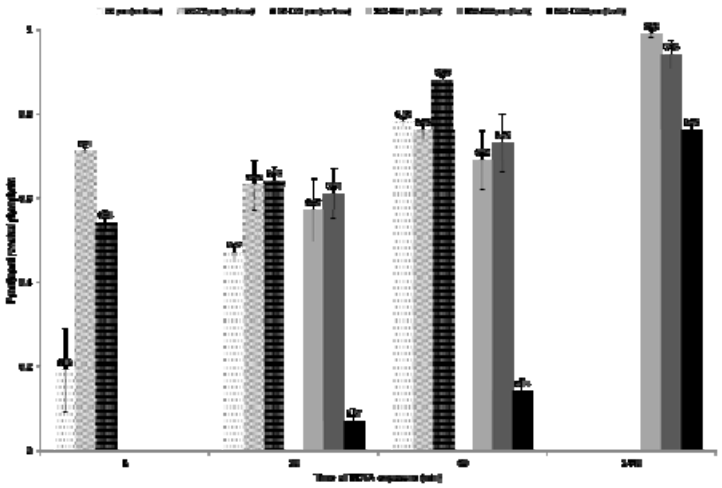

**Figure 5. The mean and 95 % confidence intervals of fraction of bulk collagen reacted with NaOCl or bulk phosphate reacted with EDTA following each step of sequential exposure to the agents and regrinding of particles.**