Staining of Carious Dentine using Dyes with Covalent and Electrostatic Binding Properties – An *in-vitro* Study

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Abstract

Aim: The aim of the present study was to evaluate the type of binding capacity (electrostatic or covalent) of different dyes to carious dentine in sectioned un-decalcified teeth utilising light-microscopic observations.

Material and methods: A stereo microscope in incident light was used to investigate the binding properties of the dyes to carious dentine. Teeth were sagittally sectioned with a thickness of 300 µm and exposed to Food Blue (FB), Acid Red (AR), Alexa Fluor 594 (AF594), Amino fluorescein (AFS) and Lucifer Yellow CH (LYCH). Two mixed solutions, Food Blue+Lucifer Yellow CH (FB+LYCH) and Food Blue+Amino fluorescein (FB+AFS), were also tested. All the dyes had the same concentration (15 mM). The tooth sections were exposed to the individual dyes for 24 hours, followed by 24 hours in a salt solution, NaCl (1 M), and subsequent exposure to an alkaline solution for 24 hours, NaOH (0.5 M). Digital images were taken of the specimens after cutting and after each exposure respectively.

Results: Based on the chemical and light-stereomicroscopic observations, dyes with covalently binding capacity bind to carious dentine in a selective way in contrast to dyes with only electrostatic binding capacity.

Conclusions: From the present study, it appears that, in order not to stain unaffected dentine, dyes with specific reactivity to caries-specific functional groups should be used in order to achieve the selective and accurate staining of carious tissue. Dyes with electrostatic bonding properties were found not to be selective in contrast to dyes carrying a hydrazine group, which bind specifically to an ester function in carious dentine.

Key Words: Covalent binding, Electrostatic binding, Caries detection, Dental caries, Hydrazine derivative

Introduction

It has been pointed out that dental clinicians may often fail to determine when the dentine should be regarded as caries free and how hard an excavated carious dentine cavity surface should be, even though the dentine is discoloured [1]. However, the discoloration of carious dentine is an imprecise indicator of whether or not the dentine is free from bacteria [2,3]. The discoloration of the dentine is less evident in acute caries and bacterial invasion is usually diffuse and extends beyond the discoloured front [2,4].

A carious lesion consists of two layers; an outer layer, which is contaminated with bacteria, and an inner layer that is partially demineralised without harbouring bacteria [5-7]. The bacterial invasion appears to occur in the outer but not the inner layer, which has previously been described [5]. Nevertheless, it is not easy clinically to define the exact endpoint of caries removal [8]. When removing the carious tissue with burs, there is a tendency for over-excavation to occur [9]. Alternative methods, such as leaving parts of the discoloration to prevent the excessive removal of caries or sound dentine, have therefore been suggested [10,11].

In addition to the tactile control of the carious dentine, which is performed in order to distinguish carious from sound dentine during the excavation process, the use of dyes as caries detectors has been suggested [12]. However, there is scepticism in the literature relating to the use of dyes, as they have been found to be non-selective and the excessive excavation of sound dentine as the result [1,8,10,13-15]. For this reason, both the excessive removal of dentine and over-staining has been reported [16]. Staining with an acid red solution, for example, has shown that the removal of all red-stained dentine has invariably led to over-excavation [10]. Other problems with caries detectors may also occur, as the removal of over-stained sound tissue cannot be ignored [16,17]. Further, dyes may also stain un-altered collagen in sound dentine [14].

Carious dentine is a highly protonated tissue, as a result of exposure to bacterial acids [18-20]. As acids produced by bacteria demineralise and soften the dental hard tissues, additional protonation might then electrostatically attract a negatively charged dye without harbouring any bacteria [3,21]. This is in line with earlier publication that showed dyes to stain demineralised collagen matrices instead of bacteria [17].

Protonated groups, such as carboxylic acid (COOH) and/or protonated free amines (NH_3^+), are formed after acid exposure to dentine or as a consequence of peptide hydrolysis [18-19,22,23]. In addition, demineralised enamel, along with demineralised dentine, holds cations (Ca^{2+}) and free protonated amines (NH_3^+) that may attract the electrostatic negatively charged groups (SO_3^-), which are common in dyes [21,24]. Normal dentine also holds charged groups to which dyes with electrostatically charged groups can also be added [25,26]. This means that many dyes that are currently used in clinics may be unspecific [8].

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The aim of the present study was to evaluate the type of binding capacity (electrostatic or covalent) of different dyes to carious dentine in sectioned un-decalcified teeth utilising light-microscopic observations.

Material and Methods

Tooth material

Permanent teeth extracted for deep dentine caries were collected from the local dental emergency clinic in the City of Gothenburg. The patients did not want to keep their extracted teeth and donated them for experimental purposes. Thus, all teeth were kindly donated by the patients of their own free will. The teeth were stored in plastic tubes under humid conditions (1% NaCl) without any identification so that none of the teeth could be traced to any specific patient. Four permanent teeth were used for the experiments. From the time of extraction,the teeth were stored in plastic tubes under humid conditions in a refrigerator (4°C) until the time came for the experiments to be conducted.

All the teeth that were used had open caries cavities accessible without any drilling. Before the experimental procedures, the outermost parts of the caries cavity were removed by hand excavation.

The teeth were mounted with cold-curing acrylate on holders for the Leica SP1600 Low Speed Microtome (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). From each tooth, five sagittal un-decalcified sections with a thickness of 300 µm were cut in the bucco-lingual direction. After cutting, digital images were taken of the specimens in a Leica M80 Stereo Microscope (Leica M80 with 8:1 zoom, x0.75, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) in incident light against a matt black background. Digital images were taken of all sections using a Leica digital camera (Leica DFC420 C, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) equipped with Leica Application Suite LAS V3.7.0 (Leica Microsystems AG, Heerbrugg, Switzerland).

The following five dyes were used: Food Blue, Acid Red, Alexa Fluor 594, Amin fluorescein and Lucifer Yellow CH. Two mixed solutions, Food Blue+Lucifer Yellow CH and Food Blue+Amino fluorescein, were also studied. The concentrations were set at 15 mM for all dyes and they all had a neutral pH.The dyes and their data are given in *Table 1*.

In order to evaluate the binding properties of the dyes to carious dentine, the tooth sections were exposed to the different dyes and dye mixtures for 24 hours, followed by 24 hours in a salt solution, NaCl (1 M), after which they were exposed to an alkaline solution for 24 hours, NaOH (0.5 M). The specimens were thoroughly rinsed with de-ionised water between the different exposure procedures. The experimental design is shown in *Figure 1*.

Two sections from two different teeth were used for each dye. Before exposure to the dyes, images of the sections were taken in the Leica M80 stereo microscope. Each section was placed in a small plastic cup, a drop of the dye (10 µl) was applied to the carious dentine in the section and the cups were sealed with a lid to prevent them drying out. After rinsing with deionised water, new images were taken of the stained sections.

The sections were then exposed to a salt solution (1 M, NaCl) for 24 hours, rinsed with deionised water, after which new images of the specimens were taken. Finally, the sections were placed in an alkaline solution (0.5 M NaOH) for 24 hours, after which they were rinsed and new images taken.

Results

Overall description of the carious dentine

The un-decalcified tooth sections were evaluated in incident light against a matt black background *(Figure 2)*. The carious dentine was seen as a multi-zoned lesion with a yellowish to brownish colour with the central part of the front of the lesion appearing darker than the surrounding carious dentine

Food blue *(Figures 3a-d)*

After exposure to Food Blue, the tooth section was completely stained with a blue-turquoise colour and some parts were more distinctly stained. The parts of the lesion surrounding the central part of the lesion and the dentinal tubules following the front of the caries progression were more saturated with staining than the dentinal tubules in the un-affected dentine. After rinsing in NaCl, most of the stain was lost, but it still remained in the zone surrounding the central part. No stain remained after NaOH treatment.

Acid Red *(Figures 4a-d)*

All parts of the section, including parts of the enamel, were stained red after 24 hours' exposure to Acid Red. Marked staining was seen in the demineralisation front of the caries lesion, with only weak staining in the central part. The stain remained after the NaCl treatment in the central part of the lesion, with an un-stained zone in the middle. The uppermost area of the cavity was unstained. The NaOH treatment removed all the red stain.

Table 1. *Data relating to the dyes used in the study (Cas. No.=Identification number; Cat. No.=Catalogue number; N/A=not applicable).*

Dye (abbrevation)	Generic name	Binding capacity	Cas. No.	Cat. No.	Company
Food Blue (FB)	Patent Blue V calcium salt	Electrostatic	3536-49-0	74748	FLUKA, USA
Acid Red (AR)	Acid Red 1	Electrostatic	3734-67-6	210633	Aldrich, USA
Alexa Fluor 594 (AF594)	Alexa Fluor 594 hydrazide, sodium salt	Covalent Electrostatic	**N/A**	A10438	Life technologies, USA
Aminofluorescein (AFS)	5-(((2-(carbohydrazino) methyl) thio)acetyl)-aminofluorescein	Covalent	**N/A**	C356	Life technologies, USA
Lucifer Yellow CH (LYCH)	Lucifer Yellow CH Dipotassium salt	Covalent Electrostatic	71206-95	L0144	Sigma, USA
Food Blue + Lucifer Yellow CH (FB+LYCH)					
Food Blue + Aminofluorescein (FB+AFS)					

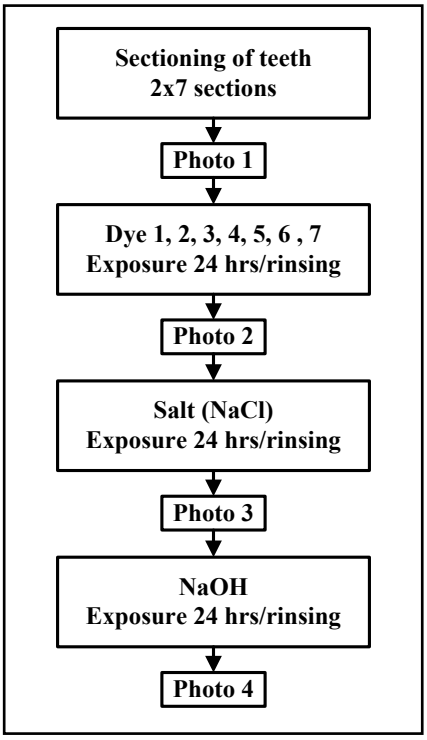


Figure 1. *Flow chart of the experimental design of the pilot study.*

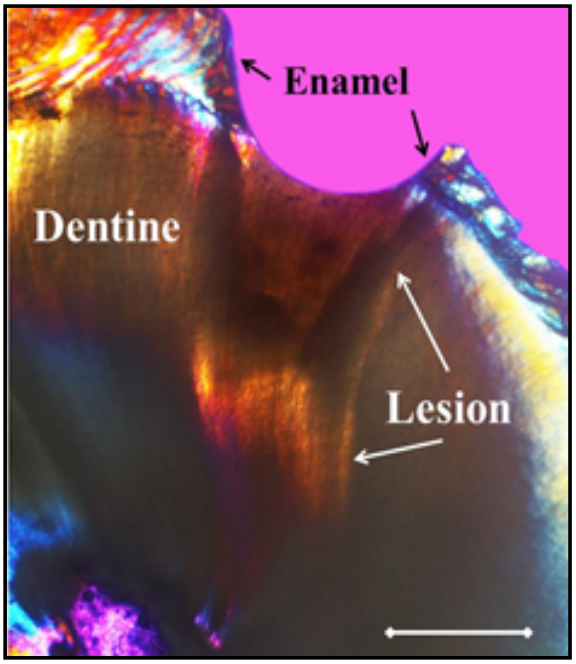


Figure 2. *Polarised light-microscopic image in incident light of an un-decalcified section from a permanent tooth with caries showing the carious dentine lesion.*

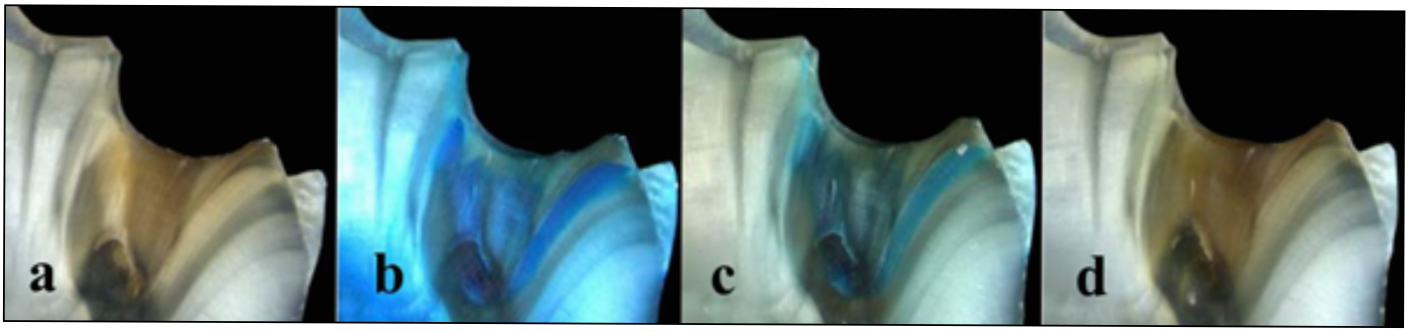


Figure 3a-d. *Light-microscopic images in incident light of an un-decalcified section from a permanent tooth with caries; a) before staining; b) after staining for 24 hours with* ***Food Blue****; c) after treatment with NaCl for 24 hours; d) after treatment with NaOH for 24 hours.*

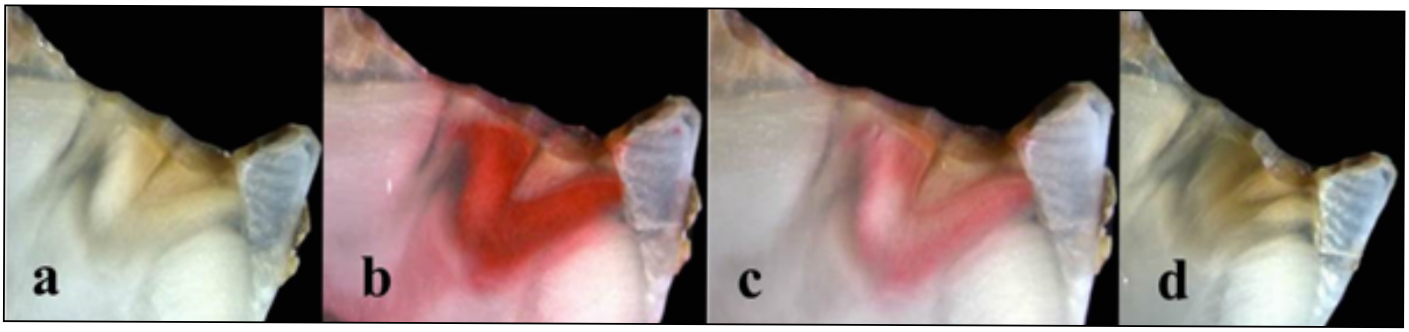


Figure 4a-d. *Light-microscopic images in incident light of an un-decalcified section from a permanent tooth with caries; a) before staining; b) after staining for 24 hours with* ***Acid Red****; c) after treatment with NaCl for 24 hours; d) after treatment with NaOH for 24 hours.*

Alexa Fluor 594 ***(Figures 5a-d)***

Alexa Fluor 594 stained the section a blue-purple colour. The zone surrounding the central part of the lesion was most heavily stained and remained stained after NaCl treatment, but with a lower intensity. Treatment with NaOH removed almost all the stain, but it could still be observed in the same areas as after NaCl treatment.

Amino fluorescein ***(Figures 6a-d)***

A yellow dye was observed, with the highest intensity in the carious lesion surrounding the central part. The intensity of the stain diminished after the NaCl treatment and the intensity remained after the NaOH treatment.

Lucifer Yellow CH ***(Figures 7a-d)***

The yellow dye with a greenish tone was observed on the entire tooth section. More intense staining was seen in the dentine carious lesion. The stain now became more yellowish after the NaCl treatment and decreased in intensity, but the highest intensity was still found within the lesion. NaOH treatment

reduced the intensity of the stain, but it still remained in the lesion.

Food Blue + Lucifer Yellow CH *(Figures 8a-d)*

The tooth section was stained a distinct green colour, but, after the NaCl treatment, it was only found in the outer part of the carious dentine lesion. After the NaCl treatment, the stain appeared bluish-green and, after the NaOH treatment, only a pale yellow colour remained.

Food Blue + Amino fluorescein *(Figures 9a-d)*

An almost fluorescent distinct green stain was observed after the staining. It was located in the central parts of the lesion, as well as in a zone in the dentine surrounding the actual lesion. The dye intensity decreased after the NaCl treatment, but it remained in the peripheral parts of the lesion. After the final NaOH treatment, no green staining could be observed. However, a zone with a yellowish colour appeared in the dentine surrounding the lesion.

Discussion

Recent work has shown that other dyes, apart from the commercially available dyes, are able to detect carious dentine by covalent binding. They appear to be more specific than the electrostatically binding dyes [27]. Also, de Almeida et al. stated that the "ideal caries-disclosing dye should solely stain the caries-infected but not the caries-affected dentine" [8].

Based on the chemical and light-stereomicroscopic observations, this study has shown that dyes with covalent binding capacity bind to carious dentine in a selective way in contrast to dyes with only electrostatic binding capacity.

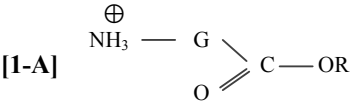
The collagen distribution and/or the bacterial content of carious dentine have been evaluated in light microscopy, where the un-decalcified sections of dental hard tissues stained with different dyes [5,7,9,14,17]. However, even if the intention was to stain only carious dentine, the undesired staining of non-carious dentine often occurred [10,17]. Furthermore, reflective light photomicrography viewed colour changes of longitudinally sectioned lesions without any addition of a dye [9]. However, as the stereomicroscopy tends to over-score the lesion, the addition of dyes is claimed to be accurate and reliable [28]. Consequently, stereomicroscopic analysis with digital photographs on sectioned lesions can be regarded as useful for the detection of dyes in dental hard tissues.

From the same tooth with dentine caries, five un-decalcified sections were prepared, making it feasible to compare the staining capacity of different stains in basically the same tissue and thereby minimising possible differences in the properties of the tooth samples.

Chemical models

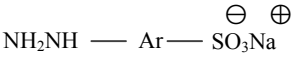
In the chemical structure schemes **[1-A]** and **[1-B],** chemical models are given. Structure **[1-A]** represents a model for carious dentine containing both an acid function, like the ammonium ion $-NH_3^+$ [19], and an ester function -COOR [27].

[1-A]



The structure **[1-B]** represents a dye containing both an $-SO_3^-$ function and a $-NHNH_2$ hydrazine function.

[1-B]



Chemical reactions

The structure schemes **[2-A]** and **[2-B]** illustrate how the stain **[1-B]** has reacted with the structure **[1-A**].The ammonium ion in **[1-A]** has reacted electrostatically with the $-SO_3^-$ groupof the stain forming an ion pair, a salt **[2-A]**. The hydrazine function $-NHNH_2$ in **[1-B]** has reacted with the ester function of **[1-A]** to form an amide **[2-B]** in a covalent bonding manner. The last reaction is thermodynamically favoured, as an amide

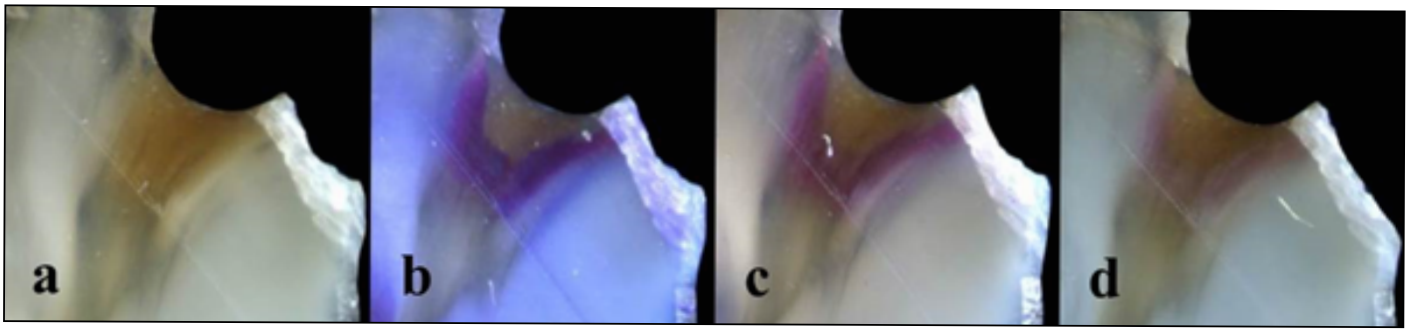


***Figure 5a-d**. Light-microscopic images in incident light of an un-decalcified section from a permanent tooth with caries; a) before staining; b) after staining for 24 hours with **Alexa Fluor 594**; c) after treatment with NaCl for 24 hours; d) after treatment with NaOH for 24 hours.*

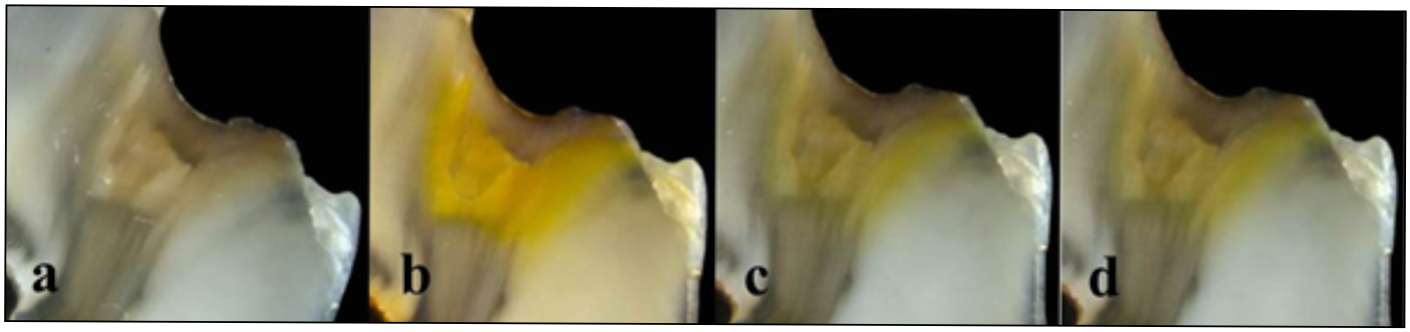


***Figure 6a-d**. Light-microscopic images in incident light of an un-decalcified section from a permanent tooth with caries; a) before staining; b) after staining for 24 hours with **Aminofluorescein**; c) after treatment with NaCl for 24 hours; d) after treatment with NaOH for 24 hours.*

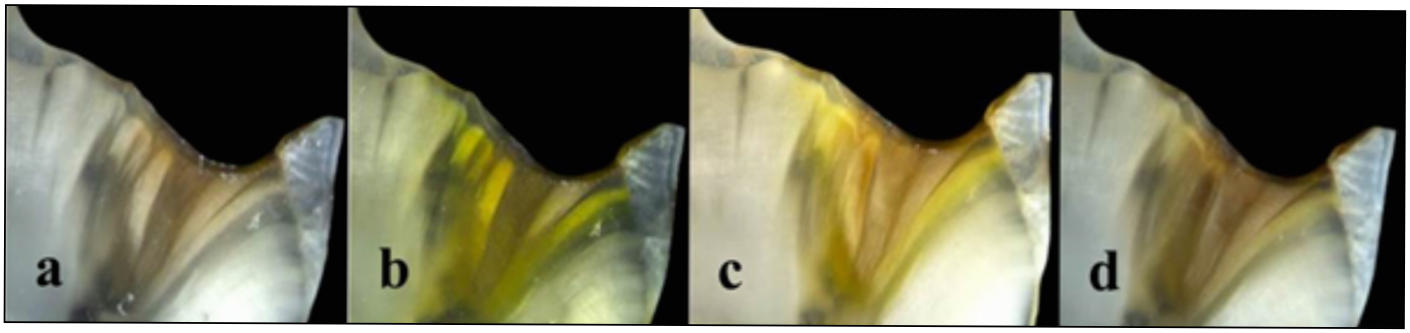


***Figure 7a-d**. Light-microscopic images in incident light of an un-decalcified section from a permanent tooth with caries; a) before staining; b) after staining for 24 hours with **Lucifer Yellow CH**; c) after treatment with NaCl for 24 hours; d) after treatment with NaOH for 24 hours.*

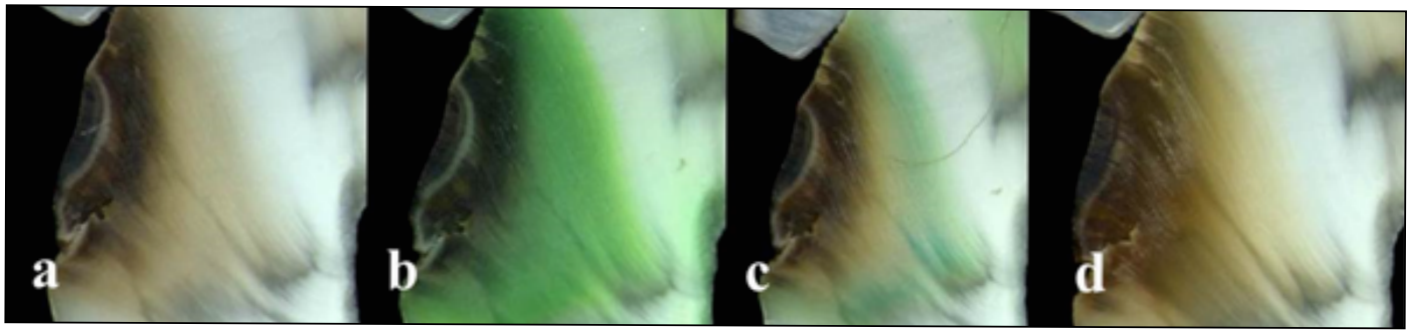


***Figure 8a-d**. Light-microscopic images in incident light of an un-decalcified section from a permanent tooth with caries; a) before staining; b) after staining for 24 hours with **Food Blue + Lucifer Yellow CH**; c) after treatment with NaCl for 24 hours; d) after treatment with NaOH for 24 hours.*

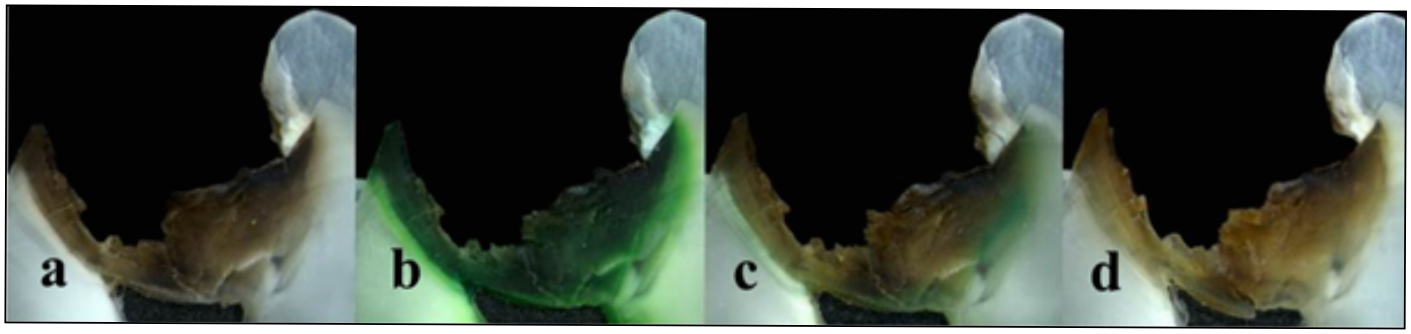


***Figure 9a-d**. L Light-microscopic images in incident light of an un-decalcified section from a permanent tooth with caries; a) before staining; b) after staining for 24 hours with **Food Blue + Aminofluorescein**; c) after treatment with NaCl for 24 hours; d) after treatment with NaOH for 24 hours.*

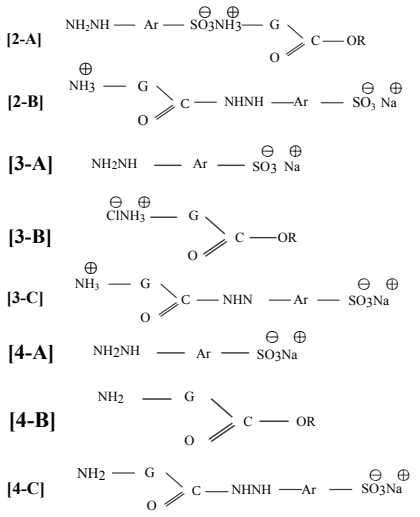
is more stable than an ester.

The addition of NaCl in a high concentration causes an ion exchange to occur in the electrostatic bond, thus exchanging SO_3^- for Cl^- in the stained carious dentine, structure **[2-A],** and consequently causing the removal of the stain from the carious tissue. The reaction products **[3-A; 3-B; 3-C]** are observed as a water-soluble un-attached stain in the supernatant **[3-A],** as de-stained hydrochloride salt of the carious dentine **[3-B]** and as stained carious dentine **[3-C]** respectively. As the ion exchange is an equilibrium reaction, all electrostatically bonded staining will not be removed in this way. The amide structure **[2-B]** will not be affected by the NaCl treatment.

In addition, as there is also an equilibrium relationship between structures **[2-A] - [3-A] - [3-B]** and the chloride ions (Cl^-), some electrostatically bonded stain will still remain in the tissue. The final treatment with NaOH will deprotonate all the ammonium groups converting the stained carious dentine, represented as structures in **[2-A]**, **[2-B]**, **[3-B]** and **[3-C]** irreversibly to structures seen in **[4-A]** (water soluble stain), **[4-B]** (un-stained de-protonated carious dentine) and to stained carious dentine **[4-C]**.

This indicates that the final removal of the electrostatically bonded dye will be carried out by the treatment with the NaOH solution, which wills deprotonate the ammonium ion ($-NH_3^+$). The positive charge for attracting the $-SO_3^-$ group will therefore no longer be available. The electrostatically bonded dye will be fully released and transferred to the water solution as structure **[1-B]**. This was also observed from the high concentration of dye in the supernatants from the electrostatically stained tissue. The amide product as seen in **[4-C]** will not be affected by the sodium hydroxide treatment and the staining from the amide product will therefore remain in the tissue.

Furthermore, stains holding both electrostatically and covalently bonding groups will react with both an electrostatic structure **[2-A]** and a covalent bonding structure **[2-B]**. Treatment with NaCl and NaOH will therefore remove the electrostatically bonded part of the stain, while the amide-bonding part will be unaffected by both the NaCl treatment and the NaOH treatment.



Summary of the reactions

The dyes used in the present study have the potential to bind either to the acidic groups of carious dentine (NH_3^+) or to the ester function groups (-COOR). This results in the formation of a salt via an electrostatic bond **[2-A]** or an amide functional group **[2-B]**. The formation of an amide requires a longer reaction time than the formation of an electrostatic bond, which is immediate.

Acid Red and Food Blue are only able to react in an electrostatic manner, as their binding groups are only of an anionic character (SO_3^-). They were therefore completely removed after the NaOH treatment. Alexa Fluor 594 and Lucifer Yellow CH hold both an electrostatic group (SO_3^-) and a hydrazine derivate group (NH_2NH-R) and, for that reason, they are able to react with both positive ions [19] and the ester functions [27] of the carious tissue. However, the hydrazine derivative, Amino fluorescein, only holds hydrazine groups and might only react with the esters in a covalent manner. This could be seen in *Figures 2-4*, where the dyes resist all the different treatments.

Mixtures of two stains, Food Blue (blue colour) with Lucifer Yellow CH (yellow colour) and Food Blue (blue colour) with Amino fluorescein (yellow colour), resulted in a green colour. The green mixture became more bluish after the NaCl treatment and yellow after the NaOH treatment. This indicates that the blue stain was concurred out by the Cl^- ion in the exchange reaction, whereas the yellow stain remained covalently bonded to the carious dentine. When comparing the initial staining with the final image after NaOH, a slight yellowish stain remained after all the treatments. Both Lucifer Yellow CH and Amino fluorescein are still present in the carious dentine after treatment with the NaOH solution. The blue stain holds more electrostatic groups than the yellow stain at stoichiometric quantities, which would explain the more pronounced bluish appearance after the NaCl treatment. The competitive staining between two dyes with different bonding properties showed that the electrostatically bonded part (the blue part of the mixture) could be rinsed off and the covalently bonded part (the yellow part of the mixture) was still attached to carious dentine after the NaCl and NaOH treatments.

Since the molecular size of a dye is less than the diameters of the dentinal tubules [29], there is always an opportunity for the dyes to be stacked between crystals. Long-term exposure of the whole sections will reduce this problem, as the reaction will have both the time and the ability to react to the sectioned tooth surfaces from all sides. Furthermore, to overcome limitations caused by the diffusion rate, a long exposure time (24 hrs.) will minimise this problem. Subsequently after 24 hours of exposure to the dyes, NaCl and NaOH solutions, respectively, equilibrium almost certainly being reached and the reaction has been accomplished.

From a clinical point of view a shorter reaction time is needed, why further evaluations of the different reactions times ought to be investigated.

Conclusions

To achieve a selective and accurate staining of carious tissue dyes having specific reactivity to carious specific functionalities should be used. Dyes having electrostatic bonding properties have been shown not to be selective in contrast to dyes carrying a hydrazine group which bind specifically to an ester- function in the carious tissue in a covalent manner.

Therefore, it can be concluded that modified proteins such as ester functional groups in carious dentine can be stained with dyes carrying a hydrazine group, thereby acting selectively in distinguishing between sound and carious dentine. Moreover, a selective and covalently bonded dye will not stain unaffected dentine and will be a support in judging the clinical end point during caries excavation.

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