FLUORIDE AS A PROTEOLYTIC ENZYME INHIBITOR IN DENTIN

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To my beloved parents, Seyit and Arife Altinci
and to my lovely brothers, Demirhan and Mustafa Altinci
for always being with me with their endless love, encouragement and support

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ABSTRACT

Proteolytic degradation of demineralized dentin occurs due to the catalytic activity of dentin matrix-bound matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs). This degradation process takes role in the progression of caries and erosive lesions, as well as the failure of resin-dentin adhesive interfaces. NaF has been previously shown as an effective inhibitor of salivary MMP-2 and -9. Therefore, in this thesis, NaF, KF and CaF_2 were systematically screened to investigate the efficacy of fluoride on the inhibition of dentin matrix-bound MMPs and CCs in order to prevent the collagenolytic breakdown of demineralized dentin matrices, and to improve the stability of adhesive interfaces. Degradation of demineralized dentin subjected to NaF, KF and CaF_2 with a wide range of concentrations has been systematically screened using total MMP activity assay, gelatin zymography, dry mass loss and the quantitative measurements of collagen degradation products involving ICTP, CTX and hydroxyproline, and scanning electron microscopy analysis. Bonding stability to phosphoric acid-etched and NaF, KF and CaF_2 treated dentin surface was examined by a microtensile strength test set-up.

According to the results, NaF, KF, or CaF_2 did not prevent the MMP-dependent degradation of demineralized dentin. High levels of NaF and KF was effective in the long-term inhibition of dentin matrix-bound CCs. However, since MMPs are the major enzyme groups responsible from the collagen degradation in dentin, only CC-inhibition was not sufficient for the prevention of demineralized dentin degradation. NaF and KF treatment of acid-etched dentin surface might be beneficial for the durability of resin-dentin adhesive interfaces by slowing down the breakdown of insufficiently resin infiltrated collagen fibrils, probably via silencing MMPs and CCs.

KEYWORDS: dentin matrix, collagen degradation, cysteine cathepsins, matrix metalloproteinases, NaF, KF, CaF_2

TIIVISTELMÄ

Demineralisoituneen dentiinin proteolyyttinen hajoaminen tapahtuu dentiinin orgaaniseen matriksiin sitoutuneiden entsyymien, matriksin metalloproteinaasien (MMP) ja kysteiini katepsiinien (CC) toimesta. Tämä hajoaminen vaikuttaa kariesleesion ja eroosion etenemiseen, kuten myös hampaan ja paikka-aineen sidosaineen liitospinnan heikkenemiseen. NaF:n on aikaisemmin osoitettu estävän tehokkaasti syljen MMP-2:n ja MMP-9:n aktiivisuutta. Tässä väitöskirjatyössä kartoitettiin systemaattisesti NaF:n, KF:n ja CaF_2:n dentiinimatriksin MMP- ja CC-estovaikutusta, tavoitteina demineralisoituneen matriksin hajoamisen estäminen ja paikka-hammasliitoksen stabiloiminen. Lukuisilla eri NaF-, KF- ja CaF_2-pitoisuuksilla käsitellyn demineralisoidun dentiinin hajoamista tarkasteltiin monipuolisesti käyttäen totaali-MMP-aktiivisuusanalyysiä, gelatiinizymografiaa, kuivamassakatoa, kollageenin hajoamistuotteiden ICTP-, CTX- ja hydroksi-proliinimittauksia sekä elektronimikroskopiaa. Happoetsatun ja NaF-, KF- ja CaF_2-käsitellyn dentiinin sidoslujuuden stabiilisuutta tutkittiin mikrovetolujuus-menetelmällä.

Tulokset osoittivat, että NaF, KF tai CaF_2 eivät estäneet MMP:ien aiheuttamaa demineralisoituneen dentiinin hajoamista. Korkeat NaF- ja KF-pitoisuudet estivät pitkäaikaisesti dentiinimatriksiin sitoutuneita CC:ja. Koska MMP:t ovat kuitenkin pääasiassa vastuussa dentiinimatriksin hajoamisesta, pelkkä CC-esto ei riittänyt estämään matriksin hajoamista. NaF- ja KF-käsittely voisi kuitenkin parantaa hampaan ja paikkamateriaalin liitosaluetta hidastamalla epätäydellisesti resiini-infiltroituneen kollageenin hajoamista, mahdollisesti MMP- ja CC-eston kautta.

HAKUTERMIT: deniinimatriksi, kollageenin hajoaminen, kysteiini katepsiinit, matriksin metalloproteinaasit, NaF, KF, CaF_2

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Abbreviations

ANOVA	Analysis of variance
AS	Artificial saliva
CaF_2	Calcium fluoride
CC	Cysteine cathepsin
CTX	C-terminal crosslinked telopeptide of type I collagen
d	Repeating unit of collagen molecule as the stagger distance of 67 nm
DMFS	Decayed/Missed/Filled Surface
EDS	Energy dispersive spectroscopy
EPA	United States Environmental Protection Agency
FDA	US Food and Drug Administration
Gly	Glycine
HEPES	Hydroxyethyl-piperazineethane-sulfonic acid buffer
HYP	Hydroxyproline
ICTP	Crosslinked carboxyterminal telopeptide of type I collagen
Isoleu	Isoleucine
KF	Potassium fluoride
kV	Kilovolt
Leu	Leucine
μl	Microliter
μm	Micrometer
μTBS	Microtensile bond strength
mM	Millimolar
MMP	Matrix metalloproteinase
MT1-MMP	Membrane type 1 metalloprotease
MT2-MMP	Membrane type 2 metalloprotease
N	Newton
NaF	Sodium fluoride
ng	Nanogram
nm	Nanometer

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I   Altinci P, Mutluay M, Seseogullari-Dirihan R, Pashley D, Tjäderhane L, Tezvergil-Mutluay A. NaF Inhibits Matrix-Bound Cathepsin-Mediated Dentin Matrix Degradation. *Caries Research*, 2016; 50: 124–132.

II   Altinci P, Mutluay M, Tjäderhane L, Tezvergil-Mutluay A. Inhibition of dentin matrix-bound cysteine cathepsins by potassium fluoride. *European Journal of Oral Sciences,* 2019; 127: 1–9.

III   Altinci P, Mutluay M, Tjäderhane L, Tezvergil-Mutluay A. Effect of calcium fluoride on the activity of dentin matrix-bound enzymes. *Archives of Oral Biology*, 2018; 96: 162–168.

IV   Altinci P, Mutluay M, Tjäderhane L, Tezvergil-Mutluay A. Microtensile Bond Strength to Phosphoric Acid-etched Dentin Treated with NaF, KF and CaF_2. *International Journal of Adhesion and Adhesives*, 2018; 85: 337–343.

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1   Introduction

Dentin organic matrix can be regarded as the protein foundation of dentin, consisting of mainly type I collagen fibrils and several non-collagenous proteins. A durable mineralized tissue is constituted with the inclusion of hydroxyapatite minerals into the collagen network. Proteolytic activities in demineralized dentin matrices, due to matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs), have been a breakthrough discovery of the recent decades in dentistry (Pashley et al. 2004). It is thought that these enzymes physiologically function during tooth development, then become fossilized with the maturation of teeth, and stay inactive in mineralized dentin (Hall et al. 1999; Martin-De Las Heras et al. 2000). The dissolution of hydroxyapatite minerals under cariogenic or erosive acid attacks, or due to the effect of adhesive bonding procedures requiring the use of acid etchants or acidic functional monomers, activate these enzymes and initiate the collagenolytic and gelatinolytic events in dentin (Nishitani et al. 2006; Kato et al. 2010, Mazzoni et al. 2012; Tjäderhane et al. 2013a,b). Consequently, MMPs and CCs play significant role in the caries progression and erosive processes, and also cause the degradation of resin-dentin adhesive interfaces, resulting in the failure of adhesively bonded restorations. To prevent these enzymatic attacks and maintain the structural integrity of demineralized dentin matrices, new strategies and treatment modalities are required in clinical practices (Tjäderhane et al. 2015). By the inhibition of dentin matrix-bound MMPs and CCs, the remineralization prospect of early caries and erosive lesions may be improved, and accordingly, restorative treatment needs can be reduced. The incorporation of anti-enzymatic measures to dentin bonding procedures may enhance the stability of adhesive interfaces, which can significantly increase the longevity of adhesively bonded restorations (Tjäderhane et al. 2013a; Sabatini and Pashley 2015; Hass et al. 2016; Zhang et al. 2017).

To date, several methods have been employed to improve the enzymatic degradation resistance of demineralized collagen fibrils, and silencing and/or inhibiting MMPs and CCs, such as the use of enzyme inhibitors, collagen crosslinkers, and biomineralization techniques (Liu et al. 2011; Niu et al. 2014). Although some inhibitor agents have given promising results, their efficacy has been found to be only limited in the short term, or their application into clinical practices

has not been considered feasible (Tjäderhane et al. 2013b; Montagner et al. 2014). Therefore, many studies are currently in search of new MMP inhibitors that can be used in a wide range of clinical applications (Tezvergil-Mutluay et al. 2011, 2013, 2015; Tjäderhane et al. 2015; Altinci et al. 2017).

Fluoride is a well-known constituent of many oral hygiene products, and incorporated into the formulations of glass ionomer-based restorative materials, several dentin adhesives, as well as composite resins due to its anticariogenic and antimicrobial properties (Buzalaf et al. 2011; Carey 2014; Lussi and Carvalho 2015). Sodium fluoride (NaF) has been also found effective in the inhibition of several bacterial enzymes, as well as salivary MMPs (Takahashi et al. 2011; Kato et al. 2014). However, it is not known whether any inhibitory effects could be achieved on the dentin matrix-bound MMPs and CCs, which are more resistant to inhibition compared to recombinant enzymes.

Within the context of this thesis, the aim was to investigate the effects of sodium fluoride (NaF), potassium fluoride (KF), and calcium fluoride (CaF_2) with a wide range of fluoride concentrations on the catalytic activity of dentin matrix-bound MMPs and CCs. Therefore, the proteolytic activities in the fluoride-treated demineralized dentin matrices were screened using indirect and direct test methods. Total MMP activity assay and gelatin zymography were selected as the tools for the direct examination of MMP and CC activities in demineralized dentin matrices. Dry mass loss and analysis of collagen degradation products, including crosslinked carboxyterminal telopeptide of type I collagen (ICTP), C-terminal crosslinked telopeptide of type I collagen (CTX), and hydroxyproline (HYP), were studied as indirect indicators of enzymatic activity in dentin. The durability of adhesive interface between the phosphoric acid-etched and fluoride-treated dentin surface, and adhesive resin was further examined by microtensile bond strength tests and scanning electron microscopy analysis.

2 Review of the Literature

2.1 Tooth structure

2.1.1 Enamel

Enamel is an aesthetic and durable tissue, which lacks the ability to repair itself. As the most mineralized tissue in the human body, its inorganic part (85%vol, or 96%wt) is constituted of mainly carbonated hydroxyapatite crystals (Ca_{10-x} Na_x $(PO_4)_{6-y}$ $(CO_3)_z$ $(OH)_{2-u}$ F_u), together with trace amounts of sodium, magnesium, potassium, and fluoride ions. Enamel organic matrix contains proteins (3%vol, or 1%wt) and loosely bound water molecules (12%vol, or 3%wt), and serves as a 'glue' for the hydroxyapatite crystals by enhancing mechanical strength and optical properties (Bachmann et al. 2004). Selectively permeable to ions, enamel can accommodate varying quantities of calcium and fluoride ions depending on the available sources from oral hygiene and dietary habits (Lacruz et al. 2017). Therefore, the physicochemical qualifications of enamel can demonstrate variance between individuals.

2.1.2 Dentin

Dentin has a tubular structure (1-2 μm in diameter) formed by hypermineralized peritubular dentin, and less mineralized intertubular dentin. The extensions of odontoblast cells accommodated in pulp extend along the dentin tubules. Dentinal fluid fills the tubules, hydrating the dentin structure and conducting external stimuli to the nerves in the pulp (Ajcharanukul *et al*. 2011). Dentin tissue can also be considered a hydrated biocomposite structured by inorganic and organic components. Similar to enamel, carbonated hydroxyapatite crystals constitute the inorganic part (47%vol, or 70%wt). However, these minerals are smaller and contain more carbonate than the apatite minerals in enamel, rendering dentin more soluble and vulnerable to acid attacks. Dentin organic matrix (33%vol, or 20%wt) contains primarily type I collagen fibrils (30%vol of mineralized dentin, and 90%vol of dentin organic matrix), as well as types III and V collagen fibrils (%1-3vol of dentin organic matrix), and several non-collagenous proteins (10%vol of dentin organic matrix)

(Goldberg *et al*. 2011). During tooth development, type I collagen fibrils are secreted by the odontoblasts, form the predentin, then crosslink, and mineralize. Non-collagenous proteins, including glycoaminoglycans, SIBLINGs (Small Integrin Binding Ligand N-Linked Glycoproteins), Fetuin-A, and non-phosphorylated proteins such as osteonectin and osteocalcin, play a role during the formation and mineralization stages of collagen fibrils by regulating the growth of hydroxyapatite crystals. The water content of mineralized dentin (10%vol, or 22%wt of dentin organic matrix) is substantially higher than the enamel (%12vol, or 3%wt) (Bachmann *et al*. 2004), and demineralized dentin matrix contain loosely bound, tightly bound, and free water molecules (Agee *et al*. 2015).

Type I collagen fibrils (100–120 nm in diameter) are constituted of tightly packed collagen microfibril bundles (10–25 nm in diameter) (Figure 1) (Takahashi *et al*. 2013). The microfibril structure (4–5 nm in diameter and 300 nm in length) contains hundreds of collagen molecules (approximately 1.4 nm in diameter) (Bertassoni *et al*. 2012; Sherman *et al*. 2015). Between each collagen molecule is a distance of approximately 1.26–1.33 nm (Bertassoni *et al*. 2012). Water fills the space between the collagen microfibrils by creating microchannels. Within a microfibril, the collagen molecules are lined up in such a manner that the staggered spaces between the ends of each consecutive collagen molecule leave a gap (0.54 *d*), and the overlap zones (0.46 *d*) appear in locations where multiple collagen molecules are superimposed (Bertassoni *et al*. 2012; Sherman *et al*. 2015). This structural organization gives the specific appearance of axial 67 nm periodicity to collagen fibrils, seen as a banded pattern when examined under electron microscopy (Varma *et al*. 2016).
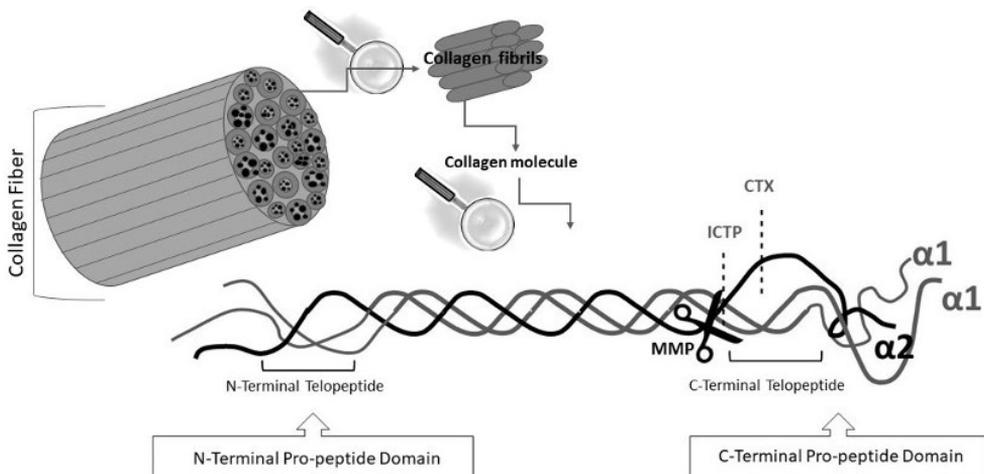
Figure 1. Type I collagen structure; collagen fiber, collagen fibril, collagen molecule and collagen triple helix. (Modified from Gelse et al. 2003)

Collagen molecules (1.4 nm in diameter and 300 nm in length) demonstrate a triple-helix polypeptide assembly of 2 α-1 chains and 1 α-2 chain (Figure 1). In each polypeptide chain, an amino acid residue with glycine repeats a motif of $(X-Y-Gly)_n$ in which mostly proline and hydroxyproline are located at the X and Y positions (Bertassoni et al. 2012). Hence, each peptide draws a turn to the left, and the three chains together make a twist to the right. Consequently, a rigid helical center of a collagen molecule with N- and C-terminal ends, named telopeptides, is formed (Figure 1). Water molecules are found tightly bound to the peptide chains, creating hydrogen bridges between peptide chains to maintain molecular integrity. Water molecules also connect the neighboring collagen molecules to each other by providing intermolecular bonds (Nomura et al. 1977). Several collagen molecules are bound by the contribution of proteoglycans, creating a collagen microfibril (Orgel et al. 2009).

2.2   Endogenous dentin enzymes

2.2.1   Matrix metalloproteinases

MMPs belong to a large family of endopeptidases requiring zinc and calcium ions for their catalytic activities. To date, 23 members of the MMP family have been identified in humans, with the ability to cleave nearly all extracellular matrix proteins as fibrillar and nonfibrillar collagens, including type I collagen (Tallant et al. 2010). MMPs regulate the composition of extracellular matrix and actively function during physiological events requiring cell turnover, such as tissue remodeling, wound healing, and angiogenesis. They are also involved in the pathogenesis of many diseases, including periodontitis, tumoral invasions and metastasis, rheumatoid arthritis, ulcers, fibrosis in the liver and lungs, as well as cardiovascular diseases (Maskos and Bode 2003, Sorsa et al. 2006). According to their substrate specificity, MMPs can be simply classified into six groups as collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and others (Tallant et al. 2010). It should be also noted that most MMPs can degrade several substrate types under in vitro conditions. Therefore, they are also classified according to their structural properties, as basic-structure MMPs, MMPs with fibronectin-domain inserts, membrane-bound MMPs, and minimal-domain MMPs (Page-McCaw 2007).

2.2.1.1   MMP structure and activation

The general structure of MMPs consists mainly of 4 domains: a prodomain with a cysteine residue; a catalytic domain with at least 2 zinc ions (1 zinc ion known as catalytic zinc located in the catalytic site, and the other one is called as structural

zinc) and 1 calcium ion; a hemopexin-like domain functioning in the substrate binding stage; and a hinge region connecting the catalytic domain to the hemopexin-like domain (Figure 2) (Bode et al. 1999; Tallant et al. 2010). The prodomain is located on the N-terminal side of the catalytic domain. This domain provides the enzyme latency via its cysteine residue linked to the catalytic zinc in the active site of the catalytic domain, preventing the binding of water molecules. Most MMPs synthesized in this inactive proform (zymogen), and become activated when the prodomain is removed or destabilized from the catalytic domain with a mechanism called 'cysteine switch'. Hence, the catalytic site becomes available for substrate binding and cleaving actions. MMP activation may occur as auto-activation, or by the action of other proteolytic enzymes, including MMPs and CCs, via chemical agents, and pH or temperature changes (Tjäderhane et al. 2013a; Christensen & Shastri 2015). MMPs can maintain their structural stability under low pH conditions; however, they require neutral pH for catalytic activities.
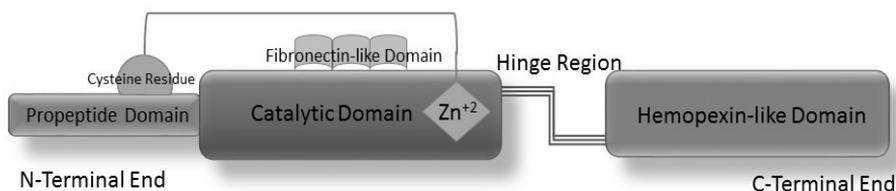
Figure 2. Basic MMP structure. (Modified from Ra and Parks 2007)

MMPs cut off a specific peptide bond of Gly-Leu/Isoleu on type-I collagen molecules, cleaving them into ¼ and ¾ fragments (Tallant et al. 2010). In this regard, MMP-1, -8, and -13 are known as true collagenases degrading native collagen fibrils (Garnero et al. 2003). Gelatinases, including MMP-2, -9, and -13, degrade denaturized collagen molecules and release crosslinked C-terminal telopeptide fragments (ICTP) as degradation product, which is regarded as an indicator of MMP-dependent proteolysis.

The activation of MMPs is regulated at the different stages: the transcriptional controlling of MMP genes, the enzymatic activation stage, their activity according the substrate specificity, and tissue inhibitors of metalloproteinases (TIMPs) or serum inhibitors (Nagase 1997). TIMPs block the active site of the catalytic domain by acting similar to the substrates.

2.2.1.2   MMPs in dentin and pulp

To date, it is known that odontoblasts secrete MMP-1, -2, -3, -8, -9, -13, -14, and -20, as well as membrane-type matrix metalloproteinase-1 and -2 (MT1-MMP and

MT1-MMP) (Sulkala et al. 2002; Palosaari et al. 2003). MMP-13 is reported to be abundantly secreted from the pulp tissues of both sound and carious teeth (Sulkala et al. 2004). During pulpitis, a significant increase has been detected in the secretion of MMP-1, -3, and -9 (Gusman et al. 2002; Shin et al. 2002), indicating that these enzymes mainly function during the pulp inflammation and healing process. Another study found that MMP-3 may also act as an anti-inflammatory agent during pulpitis (Eba et al. 2012). The functional roles of MMPs can be listed as follows; secondary and tertiary dentin matrix formation and mineralization, matrix degradation during dentin injury, collagen degradation during caries formation, and pulp inflammation (Palosaari et al. 2000, 2003).

2.2.2   Cysteine cathepsins

Cysteine cathepsins are the members of C1 subfamily of papain-like enzymes. Among 11 types of CCs (B, C, F, H, K, L, O, S, V, X, and W), cathepsin B, H, L, C, X, F, O, and V have been isolated in human tissues together with the tissue-specific cathepsins, including cathepsin K (predominantly expressed by osteoclasts), S (by antigen-presenting cells), and W (CD8+ lymphocytes and natural killer cells) (Turk et al. 2012). They are secreted as inactive zymogens in cells, and transferred and activated in endosomes via endosomal acidification or other proteases.

2.2.2.1   Structure and function of CCs

All cysteine cathepsins contain a signal peptide, a propeptide, and a catalytic domain. Signal peptides function in the transfer of the enzyme to endoplasmic reticulum following mRNA translation. The propeptide part acts as a guide during the transportation of proenzyme to endosome or lysosome, preventing the early activation of the enzyme, and behaves as a scaffold for catalytic domain folding. The catalytic domain contains active sites with a cysteine and histidine residue, which together form a catalytic thiolate-imidazolium ion pair and an asparagine residue (Polgár and Csoma 1987). The thiol group behaves as a nucleophile when attacking to the covalent peptide bonds for cleavage. The folded structure of cathepsins involves L domain, in which the active site cysteine is located, and R domain containing histidine residue.

In general, CCs function in an acidic environment with the optimum pH at 5.5 (Turk et al. 1995), except cathepsin B, the optimal pH of which is 7.4, and cathepsin S, which can function even in an alkaline environment (Khouri et al. 1991; Turk et al. 1995). During normal tissue modulation or pathological events, CCs become activated and can activate other proteases, including MMPs, and degrade collagen fibrils, elastin, and proteoglicans (Lutgens et al. 2007). They can function either as

endopeptidases (cathepsins K, L, S), exopeptidases (cathepsins C and H), or both (cathepsin B). Cathepsins can cleave ICTP fragments to eight amino acid chains, called C-terminal crosslinked telopeptide of type I collagen, or CTX, fragments. Cathepsin K is the only cathepsin with the ability to cleave both the triple helical region of collagen and telopeptides, whereas cathepsins L and S cleave the non-helical telopeptide extensions (Garnero et al. 1998).

2.2.2.2   CCs in dentin and pulp

Most CCs can be expressed by odontoloblasts and pulp cells (Tersariol et al. 2010). To date, active and preforms of cathepsins B, K, and L have been isolated from dentin organic matrices (Vidal et al. 2014; Scaffa et al. 2012). Similarly to MMPs, it is thought that they are trapped in dentin matrix following dentin mineralization. CCs detected in deep dentin layers have indicated that these enzymes can pass through dentin tubules after being released from the pulp (Nascimento et al. 2011). CC intensity shows differences through dentin depending on several factors, such as sound or carious dentin, as well as the localization and activity level of the caries lesion (Nascimento et al. 2011; Vidal et al. 2014; Scaffa et al. 2017).

2.3   Proteolytic activity in demineralized dentin matrices

It is thought that MMPs and CCs have a physiological role in enamel and dentin formation by modulating the contents of the extracellular matrix (Satoyoshi et al. 2001; Tersariol et al. 2010; Chaussain et al. 2013). After amelogenesis, MMP-20 (enamelysin) is secreted only by mature odontoblast cells (Grant et al. 1999). As a gelatinase, MMP-2 is also involved in the enamel formation process by further degrading the collagen fragments. Likewise, endogenous dentin MMPs, MMP-2 and -9 (gelatinases), MMP-8 (collagenase-2), MMP-3 (stromelysin-1), MMP-13 (collagenase-3), and MMP-20 physiologically function during dentinogenesis. However, they become trapped with dentin mineralization, and lose their activity. In recent decades, many published studies have shown that MMPs and CCs become activated following dentin demineralization and cause the degradation of dentin organic matrices (Kato et al. 20012; Tezvergil-Mutluay et al. 2013; Vidal et al. 2014; Zarella et al. 2015; Zhang et al. 2017).

2.3.1   Caries progression

In previous years, it was thought that bacterial enzymes break down dentin, leading to cavity formation. However, in vitro studies have shown that the enzymes sourced

by cariogenic bacteria, including S. Mutans, are not able to cut off the hydrogen bonds within the triple helical structure of collagen molecules, and collagen hydrolysis occurs without the presence of bacteria or additional enzymes (Hashimoto et al. 2003, 2010b). As early as 1983, it was reported that the collagenolytic activity increase in caries lesions was comparable to sound dentin, indicating the presence of collagenases in dentin matrix (Dayan et al. 1983). With the discovery of dentin matrix-bound MMPs, the mechanism behind the destruction of dentin during caries progression could be explained (Tjäderhane et al. 1998; Pashley et al. 2004; Tjäderhane et al. 2015). Later, studies have shown higher MMP and CC activities in caries-affected (Vidal et al. 2014) and caries-infected (Nascimento et al. 2011) dentin. Under cariogenic conditions that cause dentin demineralization, the latent MMPs bound to dentin matrix, and the MMPs sourced from saliva, gingival crevicular fluid, and secreted from odontoblasts, are activated due to the low pH of the environment, and by the action of other enzymes, including MMPs and cathepsins (Nagase 1997). Additionally, TIMP-inhibited MMPs are also activated by the phosphorylated proteins liberated as a result of bacterial acids from collagen fibrils (Fedarko et al. 2004). However, MMPs require neutral pH for catalytic activity, which is later provided by the salivary and dentinal buffering systems. Thus, the collagen molecules can be cut off into ICTP fragments by the MMPs and further degraded into CTX fragments by the CCs.

2.3.2   Dentin erosion

Dentin erosion is defined as the dissolution of tooth structure due to the numerous cycles of acid exposure without the involvement of acidogenic bacteria (Buzalaf et al. 2012, 2015). Similarly as with the carious process, the mineral content of dentin matrix is dissolved by gastric or dietary acids. In fact, the superficial collagen fibril mesh has been found to be resistant to mechanical abrasion (Ganss et al. 2007; Shellis et al. 2014). However, due to demineralization and low pH, MMPs and CCs become activated in dentin (Kato et al. 2010; Buzalaf et al. 2012; Zarella et al. 2015). With the neutralization of the pH by the buffering system of saliva, collagen fibrils are degraded by both the salivary and dentin matrix-bound proteolytic enzymes, causing tissue loss. A study showed that MMPs are the main enzymes responsible for tissue loss during the erosive process (Zarella et al. 2015). Since CCs can also activate MMPs, it is thought that they play a secondary role in the pathogenesis of dentin erosion (Buzalaf et al. 2012; Zarella et al. 2015).

2.3.3   Degradation of resin-dentin bonds

Bonding to dentin occurs mainly through the infiltration of resin monomers into collagen fibrils exposed by phosphoric acid etching or acidic primers. Alternatively, more simplified resin adhesives use acidic functional resin monomers for the simultaneous dentin etching and resin infiltration steps in order to reduce the clinical technique sensitivity of the conventional multistep bonding agents. The strength and durability of the bond between dentin and restoration are determined by the quality and stability of the adhesive interface, in particular, the hybrid layer which is composed of collagen fibrils, residual inorganic minerals, and resin monomers (Liu et al. 2011; Tjäderhane et al. 2013a,b). Ideally, the main goal of adhesive bonding is the coverage of all exposed collagen fibrils by resin monomers. However, the bonding process may be impaired by several factors, such as the use of hydrophilic adhesive systems, inadequate resin infiltration into the demineralized dentin, insufficient resin polymerization, and the activation of MMPs and CCs during bonding procedures (Oliveria et al. 2004; Tjäderhane et al. 2013a,b). Water sourced by dentinal tubules, bonding agent components, as well as the water molecules bound to the collagen fibrils also cause the hydrolysis of resin monomers (Carrilho et al. 2005; Hashimoto et al. 2010a) and collagen fibrils in the adhesive interface (Hashimoto 2010a,b; Tjäderhane et al. 2013a; Frassetto et al. 2016).

Collagenolytic and gelatinolytic activities in acid-etched dentin without the involvement of any bacteria were firstly demonstrated by Pashley et al. in 2004. Later studies have confirmed collagen fibril degradation within adhesive interfaces due to catalytic activity of MMPs and CCs, and the resultant decreasing bond strength to dentin over time (Hu et al. 2015). It has also been reported that MMPs are the major group of enzymes responsible for demineralized dentin matrix degradation, causing degradation 10 times higher compared to CCs (Tezvergil-Mutluay et al. 2013; Zarella et al. 2015; Scaffa et al. 2017).

The dissolution of hydroxyapatite minerals similar to the cariogenic and erosive processes has been shown as the main factor causing the activation of endogenous dentin enzymes, and thus the degradation of the adhesive interface. In this regard, varying levels of proteolytic activity have been detected at the adhesive interface depending on the pH of the adhesive system (Mazzoni et al. 2006; Nishitani et al. 2006). Owing to their low acidity (pH=0.17), phosphoric acid gels may partially denature endogenous enzymes; however, they can be reactivated with the resin monomer application (Mazzoni et al. 2011b, 2012). Mildly acidic self-etch adhesive systems (pH=1.5–2.7) can arouse high levels of enzymatic activity without denaturing the enzymes (Apolonio et al. 2017). Moreover, self-etch adhesives can also increase the gelatinase (MMP-2 and MMP-9) expression from the odontoblasts (Nishitani et al. 2006; Lehmann et al. 2009). Among the currently detected proteases in dentin, MMP-2 has been reported as the main protease in the hydrolytic deg-

radation of hybrid layers (Mazzoni et al. 2011b). As direct evidence of gelatinolytic activity in the hybrid layer, intensive MMP-2 and MMP-9 activities have also been demonstrated at the bottom of the hybrid layer via in situ zymography (Mazzoni et al. 2012).

The inhibition of endogenous dentin enzymes can prevent the breakdown of demineralized dentin organic matrices, thereby possibly enhancing the remineralization prospect of early caries and erosive lesions (Kato et al. 2012; Giacomini et al. 2017). Durability of adhesive interfaces can also be improved, increasing the clinical longevity of restorations. Therefore, the introduction of anti-enzymatic measures into clinical practices carries great importance for the prevention of dentin tissue loss. Several approaches are currently under investigation, including the use of enzyme inhibitors such as chlorhexidine, and cationic metal ions such as zinc and iron (Kato et al. 2012; Altinci et al. 2017), benzalkonium chloride (Tezvergil-Mutluay et al. 2011) and MMP inhibitor monomers and solvents (Tezvergil-Mutluay et al. 2015). In addition, crosslinking agents such as curcumin, epigallocatechin gallate, and riboflavin, increasing the degradation resistance of collagen fibrils have been also studied (Hiraishi et al. 2013; Frassetto et al. 2016; Seseogullari-Dirihan et al. 2016). The biomineralization of collagen fibrils vulnerable to enzymatic attacks have also been suggested as a promising method (Niu et al. 2014; Xie et al. 2016).

2.4 Fluorides in dentistry

2.4.1 Fluoride properties

Fluorine is a chemical element with the symbol F, atomic number 9, and atomic weight of 18.99. As the lightest halogen, it is located in group 17, and period 2 of the periodic table. It is found in highly toxic, diatomic gas form at room temperature. As the most electronegative element, fluorine easily reacts with all other elements, including metals, nonmetals, metalloids, and most noble gases, and is never found in the elemental form in nature. Due to its high electronegativity, fluorine creates mostly ionic bonds in its compounds, although it can form polar covalent bonds as well (Bizet and Cahard 2014).

Fluoride with the chemical formula F^- is the monatomic and the simplest anion of fluorine. When reacted with alkali metals such as sodium and potassium, fluoride creates monofluoride salts, which are highly soluble and ionic in water. On the other hand, highly ionic and water-insoluble difluoride compounds are formed with alkaline earth metals (beryllium, magnesium, calcium, strontium, barium, and radium). The water solubility of NaF and CaF_2 at 20°C, and KF at 18°C, are 40 g/L,

0.0016 g/100mL, and 92g/100mL, respectively (Sanz and Vega 2007; Reynolds and Belshner 2017).

Fluoride is classified as moderately toxic, and water-soluble fluoride compounds may even be lethal (Usuda et al. 2015). In water-based solutions, it behaves as a weak base (pKb =10.8) and mostly remains in the ionic state, rather than forming hydrogen fluoride (HF) by reacting with protons (H^+) (Toteva and Richard 2002). Many fluoride compounds are widely used in various fields, for example in industry for metal smelting and welding, in the form of hydrofluoric acid or hydrogen fluoride; in the production of Teflon utensils and several pharmaceuticals (Yerien et al. 2016); as well as in a medical imaging method known as positron emission tomography (PET) (Sharma et al. 2017). Several fluoride compounds are widely used in caries preventive regimes in dentistry due to their remineralizing and antimicrobial effects (Dionysopoulos et al. 2013; Carey 2014; Maguire 2014; Guedes et al. 2016).

Fluoride can be stored in human tissues and body fluids (gingival crevicular fluid, ductal saliva, and urine) as ionic fluoride or bound to proteins and tissues (Buzalaf and Whitford 2011; Sharma et al. 2017). Bone and tooth tissues contain nearly 99% of the total fluoride in the human body, firmly and reversibly bound to apatite and calcium phosphate compounds (Whitford 1994). The fluoride content of enamel remains constant after tooth maturation, and only the superficial layer may demonstrate changes depending on the available amount of fluoride in the oral environment (Kotsanos and Darling 1991). Dentin layer peripheral to pulp and secondary dentin may also show changes in fluoride content after tooth eruption (Yoon et al. 1960).

2.4.2   Mechanisms of fluoride action in dentistry

There are numerous studies reporting that fluoride prevents caries formation by inhibiting demineralization and enhancing remineralization processes (Baysan et al. 2001; Benson et al. 2013; Carey et al. 2014; Bonetti and Clarkson 2016; Marinho et al. 2016; Nóbrega et al. 2016). Authors stated that free fluoride available in the vicinity of dental tissues during demineralization is more effective in caries prevention than structurally bound fluoride, which is explained by the different interaction modes of fluoride with tooth tissues depending on fluoride concentration and duration of exposure (Ogaard et al. 1988; Rošin-Grget et al. 2013). By exchanging with hydroxyl groups in apatite structure, fluoride ions can render hydroxyapatites to fluorohydroxyapatite, which are also more insoluble, and thus more resistant to acid attacks. If the oral environment is supersaturated with calcium, phosphate, and fluoride ions, fluorapatite crystal growth can occur on tooth surface. The high levels of fluoride treatment ranging from 100 ppm to 10,000 ppm cause the

formation of CaF_2 or CaF_2-like minerals on the tooth surface, or in dental plaque and saliva (Aoba 1997; Buzalaf et al. 2011). CaF_2-like minerals can also serve as fluoride reservoirs in case pH drops below the critical level of 5.5; in the same way, fluoride can also prevent the erosion process by interfering with demineralization in enamel and dentin (Castilho et al. 2015; Alexandria et al. 2017).

Fluoride ions can directly or indirectly show antimicrobial effects (van Loveren et al. 2008; Nakajo et al. 2009; Takahashi & Washio 2011; Buzalaf et al. 2011). It has been shown that fluoride can inhibit the carbohydrate metabolism and the acid production of cariogenic bacteria by inhibiting the bacterial enolase and proton-extruding adenosine tri-phosphatase activities (van Loveren et al. 2008; Nakajo et al. 2009; Takahashi & Washio 2011), thereby demonstrating anti-cariogenic activity on biofilm composition (Chau et al. 2015). Accordingly, it has been recently reported that S. Mutans levels in high-caries-risk children can be reduced by the use of fluoride rinses (Perala & Bhupathiraju 2016). However, it should also be noted that the antimicrobial efficacy of fluoride on biofilm species may be reversible and may decrease over time (Chau et al. 2014; Dang et al. 2016). Thus, these aspects require further investigations.

2.4.3   Clinical applications of fluoride in dentistry

Fluoride is the only anticariogenic drug recognized by the US Food and Drug Administration (FDA). Community water fluoridation has been reported to be a highly effective method in caries prevention for children, and possibly for adults as well. More than 70 years ago, fluoride began to be incorporated into community water as an anticariogenic measure, and is currently still in use in approximately 27 countries, including Australia, Canada, and the United States (Harding and O'Mullane 2013). There are also countries in which the drinking water naturally contains fluoride, including some regions of Sweden, Finland, and Turkey. On the other hand, in nearly 98% of Continental Europe, drinking water is not fluoridated either due to healthcare strategies or because topical fluoridation is already common via oral hygiene products. The United States Environmental Protection Agency (EPA) currently recommends the 4.0 mg/L standard fluoride level in drinking water as a safe public health measure for caries prevention in children and adults in the United States. The US Public Health Service and the American Dental Association state that the optimal fluoride in water should be in the range of 0.7 ppm to 1.2 ppm (Sharma et al. 2017), and this level is adjusted using sodium fluoride, sodium fluorosilicate, and fluorosilicic acid in fluoride-deficient water.

Fluoride must ideally be available in the vicinity of teeth 24 hours a day to obtain the maximum anticariogenic benefit, which requires the use of topical oral hygiene products containing fluoride as well as patient compliance (Buzalaf et al. 2011;

Rošin-Grget et al. 2013). The simplest and most affordable way is to use toothpastes fluoridated with sodium fluoride (NaF), sodium monofluorophosphate (Na_2FPO_3), amine fluoride ($C_{27}H_{60}F_2N_2O_3$), or stannous fluoride (SnF_2), and their combinations depending on the used abrasives, non-active agents, and detergents in the formula. Hence, free fluoride ions, and profluoride compounds that are hydrolyzed to free ionic fluoride by the salivary enzymes, can be constantly provided. In Europe, the maximum fluoride level permitted in toothpastes is 1500 ppm, and lower in the U.S. at 1,100 ppm (ten Cate et al. 2013). There are also low-fluoride toothpastes with 500 ppm F that are reported to be as effective as the 1100 ppm F toothpastes in caries-inactive children and caries-active teenagers (Stookey et al. 2004; Lima et al. 2008), and high-fluoride toothpastes with 2800 ppm and 5000 ppm that can be prescribed to adults. Regarding their clinical efficacy, there are only a few studies reporting that these high-fluoride toothpastes can be more protective against caries development for 14–16-year-olds, and DMFS$\geq$ 5 patients, and against root caries (Baysan et al. 2001; Ekstrand et al. 2008; Nordström and Birkhed 2010; Srinivasan et al. 2014).

Fluoride-releasing rinses, varnishes, and gels as topical fluoride treatment agents can be beneficial in patients with moderate or high caries risk (Twetman et al. 2016). Although there is limited evidence regarding the long-term anticariogenic efficacy of fluoride varnishes containing as much as 50000 ppm NaF, the American Dental Association recommends the fluoride-varnish treatment at 6-month intervals for moderate- and high-caries-risk patients (Maguire 2014).

In order to prevent secondary caries formation and improve the clinical longevity of restorations, various forms of fluoride have been incorporated into the composition of dental restorative materials, including glass ionomer-based restoratives, resin-based adhesive systems, and composite resins (Wiegand et al. 2007; Al-Saud and Al-Nahedh 2012; El-Deeb et al. 2013; Amaechi and van Loveren 2013; Lussi and Carvalho 2015; Guedes et al. 2016). It is thought that the microgaps between restoration margins and tooth structure can be sealed due to the acid-resistant minerals formed by the interaction of fluoride ions with the residual minerals in enamel and dentin. Depending on the fluoride compound and concentration, the studies showed promising results with fluoride-incorporated restorative materials in terms of secondary caries prevention, adhesive bonding durability, antibacterial efficacy, and fluoride release and recharge capabilities (Xu and Burgess 2003; Peris et al. 2007; Wiegand et al. 2007; Shinohara et al. 2009; Dionysopoulos et al. 2013; El-Deeb et al. 2013).

Adhesive bonding to dentin requires the demineralization of the superficial layer to expose collagen fibrils for resin monomer infiltration, or the use of acidic-functional resin monomers for simultaneous dentin demineralization and resin monomer infiltration. However, due to the technique sensitivity of clinical application techniques, heterogeneous structure of dentin tissue, and hydrophilicity

of current simplified-adhesive systems, hydrolytic degradation occurs at the adhesive interface between dentin and restoration. Two degradation mechanisms have been described at the adhesive interface, involving the enzymatic breakdown of collagen fibrils and the hydrolysis of resin monomers (Tjäderhane et al. 2013a,b). Fluoride has been suggested as a measure for improving the mineral support of deficiently resin-infiltrated collagen fibrils, since fluoride ions can react with residual ions in the deep demineralized dentin after etching procedures, due to the newly formed minerals. Moreover, considering the inhibitory effects of NaF on the catalytic activity of salivary MMPs (Kato et al. 2014), fluoride compounds may be potential inhibitors of dentin matrix-bound MMPs and CCs, improving adhesive interface durability. In addition, since these enzymes also have role in cariogenic and erosive processes, more conservative treatment alternatives may be possible for the early lesions via fluoride use (Kato et al. 2012).

3   Aims

The aim of these study series was to evaluate the effects of fluoride on the catalytic activity of dentin matrix-bound MMPs and CCs for the prevention of proteolytic degradation of demineralized dentin. As fluoride agents commonly used in dental practices, the inhibitory efficacy of NaF, KF, and CaF_2 was investigated to analyze the liberation of collagen peptides from insoluble dentin organic matrices due to MMPs and CCs.

The specific aims of the studies were:

1.   To evaluate the inhibitory effects of NaF, KF, and CaF_2 on the dentin matrix-bound MMPs. (Studies I, II, III)

2.   To detect the catalytic activity of dentin matrix-bound CCs on dentin collagen when subjected to NaF, KF, and CaF_2. (Studies I, II, III)

3.   To assess the short- and long-term effects of NaF, KF, and CaF_2 treatment on demineralized dentin matrix degradation. (Studies I, II, III)

4.   To determine long-term bonding durability to phosphoric acid-etched and fluoride-treated dentin surface. (Study IV)

4 Materials and Methods

4.1 Preparation of dentin samples

4.1.1 Dentin beam preparation (Studies I, II, III)

Freshly extracted, sound human molars were collected according to the approval of the Ethical Committee of the Faculty of Medicine, University of Oulu (#19/2006). The teeth were stored in 0.02% NaN_3 solution until use. At first, dentin disks were prepared by removal of occlusal and deep dentin under water cooling with a low-speed diamond saw (Isomet saw, Buehler Ltd., Lake Bluff, IL, USA). Then, the disks were sectioned into dentin beams sized 1 mm × 2 mm × 6 mm. After demineralization in 10% phosphoric acid for 24 h, the beams were placed in tubes containing distilled water, rinsed under constant stirring at 4°C for 8 h, and dried to a constant weight in a desiccator containing dry silica beads under vacuum for 72 h. After the baseline dry mass of each beam was measured to the nearest 0.001 mg with a digital microbalance (Mettler XP6 Microbalance, Mettler Toledo, Hightstown, NJ, USA), they were allocated into test groups (n=10/group).

Before incubation, the dried beams were immersed in distilled water at 4°C for 2 h for rehydration, then placed in hermetically sealed polypropylene tubes labeled by corresponding group. As incubation medium, 1 mL of buffered solution containing 5 mM HEPES, 2.5 mM $CaCl_2.H_2O$, 0.02 mM $ZnCl_2$ and NaF, KF, and CaF_2 in the F concentrations of 6, 12, 24, 48, 120, 179, and 238 mM at pH 7.4 was used (Table 1). The beams incubated in the medium without fluoride served as controls. The fluoride level of each incubation medium was measured directly by a fluoride ion-selective electrode (DX219-F, Mettler Toledo, Urdorf, Switzerland).

The incubation processes were implemented at constant pH in a shaking bath (OLS200, Grant Instruments, Cambridge, UK) for 1, 7, or 21 days at 60 cycles/min and at 37°C. Then, all beams were further incubated in AS only for 6 months. After each timepoint, the incubation medium of each beam was recollected and stored at -70°C until analyses were performed for liberated collagen degradation products.

**Table 1.** Fluoride compounds and concentrations used in the study series

Fluoride mM	Fluoride ppm	NaF ppm	KF ppm	CaF$_2$ ppm
238	4524	10000	13835	9297
179	3394	7500	10376	6974
120	2261	5000	6915	4646
48	904	2000	2766	1858
24	452	1000	1383	929
12	226	500	692	464
6	112	250	343	230

4.1.2 Mineralized dentin powder preparation (Studies I, II, III)

Coronal dentin pieces were obtained by removing the roots, coronal enamel, and intra-pulpal dentin of sound molars with high-speed burs under water cooling, and placed into metal containers in a cryomilling machine together with 2 metal balls (Retsch MM400, Retsch, Haan, Germany). After the metal lids were tightened, the container was dipped into liquid nitrogen for 5 min, and then inserted onto the machine and shaken for 2.5 min. Then, the obtained fine-mineralized dentin was collected in tubes, and stored at -70°C until use.

4.2 Methods

The proteolytic degradation of demineralized dentin matrix was studied by the investigation of direct and indirect indicators for the MMP- and CC-activities as seen in Figure 3. In addition, the effects of F treatment on the dentin-resin interface were evaluated using microtensile (µTBS) bond strength test set-up and scanning electron microscopy analysis.

Research Methods

Proteolytic Degradation in Demineralized Dentin Matrix

- **Direct Indicators**

Total MMP Activity Assay (Studies I, II, III)

Gelatin Zymography (Study I,II,III)

- **Indirect Indicators**

Dry Mass Loss (Studies I, II, III)

ICTP Assay (Study I)

CTX Assay (Study I,II,III)

Hydroxyproline Assay (Studies I,II,III)

Durability of Dentin-Resin Adhesive Interface

Microtensile Bond Strength Test (Study IV)

Scanning Electron Microscopy Analysis (IV)

Figure 3. Research methods applied in this thesis.

4.2.1 Total MMP activity assay (Studies I, II, III)

Dentin beams were used as MMP source for the generic colorimetric MMP assay (Sensolyte Generic MMP assay; Anaspec, San Jose, CA, USA) for the assessment of the catalytic activity of dentin matrix-bound MMPs. Dentin beams demineralized with 10% phosphoric acid, as previously described, were placed in 96-well plates, and 300 µL of chromogenic thiopeptide substrate and assay buffer (60 min at 25°C) was pipetted. The beams were removed after 60 min incubation, and the absorbance values were measured as a baseline reading at 412 nm (Ozcan et al. 2015). After being rinsed free of assay substrate, the beams were allocated to the groups based on similar mean baseline MMP activity (n=10/group), and the incubation processes were performed. After each incubation time-point, the MMP assay was repeated as explained above. The total MMP activity was calculated as a percentage of baseline reading in order to detect the percent of inhibition or activation for each group.

4.2.2 Gelatin zymography (Study I, II, III)

Gelatin zymography assay was performed to screen the MMP-2 and -9 activities in demineralized and fluoride-treated dentin according to the protocol described by Mazzoni et al. (2013). Briefly, dentin powder batches of 200 mg/group were demineralized with 10% phosphoric acid for 10 min, and then treated with 100 µL NaF, KF, and CaF_2 dissolved in distilled water according to the F concentrations of 6, 12, 24, 48, 120, 179, and 238 mM (pH=7) for 5 min at 4°C, centrifuged for 20 min (12000 g); the supernatants were discarded. The samples were rinsed with distilled water and suspended in extraction buffer (50 mM Tris–HCl pH 6, containing 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100, 0.1% nonionic detergent P-40, 0.1 mM $ZnCl_2$, 0.02% NaN_3) for 24 h at 4°C, sonicated twice for (10 min), and centrifuged (12000 g) for 20 min at 4°C. The supernatants were collected and concentrated (10000 g, 20 min at 4°C) to 50 µl total volume via a Vivaspin centrifugal concentrator (10000 kDa cut off; Vivaspin, Sartorius Stedim Biotech, Goettingen, Germany). Bradford assay was applied to detect the total protein concentration of the extracts (Bradford 1976). Then, the protein aliquots of 100 µg were diluted (1/4 ratio) in Laemmli sample buffer. After the electrophoresis performed in 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE containing 1 mg/ml fluorescein-labeled gelatin), and washing in 2% Triton X-100 for 1 h, the gel was incubated in zymography activation buffer (50 mmol/L Tris–HCl, 5 mmol/L $CaCl_2$, pH 7.4) for 48 h. Prestained low-range molecular weight SDS-PAGE standards (Bio-Rad, Hercules, CA, USA) were used as molecular-weight markers. The gelatinase activity in the demineralized dentin powder was also analyzed. The results were monitored using a long-wave UV light scanner (Chemi-Doc Universal Hood, Bio-Rad, Hercules, CA, USA). The entire experiment was performed in duplicate.

4.2.3   Dry mass loss (Studies I, II, III)

Dry mass loss changes of the beams after each incubation timepoint were taken as an indirect indicator of demineralized dentin matrix degradation (Tezvergil-Mutluay et al. 2013). The incubated beams were rinsed in distilled water (at 4°C for 8 h) at each timepoint, and dried in the desiccator to a constant weight for 72 h. The dry masses were weighed with the digital microbalance, and the percentage of dry mass changes were calculated according to the respective baseline dry mass of each beam. After rehydration in distilled water at 4°C for 2 h, the beams were taken to the corresponding fresh incubation medium and incubated until the next timepoint.

4.2.4   Measurement of collagen degradation products

4.2.4.1   ICTP assay (Study I)

Crosslinked carboxyterminal telopeptide of type I collagen (ICTP) is the specific marker for MMP-dependent degradation of type I collagen (Garnero et al. 2003; Tezvergil-Mutluay et al. 2015). Therefore, the incubation medium of each beam was analyzed using human serum ICTP assay kits (UniQ EIA, Orion Diagnostica, Espoo, Finland) and a plate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). The assays were implemented in triplicate.

4.2.4.2   CTX assay (Study I, II, III)

C-terminal crosslinked telopeptide of type I collagen (CTX) fragments liberated to the incubation medium of dentin beams can be used as an indirect indicator for CC-dependent degradation of dentin matrix (Garnero et al. 2003; Tezvergil-Mutluay et al. 2015). For this purpose, the incubation mediums were analyzed using human serum CTX assay kits (Serum CrossLaps ELISA, Immunodiagnostic System, Farminton, UK) with a plate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). The assays were implemented in triplicate.

4.2.4.3   Hydroxyproline assay (Studies I, II, III)

The amino acid constituents of type I collagen involve hydroxyproline (9.6%). To detect the total amount of collagen fragments liberated by MMPs and CCs into the incubation medium, hydroxyproline assay was performed according to the protocol described by Tezvergil-Mutluay et al. (2010). Briefly, for each beam, the incubation medium (400 µL) and HCl (400 µL) were mixed in glass ampules (Wheaton, Millville, NJ, USA) to the final concentration of 6N HCl. The ampules

were sealed by flame (Ampulmatic, Bioscience, Inc., Allentown, PA, USA), and incubated at 118°C for 18 h, and reopened after cooling to room temperature. After the hydrolyzed contents were dried in the desiccator, the colorimetric assay protocol of Jamall et al. (1981) was performed as follows; 50% isopropanol in water (1.2 mL) and chloramine-T solution (0.2 ml) with acetate citrate buffer were added to the ampules. After 10 mins, 1 mL of Ehrlich's solution (4-dimethylamineobenzaldehyde, 60% perchloric acid, and 100% isopropanol) was pipetted. Then, the ampules were incubated at 50°C for 90 mins. The absorbance values of the liquids were measured by spectrophotometer (Model UV-A180, Shimadzu, Tokyo Japan) at 558 nm. The results were presented as µg of hydroxyproline/mg of the dry mass of demineralized dentin before incubation.

4.2.5   SEM-EDS analysis (Studies I, II, III)

Scanning Electron Microscopy (SEM) analysis was used in order to evaluate the physical interaction of F compounds with demineralized dentin (Studies I, II, III). Representative dentin beams selected from each group after 21-day and 6-month incubation periods were gold-sputtered (SC7620 Sputter Coater, Quorum, East Sussex, UK) and examined under scanning electron microscopy (SEM, Phenom-World, Eindhoven, The Netherlands) operating at 10 kV. Fractured beams were used for the elemental analysis with an EDS detector integrated to SEM device at 15 kV. Line scan function of the analysis software was used to evaluate the fluoride penetration inside the beams up to approximately 100 µm in depth.

4.2.6   Microtensile bond strength test (Study IV)

The effects of fluoride treatment on the durability of resin-dentin adhesive interfaces was investigated via microtensile bond strength (µTBS) test set-up. The occlusal enamel and dentin of sound molars were removed under water cooling with a low-speed diamond saw (Isomet saw, Buehler Ltd., Lake Bluff, IL, USA) to expose mid-coronal dentin for adhesive bonding procedures with a three-step etch-and-rinse adhesive system (Adper Scotchbond Multi-Purpose, 3M ESPE). After the standard smear layer was created by wet-grinding with 600-grit SiC paper (60 s), the teeth were randomly divided into 11 groups (n=9/group): no F treatment (control); treatment with NaF, KF, or CaF_2 solutions with the F contents of 6, 24, and 179 mM F; a two-step, single-component bonding agent containing approximately 195 mM KF (positive control) (Excite F, soft-touch single dose, Ivoclar Vivadent). The fluoride solutions were prepared by mixing the respective amount of F compound with distilled water, and the pH was adjusted to 7 before application.

The materials and their application protocols are given in Table 1. Bonding surfaces were etched with 32%wt phosphoric acid gel (Scotchbond Universal Etchant, 3M ESPE), rinsed with water spray for 15 s, and blot-dried with cotton pellets. Then, the surface was treated with F solution using a microbrush, and blot-dried after 30 s. According to the manufacturer's instructions (Table 1), the three-step and two-step etch-and-rinse adhesive systems were applied according to the test group, and light-polymerized (Elipar S10, 3M ESPE AG, Seefeld, Germany; 1186 Mw/cm^2). Build-ups in 4 mm thickness were layered with composite resin (Filtek Supreme XTE, 3M ESPE), and light-polymerized for 20 s per layer. After storage in distilled water at 37°C for 24 h, the specimens were sectioned into the beams (0.9 mm x 0.9 mm x 8 mm), and stored in artificial saliva (5 mM HEPES, 2.5 mM CaCl$_2$.H$_2$O, 0.02 mM ZnCl$_2$ and 0.3 mM NaN$_3$) (Tezvergil-Mutluay et al. 2010) at 37°C until the testing after 24 h (immediate), 6 months or 12 months. The µTBS test were performed using a microtensile tester (Bisco Microtensile Tester; Bisco, Schaum-burg, IL, USA) at a crosshead speed of 0.5 mm/min. The microtensile bond strength (MPa) was calculated by dividing the load at failure (N) by the bonding surface area of the beam (mm^2).

In Study IV, the fractured interfaces were sputter coated with gold (SC7620 Sputter Coater, Quorum, East Sussex, UK) and examined using scanning electron microscopy (SEM, Phenom-World, Eindhoven, The Netherlands) at 10 kV. The fracture types were classified as 'cohesive in composite', 'cohesive in dentin', 'adhesive failure at the resin-dentin interface', and 'mixed'; a mixture of adhesive and cohesive failure within the resin-dentin interface (Armstrong et al. 2017).

Table 2. The materials and their application protocols used in µTBS test. (From original publication IV)

Material	Chemical Composition	Application Protocol	Manufacturer	Lot no
Scotchbond Universal Etchant	Phosphoric acid 32% by weight	Etch dentin surface for 15 s; rinse with water for 15 s; remove excess water by air syringe or blotting.	3M ESPE AG, Neuss, Germany	515105
Adper Scotchbond Multi-Purpose Primer	HEMA, PAMA, GPDM, polyalkenoic acid, water	Apply the primer; gently dry for 5 s.	3M ESPE AG, Seefeld, Germany	N325842
Adper Scotchbond Multi-Purpose Adhesive	Bis-GMA, HEMA, dimethacrylates, polyalkenoic acid, initiators	Apply; light-cure for 10 s.	3M ESPE AG, Seefeld, Germany	N329945
Excite F	HEMA, dimethacrylate, phosphonic acid acrylate, silicone dioxide, initiators, stabilizers and KF (≤2.5%, approximately 195 mM KF), ethanol	Apply on dentin etched for 15s, and agitate the surface for at least 10 s; disperse to a thin layer with a weak stream of air; light-cure for 10 s.	Ivoclar Vivadent Schaan, Liechtenstein	R54345
Filtek Supreme XT (A2 Shade)	Bis-GMA, UDMA, TEGDMA, and bis-EMA(6), PEGDMA resins and the combination of nano-size silica, zirconia and zirconia/silica filler particles.	Place in 2 mm increments; light-cure for 20 s.	3M ESPE AG, Seefeld, Germany	N474941

Abbreviations: Bis-EMA: Bisphenol-A-polyetheylene glycol dimethacrylate; BisGMA: Bisphenol-A-diglycidyl dimethacrylate; GPDM: glycero-phosphate dimethacrylate; HEMA: 2-hydroxyethyl methacrylate; UDMA: Urethandimethacrylate; KF: potassium fluoride; PEGDMA: Poly(ethylene glycol) dimethacrylate; PAMA; phtalic acid monomethacrylate; TEGDMA: Triethyleneglycol dimethacrylate.

4.3　　Statistical analysis

In Studies I, II, III, and IV, the data of dry mass loss; total MMP-activity; ICTP, CTX, and HYP assays obtained after 1, 7, and 21 days, and 6 months; and the data of µTBS test after 24 h, and 6 and 12 months, were evaluated separately using Shapiro-Wilk test for normality and with Brown-Forsythe test for equal-variance assumptions. If these parameters were satisfied, the data were subjected to parametrical statistical tests. Repeated-measures ANOVA was performed for each data set with "pretreatment" condition as the group variable and "timepoint" as the repeated factor. If significant interactions were found, the pretreatment groups were compared separately at each timepoint, and timepoints compared separately for each group. The differences between the groups were compared with one-way ANOVA and TUKEY HSD post hoc tests. In case one of the parametrical test assumptions failed, ANOVA on ranks with Dunn's post hoc tests were performed.

The 6-month data of dry mass loss, total MMP-activity, and ICTP, CTX, and HYP assays in Studies I, II, and III were analyzed with one-way ANOVA and TUKEY HSD post hoc tests.

In Study IV, the µTBS were analyzed with three-factor ANOVA to detect the significant effects between the test parameters of 'pretreatment', 'concentration', and 'storage time'. The significant interactions between each test parameter were evaluated with two-way ANOVA and Sheffe's post hoc tests. The data of fracture types were analyzed using Pearson Chi-Square test.

For the statistical tests, SigmaPlot Version 12.5 software (Systat Software Inc., San Jose, CA, USA) and SPSS Statistics 23.0 software (IBM Corporation, Armonk, NY, USA) were used, and p value was set to 0.05.

5 Results

5.1 Total MMP activity (Studies I, II, III)

The total MMP activity of the beams was not significantly different among the groups of NaF, KF, and CaF_2 after 60 min of incubation ($p > 0.05$) As seen in Figure 1, the total MMP activity of fluoride-treated beams after 1-day incubation was lower in KF ≥ 179 mM and CaF_2 238 mM groups compared to the controls ($p < 0.05$), whereas there was no difference among NaF groups both after 1 and 7 days ($p > 0.05$). After 21 days, NaF ≥ 120 mM, KF ≥ 24 mM, and CaF_2 179 mM showed significantly less total MMP activity than the respective controls ($p < 0.05$).

After 6-month incubation only in AS, the total MMP activity in KF 24 and 48 mM groups was significantly higher than the control ($p < 0.05$); whereas there was no significant difference for the NaF and CaF_2 groups compared to the controls ($p > 0.05$).
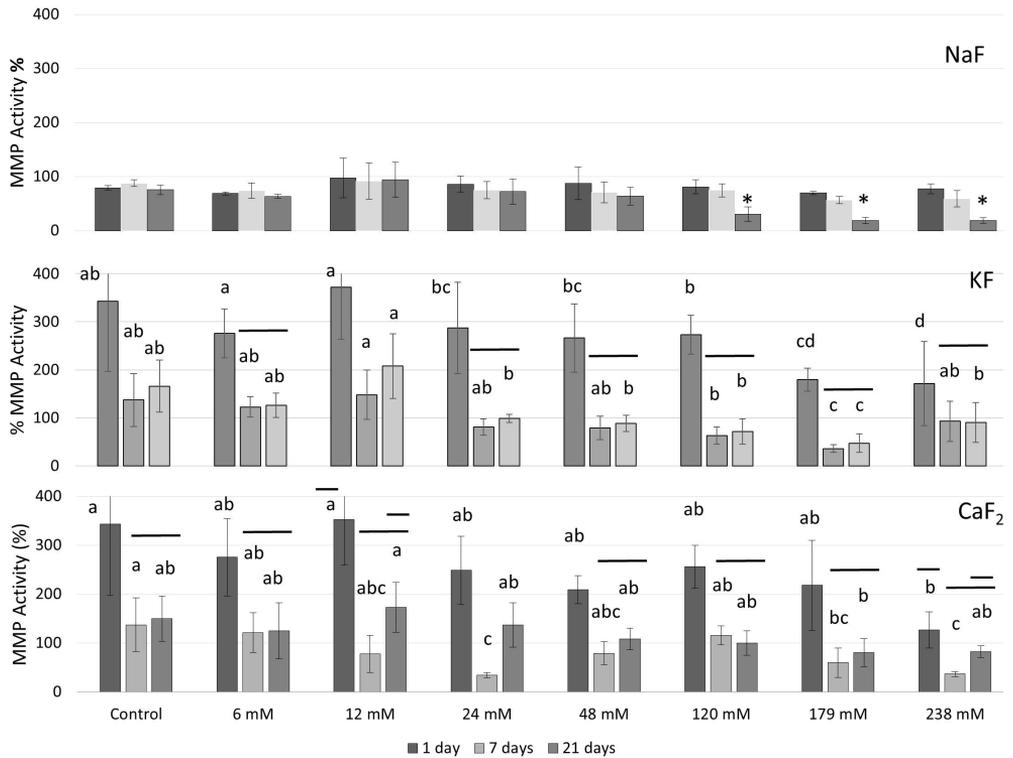
Figure 4. Bar charts comparing the total MMP-activity of the demineralized dentin beams measured after incubated in AS (control), and AS with the increasing NaF, KF and CaF$_2$ concentrations (6-238 mM) for 1, 7 and 21 days. The MMP-activity rate of each beam was calculated as a percentage of the baseline MMP-activity measured before incubations. Among NaF groups, significant differences were marked with an asterisk (*) (p < 0.05). At each time period, KF and CaF$_2$ groups marked with the same lowercase letters, and at each concentration the time period columns connected by a solid black bar are not statistically significant (p > 0.05). (Modified from original publication I, II, and III)
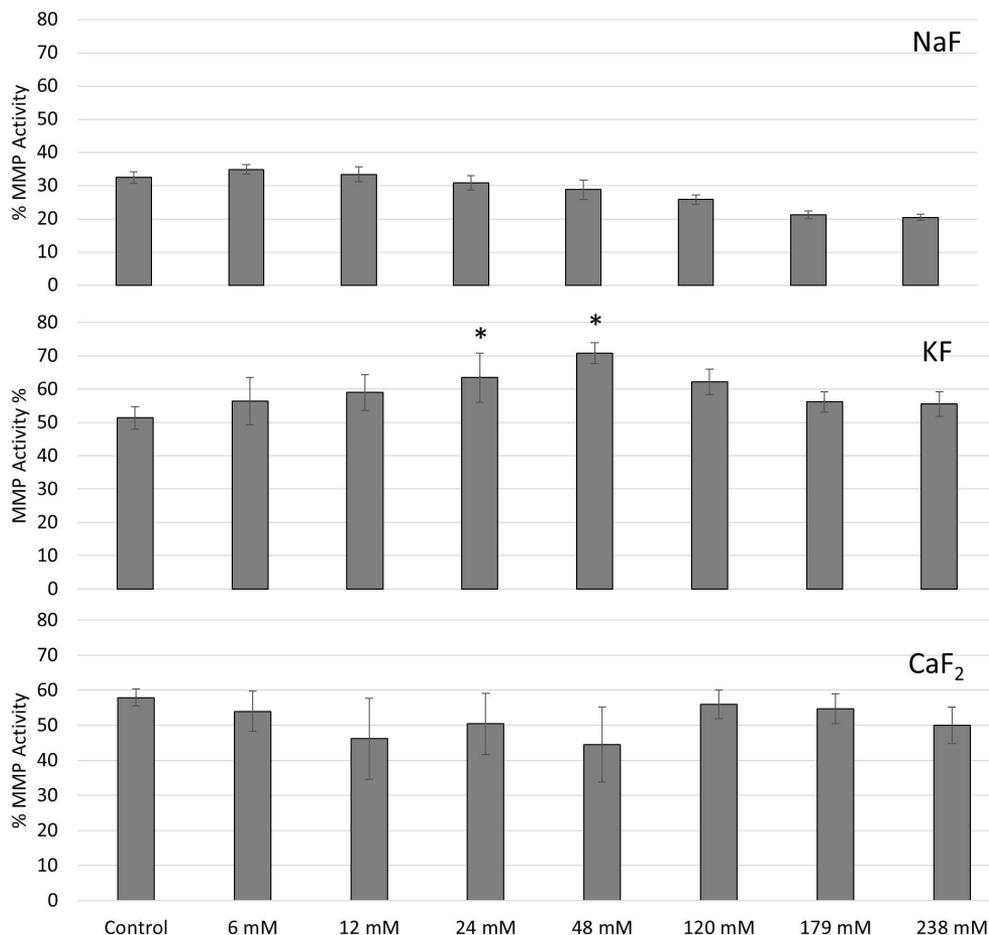
Figure 5. Bar charts comparing the total MMP-activity of the controls and NaF, KF CaF$_2$ groups (6-238 mM fluoride) after incubated in AS only for 6 months. Among KF groups, significant differences were marked with an asterisk (*) (p < 0.05). There was no significant difference between the NaF and CaF$_2$ groups and their respective controls (P > 0.05). (Modified from original publication I, II, and III)

5.2   Gelatin zymography (Studies I, II, III)

The gelatin zymography results are given in Figure 6. In demineralized dentin, the gelatinolytic activity was detected at 95 kDa, corresponding to proMMP-9 molecular weight, and at 68–72 kDa, corresponding to active and proforms of MMP-2, respectively. NaF- and KF-treated dentin samples showed relatively weaker bands at NaF 24–238 mM, and KF 12, 48, and 120 mM groups; however, the bands were still traceable. In CaF$_2$ groups, the bands remained as remarkable as the demineralized dentin at all concentrations.
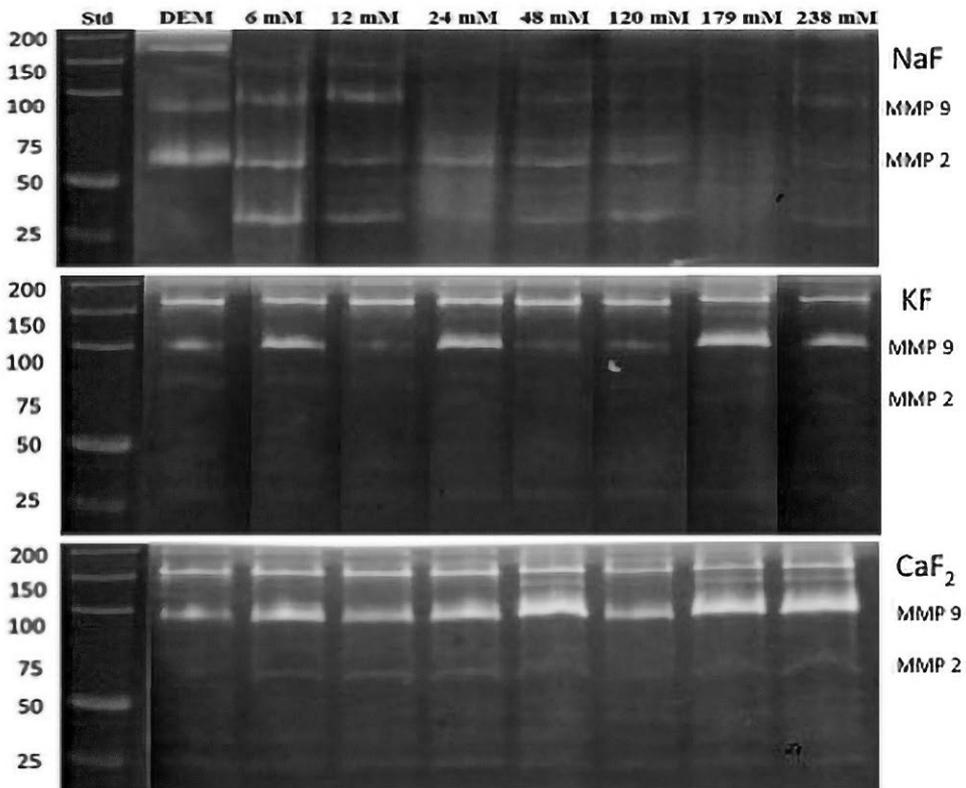
Figure 6. Gelatin zymography images of dentin protein extracted from demineralized dentin powder. Lane 1. Molecular-weight markers; Lane 2 (DEM): proteins extracted from dentin powder demineralized with 10% phosphoric acid, showing the presence of MMP-2 and MMP-9; Lane 3-9: demineralized dentin powder treated with increasing concentrations of NaF, KF and CaF_2 with 6-238 mM fluoride. (Modified from original publication I, II, and III)

5.3   Dry mass loss (Studies I, II, III)

The mean (±SD) dry mass loss of demineralized dentin beams incubated in AS or AS-containing NaF, KF, and CaF_2 in the fluoride concentrations of 6–238 mM for 1, 7, and 21 days are depicted in Figure 7. Among NaF, KF, and CaF_2, after 1 day of incubation, only CaF_2 12-48 mM and 238 mM groups showed less mass loss than the respective control ($p < 0.05$). After 21 days, NaF $\geq$ 12 mM, KF $\geq$ 24 mM, and $CaF_2 \geq$ 48 mM groups demonstrated less mass loss compared to the controls ($p < 0.05$). As seen in Figure 8, there was no significant difference between the mass loss percentages of fluoride groups and the controls after 6-month incubation only in AS ($p > 0.05$).
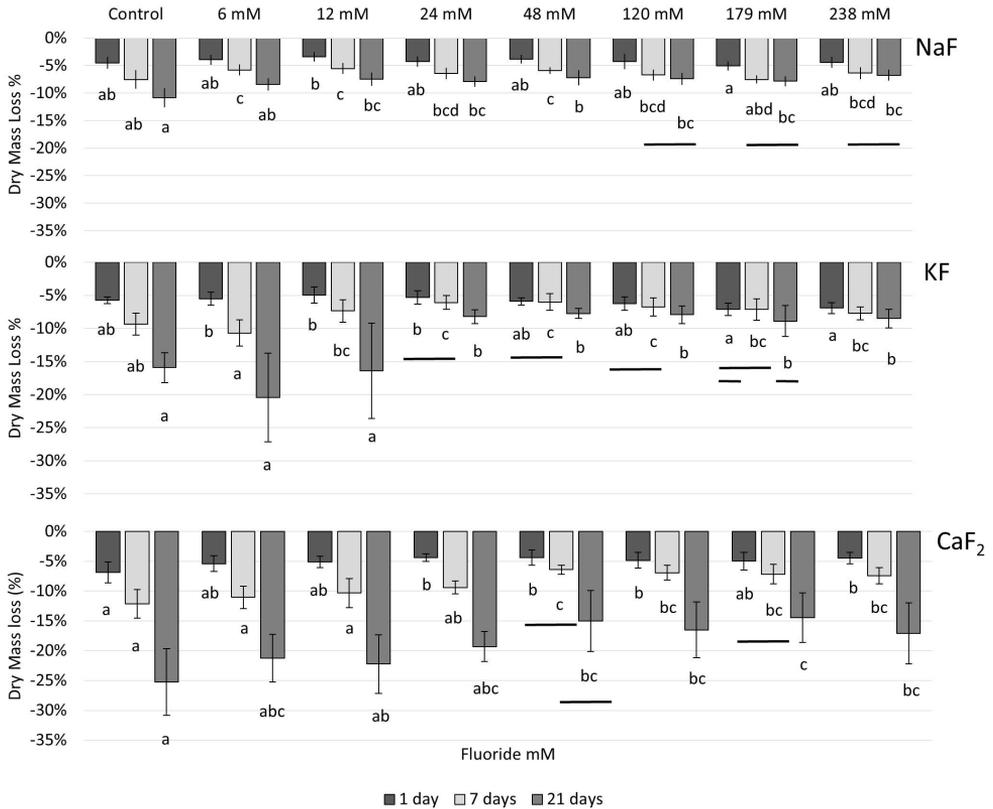
Figure 7. Bar charts comparing the dry mass loss of the demineralized dentin beams measured after incubated in AS (control), and AS with the increasing NaF, KF and CaF$_2$ concentrations (6-238 mM) for 1, 7 and 21 days. The loss of dry mass from each beam was calculated as a percentage of the original dry mass of that beam at baseline. For each time period, the groups with the same lowercase letters are not statistically significant ($p > 0.05$). For each concentration group of NaF, KF and CaF$_2$ and the control, time period columns connected by a solid black bar are not statistically significant ($p > 0.05$). (Modified from original publication I, II, and III)
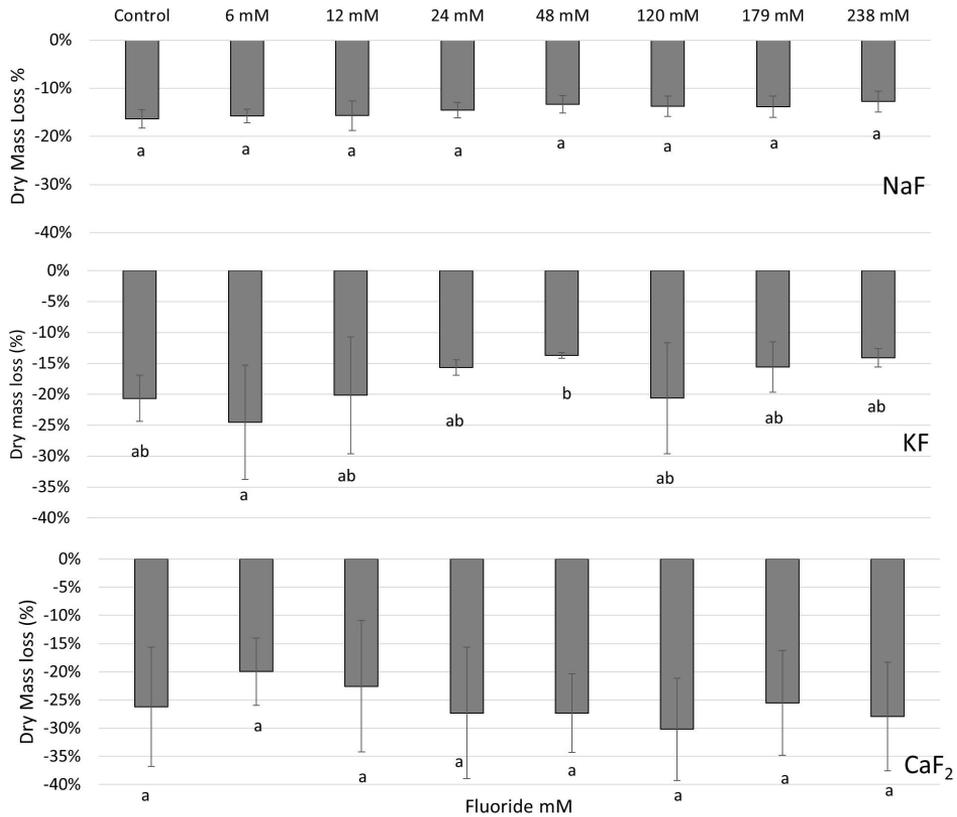
Figure 8. Bar charts comparing the dry mass loss of the controls and NaF, KF CaF$_2$ groups (6-238 mM fluoride) after incubated in AS only for 6 months. The groups with the same uppercase letters are not statistically significant (p > 0.05). (Modified from original publication I, II, and III)

5.4 ICTP assay (Study I)

The mean (±SD) ICTP levels measured from the medium of the NaF groups and the control after 1, 7, and 21 days of incubation are demonstrated in Figure 9. After 1 day, the ICTP levels of NaF 6–24 mM groups were less than the control (p < 0.05), showing a gradual increase with each higher fluoride level. After 7 days, NaF ≥ 120 mM showed less ICTP release than the control (p > 0.05). But after 21 days, the ICTP levels of NaF ≥ 12 mM were significantly higher than the control (p < 0.05).
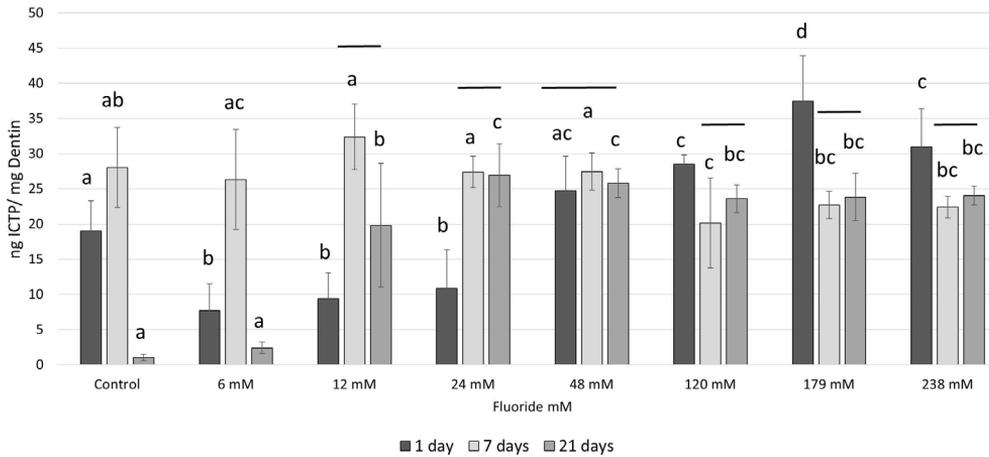
Figure 9. Bar charts comparing the mean (±SD) ICTP levels of demineralized dentin beams after incubated in AS with the increasing NaF concentrations (6-238 mM) or in only AS (control) for 1, 7 and 21 days. For each time period, the groups with the same lowercase letters are not statistically significant (p > 0.05). For each fluoride concentration as well as the control, time period columns connected by a solid black bar are not statistically significant (p > 0.05). (From original publication I)

5.5   CTX assay (Studies I, II, III)

Figure 10 shows the mean (±SD) CTX levels liberated to the incubation medium of demineralized dentin beams incubated in AS or AS containing NaF, KF, and CaF_2 at fluoride concentrations of 6–238 mM for 1, 7, and 21 days. After 7-day incubation, NaF 238 mM, KF $\geq$ 24 mM demonstrated significantly lower CTX levels than the controls (p < 0.05), whereas there was no decrease in CaF_2 groups compared to the control (p > 0.05). After 21 days, NaF $\geq$ 48 mM, KF $\geq$ 24 mM, and $CaF_2 \geq$ 120 mM groups showed less CTX release than the controls (p < 0.05). After 6-month incubation in only AS, the CTX levels of NaF $\geq$ 24 mM and KF $\geq$ 48 mM groups were significantly lower than the controls. On the other hand, the CTX release in CaF_2 24 mM group was significantly higher than in the control while there was no difference between the control and other CaF_2 groups (p > 0.05).
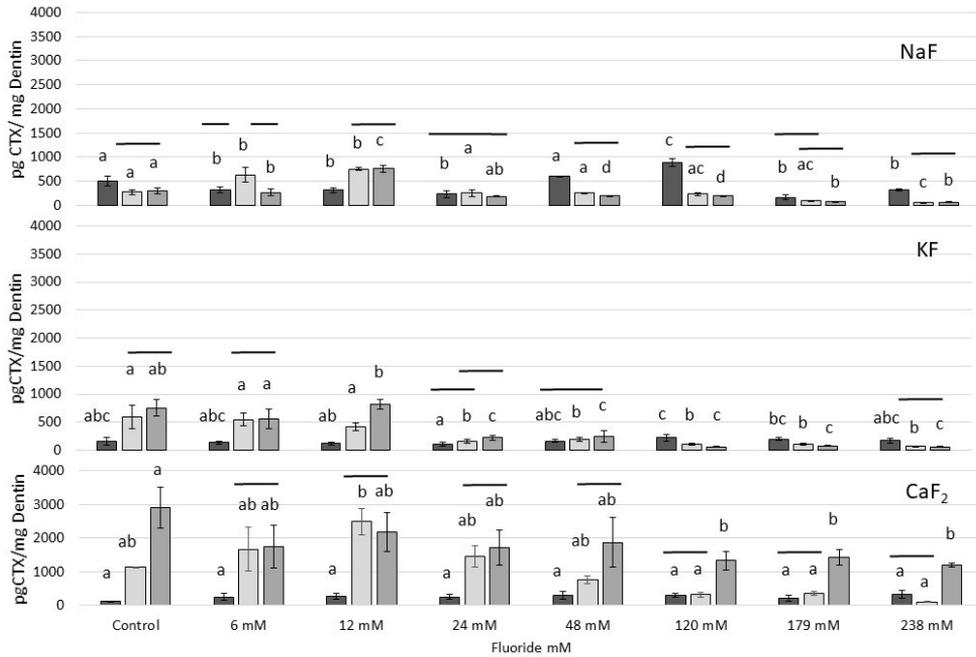
Figure 10. Bar charts comparing the mean (±SD) CTX levels of the demineralized dentin beams measured after incubated in AS (control), and AS with the increasing NaF, KF and CaF_2 concentrations (6-238 mM) for 1, 7 and 21 days. For each time period, the groups with the same lowercase letters are not statistically significant ($p > 0.05$). For each concentration group of NaF, KF and CaF_2 and the control, time period columns connected by a solid black bar are not statistically significant ($p > 0.05$). (Modified from original publication I, II, and III)
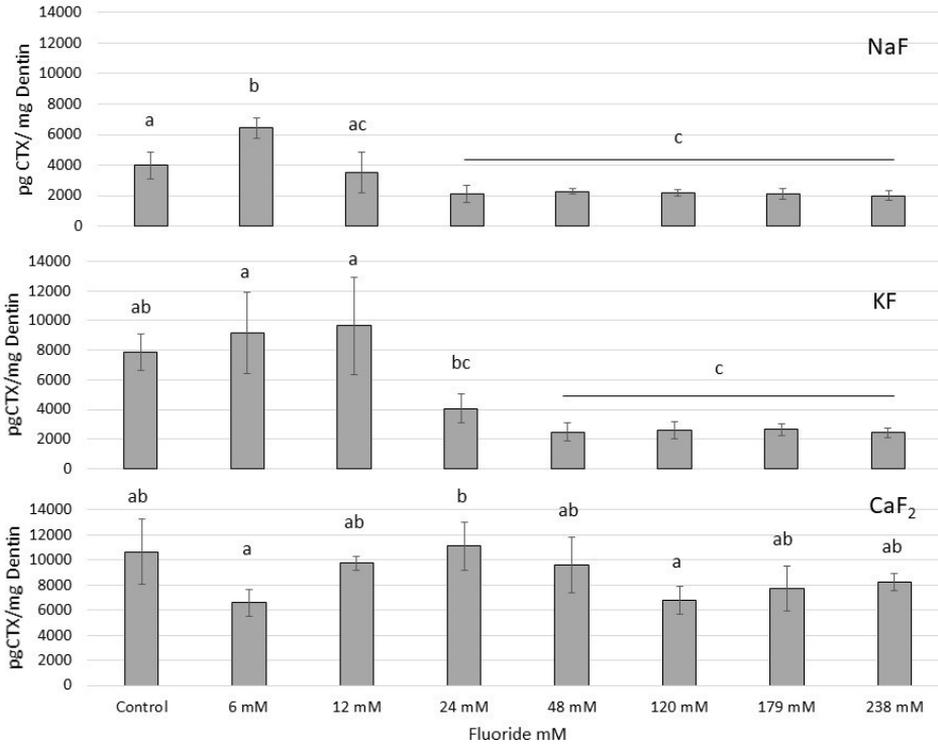
Figure 21. Bar charts comparing the mean (±SD) CTX levels of the controls and NaF, KF CaF$_2$ groups (6-238 mM fluoride) after incubated in AS only for 6 months. The groups with the same uppercase letters are not statistically significant (p > 0.05). (Modified from original publication I, II, and III)

5.6   HYP assay (Studies I, II, III)

The mean (±SD) HYP levels measured in the incubation medium of demineralized dentin beams incubated in AS or AS containing NaF, KF, and CaF$_2$ at fluoride concentrations of 6–238 mM for 1, 7, and 21 days, and detected after all groups were incubated in only AS for 6 months, are shown in Figures 12 and 13, respectively. For each incubation timepoint, there was no decrease between the HYP levels of the fluoride groups and the respective controls ($p < 0.05$).
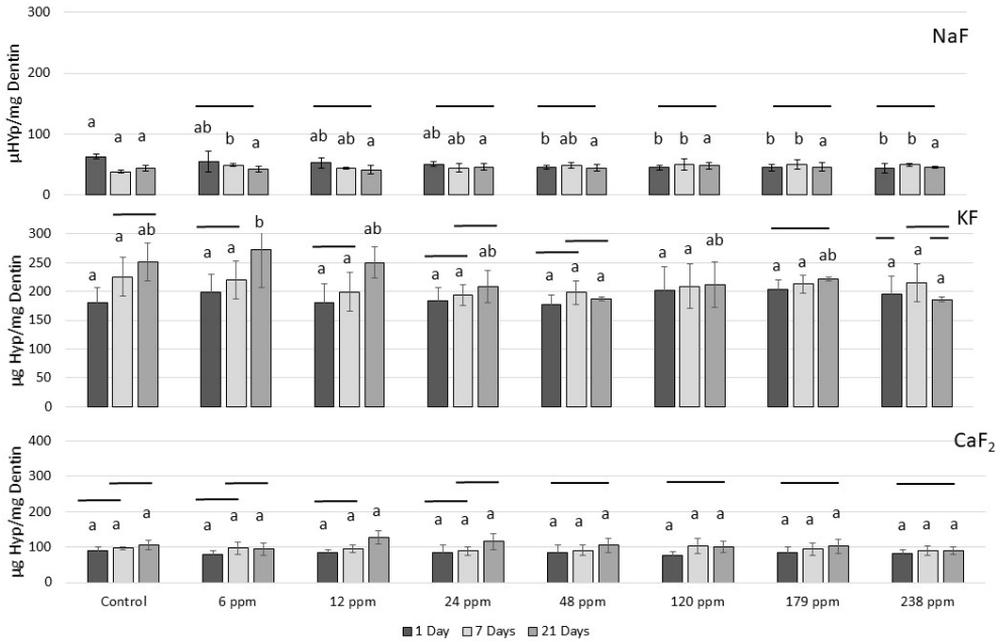
Figure 32. Bar charts comparing the mean(±SD) HYP levels of the demineralized dentin beams measured after incubated in AS (control), and AS with the increasing NaF, KF and CaF$_2$ concentrations (6-238 mM) for 1, 7 and 21 days. For each time period, the groups with the same lowercase letters are not statistically significant ($p > 0.05$). For each concentration group of NaF, KF and CaF$_2$ and the control, time period columns connected by a solid black bar are not statistically significant ($p > 0.05$). (Modified from original publication I, II, and III)
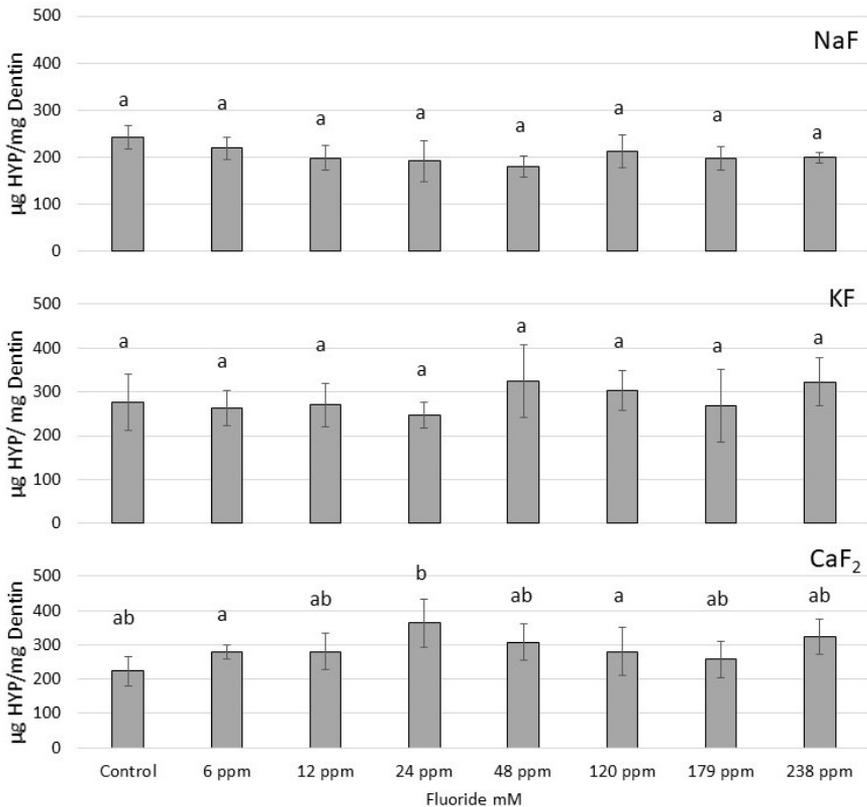
Figure 43. Bar charts comparing the mean (±SD) HYP levels of the controls and NaF, KF and CaF$_2$ groups (6-238 mM fluoride) after incubated in AS only for 6 months. The groups with the same uppercase letters are not statistically significant (p > 0.05). (Modified from original publication I, II, and III)

5.7 SEM-EDS examination (Studies I, II, III)

Figures 14 and 15 present the SEM micrographs of representative dentin beams selected from low and high fluoride concentrations of NaF, KF, and CaF$_2$ after 21-day and 6-month incubation, respectively. CaF$_2$-like mineral precipitations concentrating around the intertubular dentin matrix were seen in NaF and KF groups up to 24 mM. In CaF$_2$ groups, the precipitations were more dispersed on dentin surface without any specific localization compared to NaF and KF groups. The precipitation intensity was increased with increasing fluoride concentrations for each fluoride compound. In the NaF 238 mM group, the dentin tubule orifices were plugged with mineral deposits. At KF levels as high as F 179 mM, rather than only precipitating, the dentin matrix demonstrated an appearance as if intensified with the minerals. High concentrations of CaF$_2$ did not give any specific precipitation pattern as NaF and KF, but the size and amount of deposits increased at 21 days. After 6-month incubation in AS only, the

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mineral deposits on NaF and KF mostly dissolved. The mineral remnants still existed at a particularly high level of KF groups. On the other hand, there was not much dissolution in the mineral precipitations of beams in the CaF_2 group.

EDS analysis confirmed the presence of fluoride and calcium ions in the precipitation compositions. For each fluoride compound, fluoride penetration was detected inside the beams at a range of approximately 30–40% near superficial dentin, and slightly decreased up to 20 µm in depth. Inside dentin beams, the fluoride level was quite constant, detected as approximately 10%.
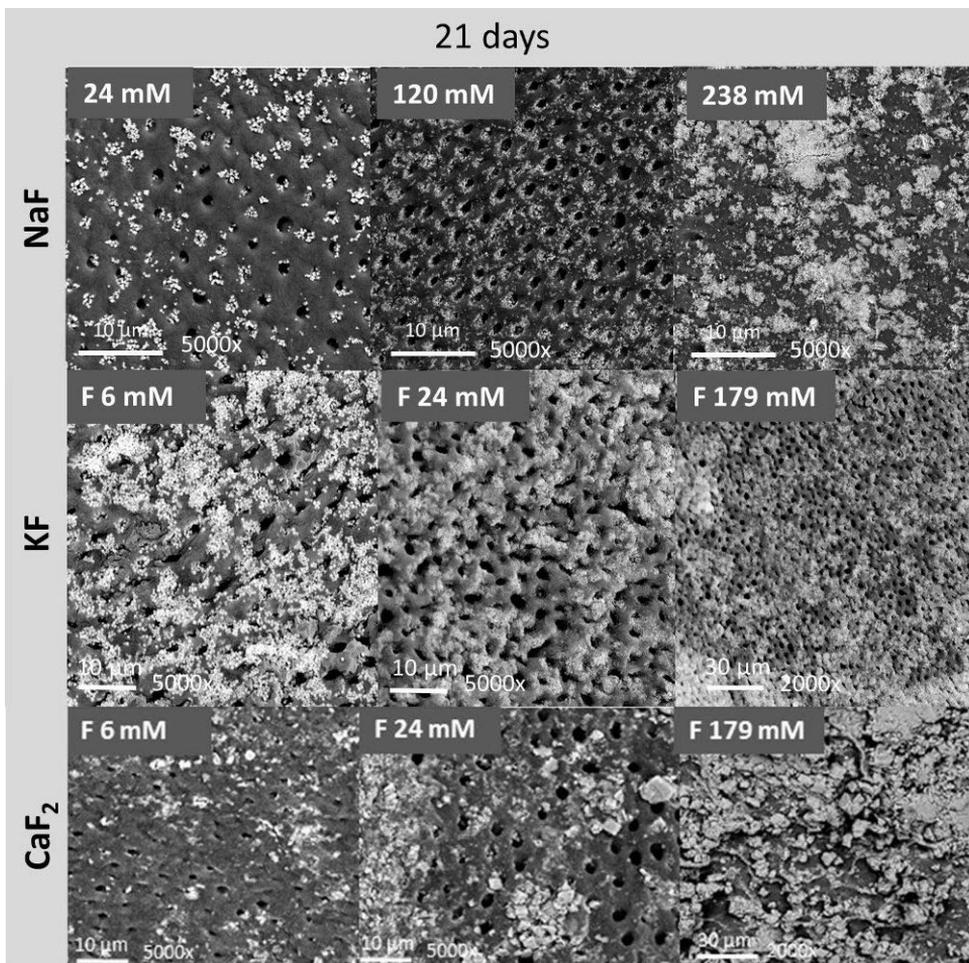
Figure 54. Representative SEM images from the surface of completely demineralized dentin beams incubated at increasingly higher fluoride concentrations of NaF, KF and CaF_2 after 1,7 and 21 days. The mineral precipitations on the beams at high fluoride levels masked the dry mass loss of the beams during incubation, possibly prevented the diffusion of thiopeptide substrate of total MMP assay into the beams, and may have caused the slow leaching of telopeptides to incubation medium. (Modified from original publication I, II, and III)
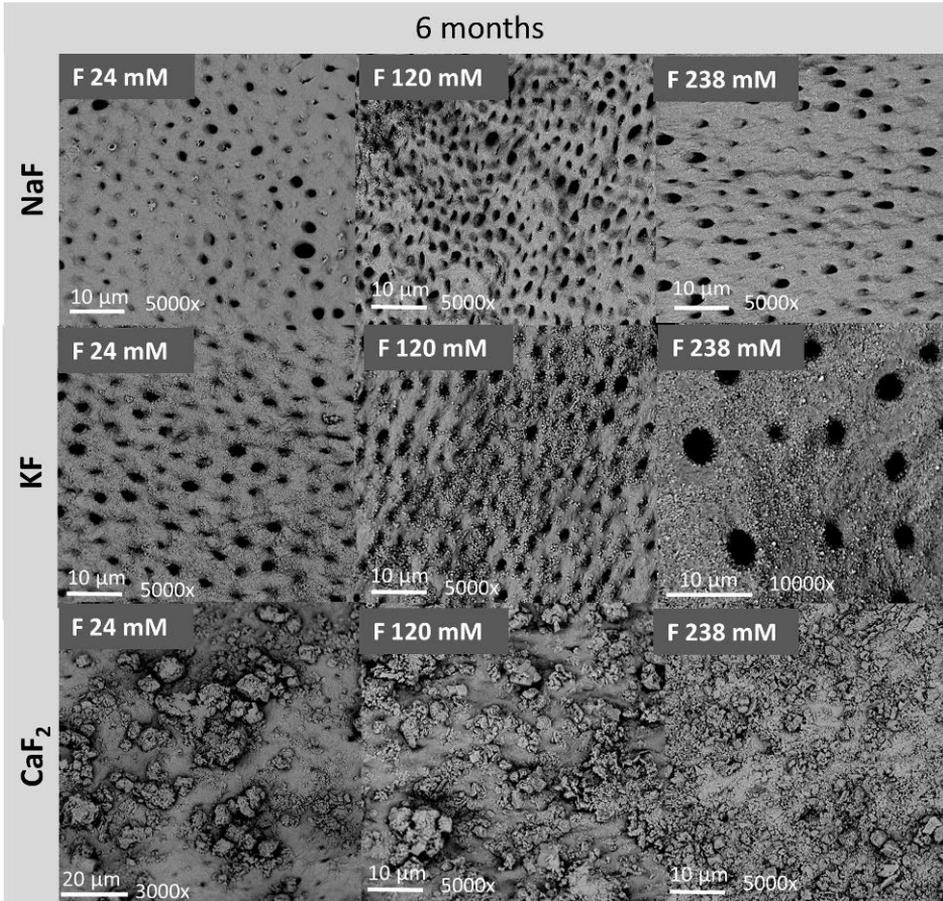
Figure 65. Representative SEM images from the surface of completely demineralized dentin beams incubated at increasingly higher fluoride concentrations of NaF, KF and CaF_2 after 6 months incubation in only AS. The mineral precipitations in NaF and KF groups were mostly dissolved whereas significant amount of precipitations still existed in CaF_2 groups. (Modified from original publication I, II, and III)

5.8 Results of µTBS test (Study IV)

Figure 16 shows the mean (±SD) µTBS results of the control, the NaF, KF, CaF_2 groups and Excite F (positive control). According to Univariate ANOVA, there were significant interactions between µTBS and each test factor as 'F compound', 'F concentration', and 'storage time' ($p < 0.05$). Based on the comparison of fluoride compounds, there was no significant difference between NaF and KF at 24 h and 12 months ($p > 0.05$). The comparison of fluoride concentrations showed no significant difference between fluoride levels of 6 and 24 mM, and 24 and 179 mM ($p < 0.05$). The analysis of storage time showed significant differences between the µTBS of 24 h and 12 months as well as 6 months and 12 months ($p < 0.05$).

After 24 h, the µTBS of NaF 24 mM was significantly higher than the control ($p < 0.05$). But, after 6-month storage, there was no significant difference between the µTBS of fluoride groups and the control. When the bond strength changes were compared between 24 h and 6 months, significant increases were detected for NaF and CaF_2 groups at 6 mM fluoride level ($p < 0.05$). However, only the bond strength of 6 mM NaF remained stable after 12 months.

The fracture types of the control group, NaF, KF, and CaF_2 groups, and Excite F are given in Figure 17. Chi-square test detected significant interactions between the test parameters of 'F compound', 'F concentration', and 'storage time' ($p < 0.05$). After 24 h, only the CaF_2 179 mM group mostly showed 'cohesive in composite' while the most common type fracture was 'mixed' in all other groups. After 6 months, all fluoride groups, except CaF_2 179 mM, showed less 'mixed' fracture compared to the control. 'Cohesive in composite' type fractures occurred predominantly in KF 24 and 179 mM groups after 12 months, while CaF_2 179 mM group demonstrated high a number of pretesting failures.

Representative SEM images of the fracture interfaces after 12-month testing are displayed in Figure 18. In all groups, including control, poorly resin-infiltrated areas were detected, which was particularly remarkable for the CaF_2 179 ppM group. On the other hand, NaF 6 mM, KF 24 mM, and Excite F groups demonstrated more homogenous resin coverage on dentin surface compared to the control and other fluoride groups.
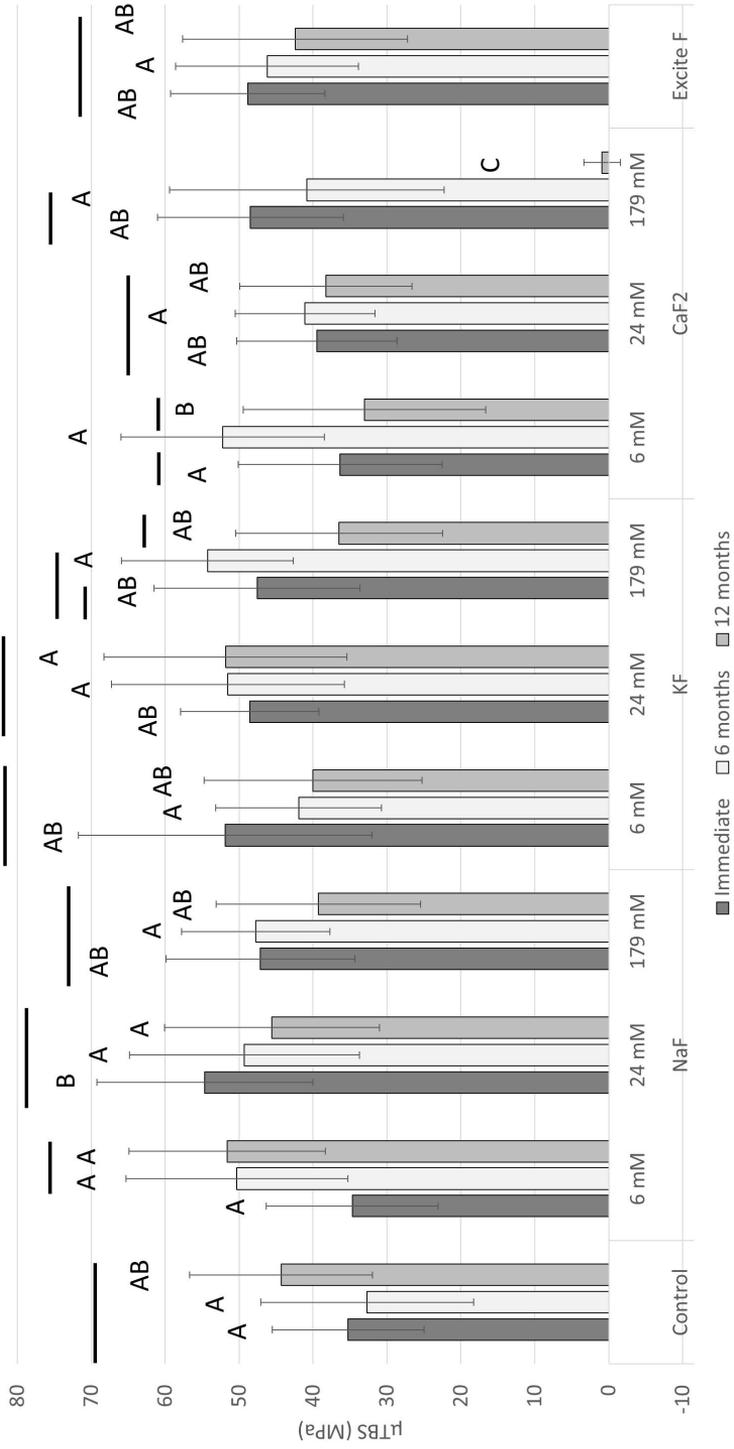
Figure 16. The µTBS to phosphoric acid-etched, and NaF, KF or CaF₂ treated dentin surface. The uppercase letters show the statistical differences between the groups. The same letters indicate no significant difference between the groups (p > 0.05). (Modified from original publication IV)
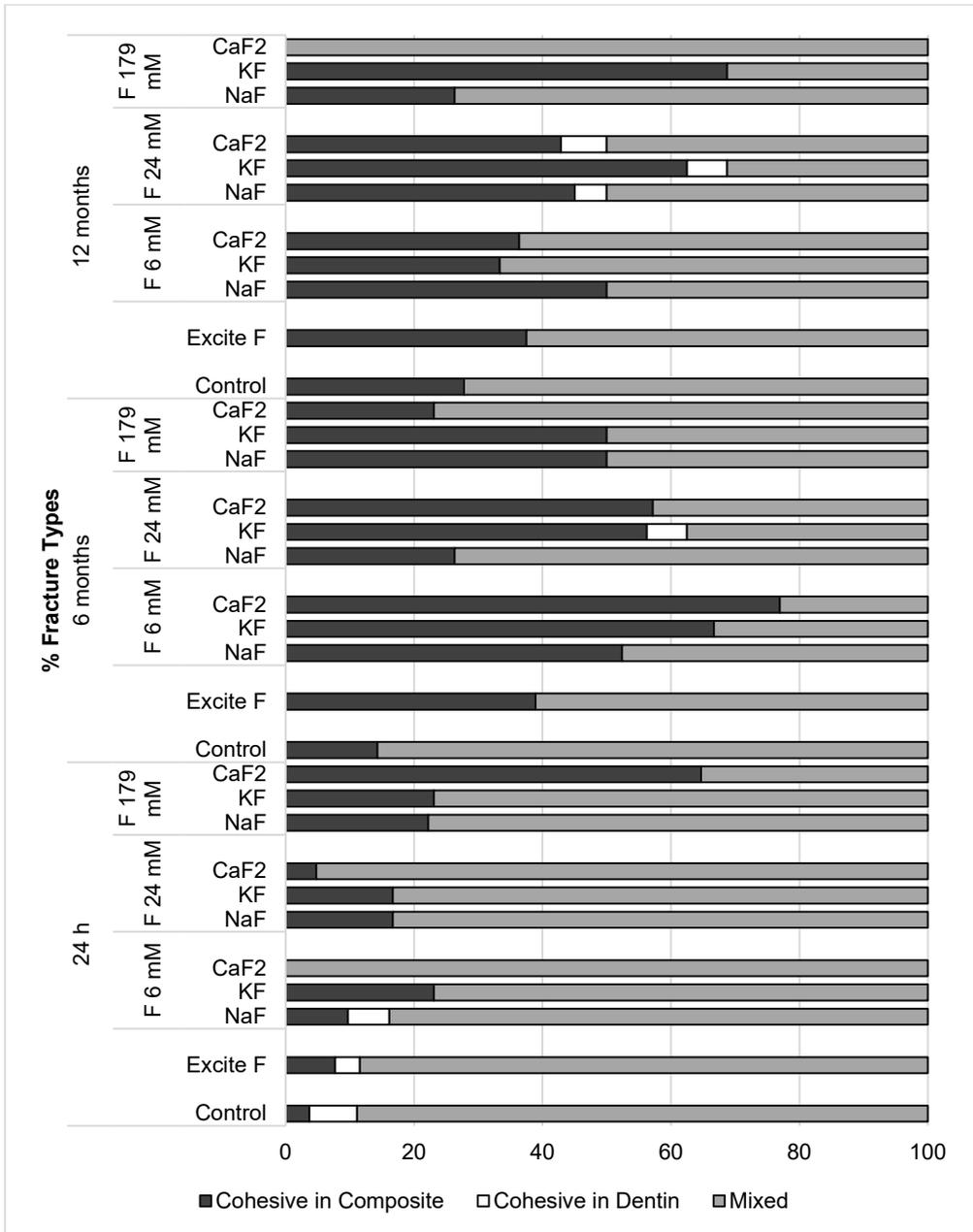
Figure 77. Fracture type analysis. (From original publication IV)
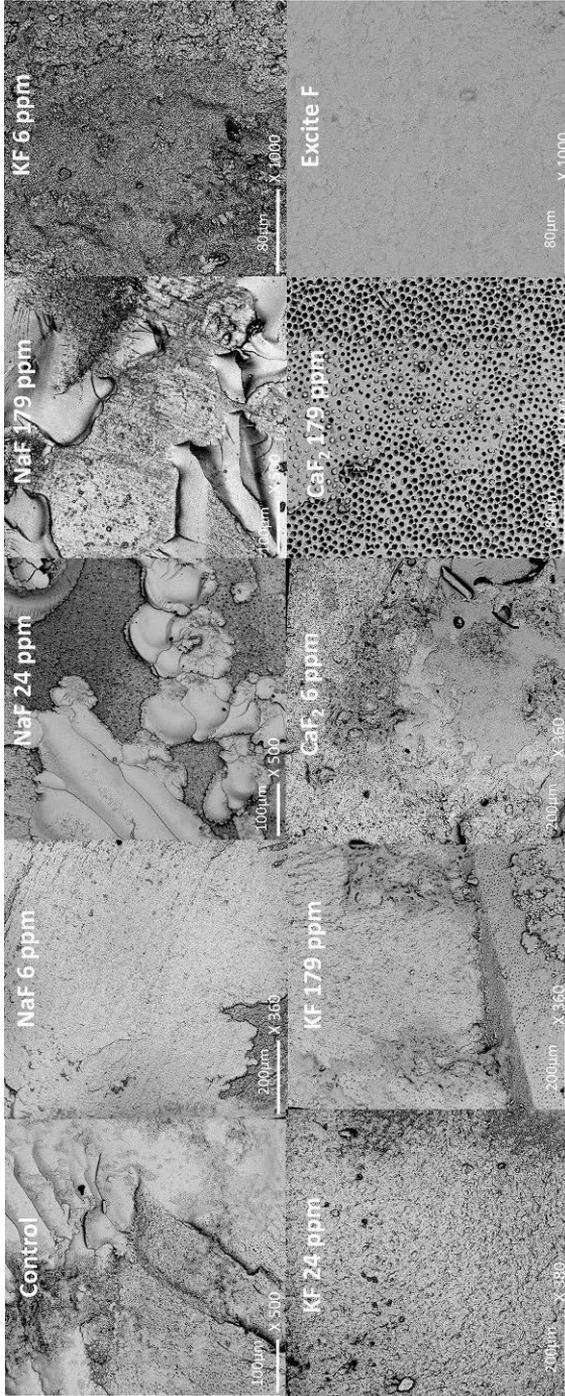
Figure 88. Representative SEM images from the fracture analysis of the specimens tested after 12 months of storage in AS. For all groups, 'mixed' fractures were common at the adhesive resin-composite interface with partial involvement of exposed dentin tubules, except CaF_2 179 mM group in which dentin tubules were not mostly infiltrated by the adhesive resin. (From original publication IV)

6   Discussion

Tooth-colored, composite resin-based restoratives have been the material of choice in current clinical practices for both anterior and posterior teeth owing to their superior aesthetic properties. Moreover, more conservative treatment options can be offered to patients by the use of resin-based adhesive systems due to the micro-mechanical and/or chemical bonding mechanism to tooth structures. However, despite the advances in the formulation of adhesive materials during the last decade, a recent study reported an approximately 10% failure rate at 10 years for posterior teeth restored with direct composite restorations (Alvanforoush et al. 2017). Secondary caries has been one of the major causes of failure particularly for posterior restorations (Opdam et al. 2014; Ástvaldsdóttir et al. 2015) while retention loss and aesthetic concerns are the main reasons for the repair or replacement of anterior restorations (Demarco et al. 2015). The degradation of resin-dentin adhesive interface caused by the hydrolysis of resin components and the enzymatic breakdown of collagen fibrils by MMPs and CCs have been indicated as the main etiological factors behind these clinical failures (Mazzoni et al. 2011b, 2012). Collagenolytic activities of dentin matrix-bound MMPs and CCs have been shown responsible for the collagen loss from demineralized dentin during cariogenic and erosive processes (Kato et al. 2010; Castilho et al. 2015; Giacomini et al. 2017).

As one of the most efficient anticariogenic agents of preventive applications in dentistry, fluoride has been incorporated into varnishes, and glass ionomer- or resin-based restorative materials and adhesive systems to create long-lasting restorations through the prevention of secondary caries development (Wiegand et al. 2007; El-Deeb et al. 2013; Chau et al. 2014, 2015). Antimicrobial and anticariogenic properties of fluoride-modified restorative materials have been the focus of many studies (Xu & Burgess 2003; Peris et al. 2007; Wiegand et al. 2007; Shinohara et al. 2009; Dionysopoulos et al. 2013; El-Deeb et al. 2013). Moreover, in recent studies, fluoride has been shown as an inhibitory agent on salivary MMP-2 and MMP-9, as well as recombinant CCs (Mei et al. 2012, 2014; Kato et al. 2014). On the other hand, it has been reported that dentin matrix-bound MMPs can be more resistant to inhibition compared to recombinant ones (Tezvergil-Mutluay et al. 2011). Therefore, considering the variance in the physical properties of fluoride compounds, this thesis

aimed to investigate the MMP and CC inhibitory efficacy of fluoride compounds, which are commonly used in the composition of resin-based restorative materials. Accordingly, Studies I, II, and III were designed to investigate the inhibitory efficacy of NaF, KF, and CaF_2 on dentin matrix-bound MMPs and CCs, respectively. In Study IV, fluoride-containing solutions were used as pretreatment agents for phosphoric acid-etched dentin surface during conventional bonding procedures in order to assess the effect of fluoride on the stability of dentin-resin adhesive interface.

6.1 Study design

Demineralized dentin matrix model was used for investigation of the proteolytic activity in dentin organic matrix, involving a series of direct and indirect analysis methods. Dentin beams in 2 mm thickness were obtained from the mid-coronal portion of sound teeth taking into account the diffusion depth of incubation medium as well as the relatively homogenous distribution of the enzymes. These beams were completely demineralized using 10% phosphoric acid, which has been shown effective in activating catalytic enzymes in dentin matrix without denaturation (Tezvergil-Mutluay et al. 2013). Demineralized beams were incubated in an incubation medium containing zinc and calcium, which are the crucial ions for the catalytic function of MMPs. The incubation process was implemented for 1, 7, and 21 days in fluoride-containing medium in order to examine the effects of fluoride ions on the catalytic activity of dentin matrix-bound MMPs and CCs in the short term. Then, all groups were further incubated in AS only, without any fluoride content, for the investigation of long-term collagenolytic activities in the previously fluoride-treated dentin organic matrices.

Considering that two distinct enzyme families function in the proteolytic degradation of demineralized dentin organic matrices, and potential enzyme inhibitor agents can further interact with other constituents of dentin matrix, i.e., residual Ca^{+2} ions, or with the collagen fibril structure, in this thesis, the activity of MMPs and CCs was investigated from various perspectives involving a series of direct and indirect examination methods. In Studies I, II, and III, total MMP activity assay and gelatin zymography were selected as direct methods and performed, and the indirect methods selected were dry mass loss and measurement of collagen degradation products in the incubation medium, including ICTP, CTX, and hydroxyproline levels. At the end of 21 days and 6 months, the representative beams from each group were additionally examined under SEM and subjected to EDS analysis.

6.1.1 Direct indicators of enzymatic activity

In the first step of the study series, the total MMP activity of demineralized dentin beams were measured by immersing the demineralized beams in a mixture of thiopeptide substrate and assay buffer (Ozcan et al. 2015). The substrate diffused into dentin and degraded by the MMPs would cause differences in absorbance values, which would reflect the inhibition percentage within F groups relative to the control without any specific indication to the inhibited MMP type. After 21 days of incubation, Study I showed that NaF $\geq$ 120 mM decreased the total MMP activity compared to the control. However, in Study II, KF was effective only in 179 mM group after 7 and 21 days, whereas in Study III there was no difference between the MMP activities of the control and CaF_2 groups after 21 days. Based on these initial examinations, it was thought that fluoride might be effective in the inhibition of dentin matrix-bound MMPs, depending on the concentration and fluoride source. These findings were in line with the study of Kato et al. (2014).

Gelatin zymography is a direct investigative method for the qualitative examination of the gelatinase activities (Mazzoni et al. 2013). In Studies I, II, and III, the bands indicating MMP-2 and MMP-9 activities in demineralized dentin samples treated with NaF, KF, and CaF_2 remained as noticeable as in the controls at all concentrations. These results were contradictory to the findings for the total MMP activity assays. Hence, considering the diversity of active enzymes playing a role in the proteolytic degradation of dentin organic matrix, further analysis was required for the validation of the inhibitory efficacy of fluoride compounds and the detection of the inhibited enzyme groups.

6.1.2 Indirect indicators of enzymatic activity

Dry mass loss changes of demineralized dentin beams before and after incubation reflect the total collagen loss due to solubilization of collagen fragments to the incubation medium without any specific indication to the activity level of MMPs or CCs (Tezvergil-Mutluay et al. 2013). After 21 days of incubation, compared to the control of each fluoride compound, the dry mass loss of the demineralized beams significantly decreased beginning from the concentrations of 12, 24, and 48 mM fluoride for NaF, KF, and CaF_2 in Studies I, II, and III, respectively. Thus, it was thought that fluoride might be an effective inhibitor of dentin matrix-bound proteolytic enzymes. In order to confirm this implication, on the next step of the studies, the collagen degradation products were measured in the incubation medium.

ICTP is regarded as the specific final product of MMP-dependent type I collagen degradation (Garnero et al., 2003). In Study I, the ICTP quantities liberated from dentin beams incubated in NaF-containing medium were determined using MMP-specific assay kits. Interestingly, according to the 1-day results, despite the inhibition

seen up to F 24 mM, there was a gradual increase in ICTP release with no inhibition effect on the catalytic activities of MMP at high fluoride levels. After 7 and 21 days of incubation, the ICTP levels of the NaF groups and the control were similar. However, considering that there was no difference in mass loss between NaF groups and the control after 1 day – whereas the dry mass loss was lower in the F $\geq$ 6 mM and F $\geq$ 12 mM groups than the control after 7 and 21 days, respectively – the MMP activities as indicated by the ICTP releases were in contradiction with the mass loss changes.

CTX is accepted as a specific marker for CC-dependent type I collagen breakdown (Garnero et al., 2003; Tezvergil-Mutluay et al., 2015). In Studies I, II, and III, the inhibition of CCs was detected for all three fluoride compounds with different efficiency concentrations after 7 days of incubation. At 21 days, NaF $\geq$ 48 mM, KF $\geq$ 24 mM, and CaF$_2$ $\geq$ 120 mM groups significantly inhibited CTX release compared to the control. At this stage of the study series, one important question was raised. Considering that CCs can have a role in the activation of MMPs (Christensen and Shastri 2015), CC inhibition could have impaired the activation of MMPs, and could be beneficial for the prevention of demineralized dentin matrix degradation. This could also explain the decreases in the mass loss of the beams incubated in fluoride-containing medium. Hence, to investigate the effect of CC inhibition on total collagen degradation, HYP analyses were performed as the indicator of total degraded collagen quantity by both MMPs and CCs. For NaF, HYP levels increased compared to the control after 7 days incubation, and after 21 days there was no significant difference between the NaF groups and the control. Likewise, KF and CaF$_2$ groups showed no significant decrease in HYP levels compared to their respective controls after 1, 7, or 21 days of incubation. Therefore, it was thought that the inhibition of CCs by fluoride was not sufficient for the prevention of proteolytic degradation of dentin organic matrices in the short term.

With the assessment of direct and indirect enzymatic activity indicators for demineralized dentin matrices, as the most significant finding from Studies I, II, and III, it was found that fluoride might be an efficient inhibitor of dentin matrix-bound CCs. However, it was not known whether this inhibition was irreversible or not in the long term. Moreover, considering the significant decreases in the total MMP activities and the dry mass loss of the dentin beams – whereas there was no decrease in HYP levels – CC inhibition alone was not enough to explain these contradictory results. For these reasons, after 21 days, the beams were examined under SEM, and EDS analysis was performed to evaluate the physical interaction between fluoride ions and demineralized dentin. Afterwards, the beams were incubated in only AS medium without any fluoride content for 6 months to screen the enzymatic activity changes.

6.1.3   SEM-EDS examination

After 21-day incubation, representative beams were examined for each fluoride compound in Studies I, II, and III, NaF, KF, and CaF_2 respectively, under SEM-EDS. CaF_2-like mineral precipitations on the beams explained the decrease in dry mass loss after incubation in fluoride-containing medium. Moreover, it was thought that these precipitations prevented the infiltration of thiopeptide substrate in the total MMP assay to dentin, therefore hindering the enzymatic digestion process. In line with the increasing density of mineral precipitations, total MMP activity assay showed decreasing level of MMP activity, leading to false MMP-inhibition results. These precipitations could also limit the easy release of degradation products to the incubation medium. Thus, their solubilization could cause increasing levels of collagen fragment accumulation in the incubation medium over time.

6.1.4   Long-term examination of proteolytic activity in dentin

Demineralized dentin beams previously incubated in NaF-, KF-, and CaF_2-containing medium were examined in terms of total MMP activity, dry mass loss, as well as CTX and HYP levels after 6-month incubation in AS only. The beams were also examined under SEM-EDS in order to assess the solubility behavior of CaF_2-like mineral precipitations. It was found that after 6 months there was no inhibition in MMP activities of fluoride groups compared to controls. In Study II, among KF groups, there was a significant increase in the MMP activity of F 24 mM and 48 mM, which could also be because of the dissolution of the mineral precipitations on the dentin beams, and the resultant infiltration of enzyme substrate to dentin.

After 6-month incubation, the dry mass loss of dentin beams was similar to that of the respective control for each fluoride compound. Compared to the controls, the CTX results showed that NaF $\geq$ 24 mM and KF $\geq$ 48 mM significantly inhibited CC activities in the long term. These were the most significant findings of Studies I and II, whereas there was no inhibition with CaF_2 in Study III. Conversely, there was no decrease in the HYP content of fluoride groups compared to controls. Under SEM, it was seen that, in NaF groups, almost all precipitations on dentin surface were dissolved during incubation. In KF and CaF_2 groups, there was partial dissolution in precipitation densities, which remained relatively dense within the latter in Study III.

6.2   Interaction of fluoride with MMPs and CCs in dentin

In a previous study, the MMP inhibitory property of NaF was reported (Kato et al. 2014). This thesis did not confirm that finding. In Studies I and II, NaF and KF showed no inhibition effect on dentin matrix-bound MMPs, while CCs were

significantly inhibited in the long term. In Study III, CaF_2 was not effective in the inhibition of either MMPs or CCs. It is known that fluoride ions can easily interact with the Ca^{2+} of dentin, creating CaF_2 or CaF_2-like minerals with fluoride levels of ≥ 100 ppm (approximately 5 mM) (Aoba 1997; Buzalaf et al. 2011). Accordingly, in Studies I, II, and III, the CaF_2-like mineral precipitations were seen on dentin beams regardless of the fluoride source, NaF, KF, and CaF_2. If one assumes that fluoride binds to the zinc and calcium ions of MMPs, causing conformational changes in the enzyme structure or converting the catalytic zone inaccessible to substrate binding, the inhibition of salivary MMPs can be explained. However, in the case of demineralized dentin, there would be free Ca^{2+} ions in the environment as the primary binding choice for fluoride. Hence, after interacting with these free Ca^{2+} ions, the remaining fluoride can further interact with the Ca^{2+} and Zn^{2+} ions in the MMP and CC structure. Considering the CC inhibition in Studies I and II, it can be suggested that CCs may be more propone to inhibition via fluoride and require lower doses than MMPs. Moreover, the relative amount of CCs in dentin can also be much lower than the MMPs, as in a previous study showing 10 times higher MMP-mediated degradation in dentin organic matrices (Tezvergil-Mutluay et al. 2013). Therefore, it can be assumed that if free fluoride ions were first consumed by the free Ca^{2+} ions in dentin matrix, the remaining ions could interact with CCs. Excessive fluoride levels did not result in MMP inhibition either, only increased the mineral precipitation on dentin beams – probably because the initial mineral accumulations served as nuclei for crystal growth (Lussi et al. 2012). In the case CaF_2 used as fluoride source, in addition to the free Ca ions already present in dentin matrix, the fluoride compound itself additionally provided extra calcium to the environment. Study III demonstrated this interaction sequence between the dentin matrix-bound enzymes, calcium ions, and fluoride, suggesting that no inhibition could be obtained with CaF_2 since fluoride ions were consumed before interacting with enzymes.

6.3   Resin-dentin adhesive interface stability

In Study IV, it was suggested that fluoride could prevent the enzymatic degradation of exposed and insufficiently resin-covered collagen fibrils in resin-dentin adhesive interfaces by directly inhibiting CCs or via indirect inhibition of MMPs, thereby enhancing adhesive bonding durability. Based on the CC inhibition of fluoride ≥ 24 mM as demonstrated in Studies I and II, acid-etched dentin surface was treated with NaF, KF, and CaF_2 with 6, 24, and 179 mM fluoride content for 30 seconds, which is a reasonable application time under clinical conditions. In general, fluoride treatment slightly increased the immediate bond strength to dentin, which was significant for NaF 24 mM compared to the control. After 12 months, apart from CaF_2 179 mM mostly showing pretesting failures, there was no significant difference

in bond strength between the fluoride groups and the control. On the other hand, the interaction between the µTBS to dentin and NaF and KF treatments was deemed significant for 6- and 24-mM fluoride levels. Furthermore, from immediate testing to 12 months, fracture types changed from 'mixed' to 'cohesive in composite resin' or 'cohesive in dentin' fractures in NaF and KF groups, which could be an indicator of durable bonding.

Considering the 1-day CTX results of Study I, II, and III, the treatment time of 30 seconds may seem inadequate for fluoride-CC interaction. However, when applied to acid-etched dentin, fluoride ions also interact with dissolved, free Ca^{2+} and phosphate ions in both superficial and deep dentin (Itota et al. 2003). In this case, it could be expected that fluoride treatment would result in the formation of new minerals on the bonding surface. Probably because of this, previous studies showed lower bond strength to dentin treated with fluoride containing desensitizing agents (Akca et al. 2007; Sarac et al. 2009; Arisu et al. 2011). Likewise, pretesting failures seen in CaF_2 with the 179 mM fluoride group can also be a result of dense mineral precipitations interfering with resin infiltration into dentin. On the other hand, after resin infiltration, naked collagen fibrils lacking resin protection can be encapsulated by these minerals, silencing the collagenolytic enzymes (Liu et al. 2011). Therefore, the conclusion in Study IV was that fluoride can enhance the stability of adhesive interfaces depending on fluoride compound and concentration, possibly by providing mineral support and indirectly inhibiting proteolytic activities.

6.4 Prospects of fluoride use against to collagenolysis in dentin matrix

Fluoride has been widely incorporated into oral hygiene products, dentin desensitizers, as well as resin-based restorative materials and adhesive systems mainly due to its anticariogenic properties. In this thesis, NaF and KF as fluoride sources gave favorable results in terms of the inhibition of dentin matrix-bound CCs at high concentrations, and the enhancement of adhesive interface stability at relatively low concentrations. Additionally, MMPs might be silenced by the inhibition of CCs, or the recovery of mineral support of collagen fibrils via the CaF_2-like precipitations. These findings showed that the fluoride compound and concentrations significantly influence the interaction of fluoride with dentin and the possible benefits. It should also be taken into account that the studies in this series were performed in neutral pH, owing to the stability of collagen fibrils. It could be suggested that lower doses of fluoride could clinically demonstrate similar CC inhibition and denser mineral precipitations under slightly acidic conditions, since more free Ca^{2+} ions would dissolve from the tooth structure. Furthermore, considering that CCs show maximal proteolytic activity at around pH 5.5, under

acidic conditions, such as erosion or active caries lesions, the anti-cathepsin efficacy of fluoride ions may vary. Therefore, these aspects require further research.

Within the limitations of this study series, NaF and KF are concentrations of F $\geq$ 24 mM can be suggested as potential CC inhibitors, used to improve the enzymatic degradation resistance of demineralized dentin matrices for the prevention of caries and erosion progression, and to enhance dentin-resin adhesive interface stability.

7   Conclusions

Within the limitations of the study series included in this thesis, following conclusions were drawn:

I. NaF, KF or CaF_2 did not prevent the MMP-dependent degradation of demineralized dentin matrices.

II. High levels of NaF and KF significantly inhibited the catalytic activity of dentin matrix-bound CCs. NaF and KF can be beneficial for the prevention of enzymatic degradation of demineralized dentin matrices by indirect MMP-inhibition owing to CC-inhibition, or via recovering the mineral support of demineralized collagen fibrils, and silencing MMPs.

III. CaF_2 did not provide CC-inhibition in demineralized dentin matrices.

IV. NaF and KF can improve the stability of resin-dentin adhesive interface depending on used concentration.

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