Optimizing dentin bond durability: control of collagen degradation by matrix metalloproteinases and cysteine cathepsins

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Abstract

Objectives—Contemporary adhesives lose their bond strength to dentin regardless of the bonding system used. This loss relates to the hydrolysis of collagen matrix of the hybrid layers. The preservation of the collagen matrix integrity is a key issue in the attempts to improve the dentin bonding durability.

Methods—Dentin contains collagenolytic enzymes, matrix metalloproteinases (MMPs) and cysteine cathepsins, which are responsible for the hydrolytic degradation of collagen matrix in the bonded interface.

Results—The identities, roles and function of collagenolytic enzymes in mineralized dentin has been gathered only within last 15 years, but they have already been demonstrated to have an important role in dental hard tissue pathologies, including the degradation of the hybrid layer. Identifying responsible enzymes facilitates the development of new, more efficient methods to improve the stability of dentin-adhesive bond and durability of bond strength.

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Significance—Understanding the nature and role of proteolytic degradation of dentin-adhesive interfaces has improved immensely and has practically grown to a scientific field of its own within only 10 years, holding excellent promise that stable resin-dentin bonds will be routinely available in a daily clinical setting already in a near future.

Introduction

Since the first study to demonstrate the rapid time-related loss of dentin bond strength [1], numerous studies have confirmed the finding both with etch-and-rinse (E&R) and SE (SE) adhesives [2]. The problem is specific for the resin-dentin bonds, as the resin –enamel bonds are very stable over time [3], and relates to the loss of of the hybrid layer collagen matrix [2].

Enzymatic degradation of the collagen matrix by host-derived enzymes plays a significant role in the destruction of the bonded interface [4,5]. To date, several matrix metalloproteinases (MMPs) and cysteine cathepsins have been identified in dentin, and suggested to be responsible for the digestion of collagen fibrils exposed at the adhesive interface. During the last 10 years, the understanding of the mechanisms involved in the proteolytic degradation of dentin-adhesive interfaces has gained immense attention and has practically grown to a scientific field of its own. The progression in this field has been rapid, and the clinical interest in measures to improve dentin bond durability is also increasing [6]. This has led to several tentative approaches to prevent such enzymatic activity. In general, the strategy to improve the durability of resin-dentin bonds relies on the ability to inhibit the activity of the enzymes. In this review, we will look at the presence, identity and function of the collagen-degrading proteolytic enzymes in dentin.

Dentin collagen: structural properties vs. bonding

Before going into details of dentinal enzymes, it is essential to briefly overview the dentin organic matrix to fully understand the complexity of the tissue in relation to the adhesion and function of the enzymes. Approximately 50 vol.% of dentin is composed of minerals, the rest being type I collagen and non-collagenous proteins (30 vol.%) and water (appr. 20 vol.%) [7]. During the mineralization of dentin and bone, collagen fibrils provide the aqueous compartment in which nanometer-sized apatite mineral crystals grow. In soft tissues (and in dentin and bone matrix prior mineralization), collagen intermolecular spaces contain highly ordered and tightly bound water molecules forming multi-layered cylinders around collagen molecules [8] (Figure 1). During the mineralization process, water within and between the fibrils is progressively replaced with minerals [9]. In mineralized dentin, there is no resin infiltration. To permit resin infiltration for adhesive retention, dental hard tissues are acid-etched by acids or acidic monomers to remove minerals that are replaced by rinse-water (E&R adhesives) or water used in SE primer-adhesives as a solvent (E&R and SE adhesives). Hydration of collagen prior to the monomer penetration is essential in order to avoid the matrix collapse due to the formation of interpeptide hydrogen bonds between collagen fibrils [10]. In addition, the ionizable moieties of acidic monomers in SE adhesives are also hydrophilic and promote water sorption over time.

During application of adhesives, solvated comonomers are expected to replace the water and penetrate into and around collagen fibrils for proper hybridization [5]. While the interfibrillar spaces between collagen fibrils in hybrid layers are wide enough to allow small hydrophilic monomers (e.g. HEMA, TEGDMA) to penetrate between the fibrils, intrafibrillar penetration has been challenged. The inter-molecular distance in the lateral packing of collagen molecules within a collagen fibril (the space supposed to be occupied by monomer molecules) is within range of 1.26 nm to 1.33 nm. Since even the adhesive monomers (such as TEGDMA) units are approximately 2 nm in diameter, the complete

infiltration of the adhesive material even at single monomer molecular level is restricted by the space available within a collagen fibril [11]. We will address this issue later in the article.

As adhesive comonomers diffuse into the water around collagen fibrils, their relatively high chemical concentrations (2–4 moles/L) lower the vapor pressure of water (Raoult's law) making it more difficult to evaporate residual water using a dental air syringe [12]. The spaces between collagen fibrils are between 20–30 nm. These spaces contain proteoglycan hydrogels [13], that may restrict the free diffusion of some of the larger adhesive monomers, such as BisGMA (512 Da) compared to small monomers such as HEMA (100 Da). Figure 2 shows a schematic of collagen fibrils cut in cross-section (large circles) in a hybrid layer being infiltrated by a mixture of BisGMA/HEMA comonomers. Note that the small black dots, representing HEMA, diffuse more deeply than the large black dots representing BisGMA. This schematic was made to illustrate what might be responsible for a decreasing concentration of BisGMA that had been reported by Spencer and Wang [14] when they examined its distribution across hybrid layers by microRaman spectroscopy.

However, as each collagen fibril is made up of hundreds of collagen molecules that are closely packed together, Bertassoni et al. [11] argued that there is not sufficient space between packed collagen molecules (ca. 1.26–1.33 nm) to accommodate adhesive monomers (Figure 2).

Physiological experiments often provide insight into how molecules interact with collagen fibrils. Toroian et al. [15] pulverized bovine bone into small particles and packed them into 2×50 cm columns to permit gel filtration column chromatography of these particles before and after demineralization. They used bone collagen particles just like investigators usually use hydrated Sephadex beads. In typical gel filtration chromatography, large molecular weight molecules like blue dextran (10^6 Da) are too large to penetrate the Sephadex beads, so they elute with the void volume ahead of molecules that can penetrate the beads and hence, whose elution is delayed. By using phosphate ions, glucose, osteocalcin (5700 Da), cytochrome (12.3 kDa), fetuin (48 kDa), hemoglobin (64 kDa) and IgG (152 kDa), Toroian et al. [15] found that molecules less than 6 kDa easily diffuse between collagen molecules and entered "collagen water", while molecules larger than 40 kDa were excluded from "collagen water". However, they found that osteocalcin (5.7 kDa, molecular diameter 1.8 nm) did penetrate into "collagen water" even though x-ray diffraction studies confirmed that the Bragg spacing between hydrated collagen molecules is 1.8 nm (Figure 2). Generally, molecules whose diameters are equal to pore sizes in barriers are not permeable because the molecule would have to fit the pore perfectly. If they touched any part of the pore, they would be reflected away and not permeate.

Toroian et al. [15] conclude that hydrated collagen molecules function as if their intermolecular size is significantly larger than 1.8 nm due molecular vibration/rotation. The results support the hypothesis "that individual collagen molecules have substantial freedom to move in the lateral plane of the fibril".

We conclude that if osteocalcin (MW 5700 Da) can equilibrate with collagen water, then BisGMA (512 Da) and HEMA (100 Da) should easily permeate into water located around and within collagen molecules. Whether water-immiscible monomers such as BisGMA can wet collagen molecules is debatable. Since BisGMA is usually solvated in ethanol or HEMA, and they are both water miscible, perhaps dimethacrylates like BisGMA can infiltrate the space between collagen molecules and polymerize to envelop them and prevent protease-induced collagen hydrolysis.

Complete coverage of the nanoscale irregularities on the collagen fibrils surface via passive monomer penetration may be difficult to achieve. These irregularities are caused by the 4 to 6 nm height difference between the fibril gap and overlap zones, and nanometer-scale microfibrils at the surface of dentin collagen fibrils [16]. Microfibrils, detected with X-ray diffraction, are discontinuous 5-mer repeats of collagen molecules with 4 to 5 nm diameters [11,17,18]. Another potential hindrance may be proteoglycans (PGs), which with their glycosaminoglycan (GAG) carbohydrates envelop the collagen surface, retaining water [11]. The ability of polymeric resins to fully displace water from the crevices present on collagen fibril surfaces may require extremely low viscosity monomeric material and precise placement of the primer components to alter the surface energy of the fibril. Nanoleakage can occur in the absence of detectable gaps in the resin-dentin interface [19,20]. These "nanovoids" can be responsible for facilitating the diffusion of water molecules with diameters of about 100 pm, from the hydrated dentin [11], allowing slow but continuous degradation of the ester bonds in adhesive polymers. Degradation of the microfibrils would lead to nanoleakage, and with increasing void size, increase in dentinal fluid seepage would speed up the hydrolysis of both resins and matrix components, with eventual loss of bond strength. Others have argued that the ability of adhesive monomers to displace water from collagen is limited by their relative concentrations. Water has a concentration of 55 moles/L, while comonomers have concentrations of 3–4 moles/L [12]. Thus, although the adhesive monomer HEMA has been shown to "fit" into the groove on water-free collagen peptides in computer models [21], there are structural and chemical reasons why this may not occur.

Other components and features of dentin matrix may also have marked impacts on the durability of dentin bonds. These include e.g. collagen cross-linking and PGs in dentin. For the sake of clarity, these aspects will be described in detail in further paragraphs.

Enzymes in dentin

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of Zn^{2+}- and Ca^{2+}-dependent enzymes, that in concert are able to degrade practically all ECM components, making them important components in many biological and pathological processes. Esterases and proteases are also classified as hydrolases. That is, they enzymatically add water across ester or peptide bonds. In humans, the MMP family contains 23 members that are frequently divided into six groups - collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs), and other MMPs - based on substrate specificity and homology [22,23]. Even though this classification is commonly used both for historical and practical reasons, it does not fully reflect the complexicity of MMP functions and biological activities, as most MMPs can degrade several substrates with variable specificity [22,23]. For example, collagenases-1 and -3 (MMP-1 and -13) can also degrade gelatin at a slow rate, and gelatinases (MMP-2 and -9) can degrade several types of collagen, especially type IV collagen (thus the old name "type IV collagenases") [22–24]. Apart from their ECM degradation, MMPs also play important roles in the conversion of noncollagenous matrix proteins to signaling molecules that affect cell survival, proliferation, and differentiation [25].

MMPs are synthesized and mostly secreted as inactive proenzymes (zymogens), in which the so-called prodomain is present, inhibiting the functional activity of catalytic domain (Figure 3). In latent (non-activated) MMPs, the unpaired cysteine in the prodomain forms a bridge with the catalytic zinc (referred to as the "cysteine switch" mechanism [26]), preventing enzymatic activity. This conserved cysteine acts as a ligand for the catalytic zinc atom in the active site, excluding water molecules and rendering the enzyme inactive. Activation occurs when this linkage is disrupted by proteolytic cleavage of the propeptide by other MMPs, cysteine cathepsins or other proteinases, or chemically e.g. by amino phenyl

mercuric acid (APMA), replacing the thiol group with water [24,25]. In addition to the pro- and catalytic domains, MMPs contain other domains responsible, for instance, for substrate specificity, recognition, and interaction [27]. Therefore, MMPs are often classified according to their molecular structures [25].

Collagen molecules contain a rigid helical center (Figure 4) with globular N- and C-terminal ends called telopeptides. In vertebrate MMP family, collagenases (MMP-1, -8, -13), MMP-2 (gelatinase A), and membrane-type 1-MMP (MT1-MMP, MMP-14) can cleave native triple-helical type I, II, and III collagens. They all cleave collagens into ¼- and ⅓-fragments at the Gly-Leu/Isoleu peptide bond where collagen peptide structure determines both specific cleavage and binding sites for MMPs [28]. The fragments denature at body temperature, and are further degraded by gelatinases and other non-specific tissue proteinases. Interstitial collagen consists of three α chains, and each chain winds around each other in a right-handed twist to form a triple helical structure with about 300 nm in length and 1.5 nm in diameter. This triple-helical structure makes interstitial collagens fibrils in their native configuration resistant to most vertebrate proteolytic enzymes. The substrate binding site of collagenases is located in a deep cleft with the entrance being only approximately 0.5 nm wide. This is not enough space to accommodate triple helical collagen with a diameter that is 3 times larger than the active site of the enzyme [27]. Currently, two mechanisms have been proposed to allow the degradation of collagen fibril by MMPs. Using a MMP-1 mutant that is unable to cleave collagen but retains the binding properties, Chung et al. [29] demonstrated that MMP-1 is capable of unwinding collagen fibril to allow the enzyme's active site to cleave individual chains in succession [27,29] (Figure 4). The role of MMP in unwinding collagen peptide helices was further supported by the cleavage of unwound fibril by MMP-3 and neutrophil elastase, which normally do not degrade fibrillar collagen [27,29]. The similar binding-unwinding mechanisms have later been suggested for MMP-8, -2 and -14 [30]. This view was at least partially challenged by Perumal and others [28], when they demonstrated that the collagen region where cleavage begins, is fully protected by the collagen C-telopeptide (the terminal end of the molecule) restricting the access of MMPs to the cleavage site of the native collagen structure [28]. They suggest that the site must be exposed by proteolytical removal of C-terminal telopetide by telopeptidase or e.g. mechanical damage, before MMP can bind to the substrate's "interaction domain" facilitating the triple-helix unwinding/dissociation function of the enzyme before collagenolysis. Alternatively, damage caused e.g. by physical loading may induce changes in fibril structure, such as breakage of cross-linkages at the C-terminus. This damage may expose the first collagen peptide chain to be cleaved by MMP, leading to a greater freedom of movement for other chains and their subsequent cleavage [28]. Collagen molecules distal to the original cleavage site would also become accessible to MMP cleavage, leading to further degradation [28]. This is an inviting hypothesis, as the damages caused to dentin collagenous matrix by demineralization in caries lesions, phosphoric acid or acidic monomers have been suggested to cause changes in the collagen molecular arrangement (e.g. breakage of cross-linkages) which may expose the catalytic binding site [11]. Interestingly, telopeptidase enzyme activity of MMP-9 has long been suggested to be important in bone matrix degradation [31]. MMP-9 is present in dentin [32], it is readily activated by acid pH changes [31,33–35], and telopeptidase activity of MMP-9 has been suggested to have a role in dentin matrix degradation in caries [35]. Alternatively, cysteine cathepsins may be involved. Cysteine cathepsins can activate MMPs [36], and this mechanism has been suggested to be involved in human dentinal caries lesions [37]. Cysteine cathepsins can also cleave C-terminal telopeptide of type I collagen [38], possibly exposing the collagenase cleavage site after being activated by acids.

Although intermolecular cross-links can occur along both helical and telopeptide regions, they are especially common in the telopeptide domains. When Garnero et al. [39] incubated

acid-etched bone powder with pure, soluble MMP-2, they saw increased liberation of soluble collagen peptides. When the peptides were isolated by SDS-PAGE electrophoresis, none of the bands were due to ¼ or ⅓ length collagen fragments. Using specific antibodies directed at various cross-linked telopeptides, they found that MMP-2 released relatively long cross-linked C-terminal telopeptide fragments called ICTP fragments. When they incubated bone powder with cathepsin K, the released telopeptides were much shorter. These were called CTX peptides. Both classes of peptides are now measured in plasma and urine in postmenopausal patients as indices of the degree of bone turnover.

When Osorio et al. [40] incubated exogenous soluble MMP-2 with completely demineralized beams of dentin, the dentin released ICTP telopeptide fragments. The control beams incubated in control media without MMP-2 also released ICTP peptide fragments, albeit at lower concentrations, indicating that the MMP-2 isolated from dentin [32,41,42] may function as a "telopeptidase". If gelatinases MMP-2 and -9 can hydrolyze the bulky telopeptides off surface collagen fibrils, they may create enough space for a collagenase to bind to the proper binding site to create unwinding of the collagen molecule that promote collagen hydrolysis. It appears that MMPs may work in concert. Gelatinases may indeed hydrolyze gelatin when it forms, but may also help initiate collagenase activity by being a "telopeptidase".

MMP activity can be regulated at multiple levels, e.g. transcription, secretion, degranulation of enzymes from intracellular granules, and by specific and non-specific inhibitors [25]. Tissue inhibitors of MMPs (TIMPs -1, to -4) are specific inhibitors that participate in controlling the local activities of MMPs in tissues. Most TIMPs inhibit active MMPs and some TIMPs can prevent pro-MMP activation. TIMPs are also involved in various cellular and tissue regulatory processes [43,44]. In body fluids, a macroglobulin protein, α2-macroglobulin, may be the primary regulator of MMP activity. α2-macroglobulin and fetuin-A (aka α-2-Heremans Schmid-glycoprotein, α-2-HS-glycoprotein, AHSG), are abundant plasma proteins that may be involved in the regulation of dentin MMPs [23]. There are also an ever-increasing number of synthetic MMP inhibitors. Most of them prevent MMP activity by chelating or replacing the active-site Zn^{2+}, or by "coating" the substrate.

Several MMPs have been identified in the dentin–pulp complex. During tooth development, MMPs participate in the organization of the ECM components and compartments [9,23]. Mature human odontoblasts synthesize at least gelatinases MMP-2 and -9, collagenases MMP-8 and -13, and enamelysin MMP-20 [45–49]. However, the gene expression profile is much larger, covering most of the MMP family members [50].

The first time collagenase in dentin was described was in the early 1980s, when Dayan et al. [51] demonstrated collagenolytic activity in carious and intact human dentin. The finding went by pretty much unexplained and unnoticed, which is not surprising, since at that time MMP research was still in its "adolescence" phase, as described by Lapière [52]. For example, the name "matrix metalloproteinases" for the family was only suggested in 1986 [52]. Recent studies have demonstrated the presence of at least gelatinases MMP-2 and -9, collagenase MMP-8 and stromelysin MMP-3 in human dentin [32,41,42,53–57]. MMP-20 is present in dentinal fluid [47], intense gelatinolytic activity has been observed in dentinal tubules with laser confocal microscopy [58] (Figure 5), and MMP-20 [47], -2 [59] and -9 [60] have been shown to increase in dentinal tubules of carious teeth. In mineralized dentin, MMP-2 is probably the most abundant MMP [23]. TIMP-1 and -2 are also found in human dentin [49,61,62]; α2-macroglobulin and fetuin-A are also present in marked quantities in dentin [23]. The physiological roles of MMPs in dentin is not well understood, but they have been suggested to participate in peritubular and tertiary dentin formation, and in the release

of dentinal growth factors [7,22,23,63–66]. The role of MMPs in collagen degradation in dentin caries progression has been well documented [35,37,67–71]. For a comprehensive overview of current knowledge of MMPs in dentin-pulp complex, the reader is referred to recent reviews [9,23].

Cysteine cathepsins

The lysosomal cysteine proteases belong to the clan CA of cysteine proteases; they are members of the C1 family of papain-like enzymes, the largest and the best characterized family of cysteine peptidases. There are 11 human cysteine cathepsins, namely cathepsins B, C, F, H, K, L, O, S, V, X and W [72–74]. Cathepsins B, H, L, C, X, F, O and V are ubiquitously expressed in human tissues, while cathepsins K, W and S are tissue-specific [75]. They are biosynthesized in the secretory route as inactive zymogens and are transported to endosomes via the mannose-6-phosphate receptor pathway [76]. Cysteine cathepsin zymogens are processed to the active forms either autocatalytically triggered by endosomal acidification or by other proteases [77]. The autocatalytic activation of cathepsins is substantially accelerated in the presence of anionic polysaccharides, such as GAGs and dextran sulfate [78,79]. The structure-function is best known for cathepsin B [75].

Cysteine cathepsins are active and stable in a slightly acidic pH and mostly unstable at neutral pH. They can be irreversibly inactivated at neutral pH [80], except cathepsin S which is stable at neutral or slightly alkaline pH [81]. Cathepsin B has mainly peptidyl-dipeptidase and carboxypeptidase activity [73,82,83]. On the other hand, cathepsin B endopeptidase activity has a pH optimum around 7.4 [84,85], which is expectable for an enzyme that is involved in many physiological processes related with extracellular matrix degradation. Unlike other cysteine proteases, cathepsin B has a flexible loop that partially occludes the active site aperture of mature enzyme and carries pH-sensitive arginine and histidine residues, and appears to play crucial role in modulating its endo- and exopepditase activities [86,87].

Cysteine proteases are often termed promiscuous proteases or those with broad substrate specificity [88]. Physiological substrates for cathepsin B, and others, are still a matter of speculation. However, cathepsin B degrades extracellular matrix components such as type IV collagen and fibronectin under both acidic and neutral pH [89].

Although cysteine cathepsins were initially considered as intracellular enzymes responsible for the non-specific proteolysis in the endosomal/lysosomal compartment, this view is rapidly changing [75]. In some (non-dental) cell types, the secretion processes of cathepsins are very well elucidated. Cathepsin B can be found in lysosomes [87] or secretory vesicles [76], or may follow the default pathway of secretion [90]. Furthermore, cathepsin B is consistently found associated with the plasma membrane of tumor cells [91]. Although lysosomes were for a long time considered to be dead-end organelles, recent developments show that these organelles or their contents in shuttle vesicles can move back and forth along microtubules rendering possible exocytosis or retrograde transport to endosomes [92].

Several cathepsins participate in bone resorption and remodeling. The most potent mammalian collagenase is cathepsin K which, in contrast to the cathepsins B, L and S, is highly expressed in osteoclasts [93] and odontoclasts [94], playing a special role in mineralized tissue resorption under normal and pathological conditions [95,96]. Indeed, in the absence of cathepsin K, osteoclasts are hampered in their capacity to digest bone collagen [97]. The biological relevance of the collagenolytic activity of cathepsin K was underlined by the finding that deficiency in this protease causes the bone-sclerosing dysplasia, pycnodysostosis, in man [98] and an osteopetrotic phenotype in mice [99]. When

osteoclasts demineralize bone matrix by lowering the pH in the resorption lacuna, the acidic environment favors the extracellular collagenolytic activity of cysteine proteases, especially cathepsin K. Later, when the pH has increased due to an increased amount of phosphate and calcium, the MMPs digest the rest of collagen [100]. Low pH around joint implants also leads to demineralization and type I collagen degradation in peri-implant bone; the acid- and cathepsin K-driven mechanisms of periprosthetic bone resorption have been described in context with loosening of hip implants [101].

Recent data have shown that odontoblasts and pulp tissue have coding DNA sequences (CDS) of most cathepsins [102], suggesting that, in terms of protein expression, the variety of cysteine cathepsins present in dentin-pulp complex can be compared to that described for MMPs [50]. Cysteine cathepsin activity has been demonstrated both in intact [102] and carious [37] dentin, and cathepsin B has been localized in dentinal tubules [102]. Once synthesized by odontoblast and/or pulp tissue, secreted cathepsins can easily reach the dentinal tubules and enter deep into dentin [102] (Figure 6). Moreover, changes in expression levels, localization and activity have been described in carious dentin [37]. The significant increase of cysteine cathepsin activity in carious dentin with increasing depth (approaching the pulp) indicates that odontoblast- or pulp-derived cysteine cathepsins may be important in active caries lesions, especially with young patients [37]. Together these findings indicate that cysteine cathepsins may have an important role during dentinal caries development. Since clinical restorations are practically always made on carious teeth with at least some degree of pulpal inflammation, it is possible that increased levels of cysteine cathepsins are present also in dentinal tubules under the restoration.

The increased expression and/or activity of cysteine cathepsins often coincide with their presence in the extracellular environment, confirming the hypothesis that pH is not sole factor responsible for their activity. Cathepsins with high collagenolytic activities are known to be mainly responsible for remodeling the extracellular matrix [75]. The extracellular matrix consists mostly of collagen and proteoglycans, which have all been identified as the cathepsin substrates [103]. Cathepsin K is the only cysteine cathepsin capable of cleaving the collagen at the triple helical region [104,105]. However, gelatin can be cleaved by several cathepsins [106] and cathepsins B and L cleave in the non-helical telopeptide extensions of collagens [107,108]. Cathepsins L and S can release GAGs from cartilage proteins such as aggrecan [109]. PGs form interfibrillar bridges that absorb water with their negatively charged GAG side chains in dentin collagenous matrix [11,23], and GAGs play a crucial role in the formation of complexes between cysteine cathepsins and their protein substrates. This will be discussed in more detail in the next section.

The interplay of dentin proteolytic enzymes and non-collagenous proteins

Since the role of MMPs in dentin pathologies first emerged less than 15 years ago [35,45], and cysteine cathepsins were detected in dentin only two years ago [37,102], it is obvious that not much is known about their potential interactions in dentin formation, physiology, or diseases, let alone in the loss of collagen matrix in the hybrid layer. However, the exceptionally wide expression profile of MMPs [50] and cysteine cathepsins [102] in mature human odontoblasts strongly indicate that the enzymes must have various tasks in the formation and maturation of mineralized dentin. This kind of wide-scale expression of two extracellular matrix–degrading enzyme families in specific tissue is quite unique, especially the cells that in general are not considered to participate in tissue remodeling under physiologic conditions. Most of the potential interactions between MMPs and cysteine cathepsins in dentin are still based on the research with other tissues, and must be considered speculative until more work has been performed in dentin.

In cartilage collagen, cathepsin B and MMPs have been suggested to have at least partially independent pathways in cartilage proteoglycan breakdown [110]. Cysteine cathepsins are able also to activate MMPs [111]. Cathepsin B from articular chondrocytes increases MMP levels and stimulates angiogenesis by proteolytic inhibition of TIMPs [112]. Moreover, procathepsin B can be activated by active MMPs [113], and conversely, cathepsin B has been shown to be responsible for activation of MMP-1 in gingival fibroblast cultures [114]. This scenario establishes a possible and reasonable conclusion that both class of proteolytic enzymes, cysteine cathepsins and MMPs, are participating synergistically in bone resorption and make cathepsin B an attractive candidate for the potential cathepsin-MMP interplay also in dentin-pulp complex (Figure 7A,B). Identification of different cathepsins at the protein level in dentin may, in the future, better elucidate the potential cathepsin-MMP interactions also in the degradation of the hybrid layer collagen.

Several MMPs (at least MMP-1, -2, -7, -8, -9 and -13) can bind to PGs and/or GAGs which may affect their binding properties, regulate enzyme activity but also protect them from auto- or other activation [115–118]. For example, pro-MMP-9-PG heteromers bind to type I collagen as well as gelatin *in vivo*, while monomeric MMP-9 has much greater affinity to gelatin [117]. Interestingly, the organomercurial compound p-aminophenylmercury acetate (APMA) does not activate pro-MMP-9-PG complex, commonly used MMP activator [117]. Instead, Ca^{2+}, known to stabilize MMP-9 and other MMPs but not induce proenzyme activation, induced an autoactivation of the pro-MMP-9 in the complex and release of activated enzyme from the complex [115]. Since the weak response of dentinal MMPs to APMA-activation is a frequent finding [42,119–121] and calcium is known to be needed for demineralized dentin autolytic degradation [121,122], it is tempting to speculate that MMP-PG/GAG complexes would also be present in dentin matrix, where MMP-3 might participate in its activation by cleaving the heteromer PG [54]. Due to these unique characteristics, pro-MMP-9-PG heteromer (and other MMP-PG/GAG complexes) may have fundamental physiological importance in spite of their presumably low relative amounts in tissues [117], because only catalytic amounts of the enzyme are required to digest physiological targets. GAGs also participate in the regulation of cysteine cathepsin activity in a very complex manner. GAG activation (e.g. [123]) and stabilization [124] of cathepsin (and possibly MMPs [116]), allows functional activity of cathepsins even in neutral pH. GAGs are able to accelerate the conversion of zymogen forms of cysteine cathepsins into mature forms at neutral pH, activating cathepsin B [123], cathepsin L [125], cathepsin S [106,126,127] and congopain [128]. The presence of GAGs in the dentin [129] and their release during acid-demineralization and subsequent proteolytic degradation of acid-demineralized dentin matrix [130] supports the possibility that GAGs are involved in *in vivo* processing of cysteine cathepsins in dentin. Interestingly, procathepsin S is capable of autocatalytic activation not only at acidic pH but also at neutral pH in the presence of related GAGs [78]. Also, cathepsin B is stabilized at pH 7.4 by interaction with heparan sulfate and heparin GAGs [124]. Of the several GAGs that can complex with cysteine cathepsin K, only chondroitin sulfates and keratan sulfates (both present in dentin [129]) (Figure 8) induce its collagenolytic activity; and chondroitin sulfates can accelerate the procathepsin B processing into active form [79]. With other cysteine cathepsins, the effect is inhibitory [106,127], or they may participate on cellular trafficking of cysteine cathepsins [131]. This highly selective inhibition/activation mechanism has been suggested to be important in the control of collagen turnover [127] (Figure 7C).

In vivo, the dentinal face of the adhesive interface is bathed in dentinal fluid, and the permeability of simplified polymerized adhesives has been long recognized [132]. The enzymes dissolved in dentinal fluid may thus participate in the degradation of hybrid layer collagen, especially *in vivo* (Figure 7D). Using the *in vitro* thick-slice human tooth model, Lehmann and others [133] demonstrated an increased MMP-2 protein expression in the

odontoblasts when the overlying dentin was treated with SE adhesive-bonded restoration. The authors concluded that odontoblasts may participate into hybrid layer degradation by increasing enzyme synthesis [133]. Boushell and others [59] demonstrated a significant increase in MMP-2 immunoreactivity in the caries-affected tubules, regardless of the level of caries severity. MMP-13 is expressed in high levels in odontoblasts and pulp tissue both in healthy and carious teeth [48,134]. MMP-1, -2 [135] and MMP-9 [136,137] levels increase in inflamed pulp, and MMP-9 has been detected in dentinal fluid of teeth with pulpitis but not in healthy teeth [60]. Polymerized adhesive disks increase MMP-2 production in pulpal fibroblasts in culture [138]. Cysteine cathepsin B is present in dentinal tubules [102] (Figure 6), and odontoblast- or pulp -derived cysteine cathepsins, delivered via dentinal fluid, have been suggested to have an important role in dentinal caries [37]. The previous data indicates that at least MMP-20 may also be functional in dentinal caries matrix degradation [47]. Since teeth are almost always restored because of caries, usually accompanied with at least mild local pulpitis, the influx of odontoblast- or pulp tissue-derived enzymes may be significant factor in the degradation of hybrid layer *in vivo*. However, the presence of resin tags in dentinal tubules may prevent the outward diffusion of cysteine cathepsins or MMPs from the pulp into peripheral dentin.

Enzymatic degradation of dentin collagen

At the turn of the millenium, morphologic evidence began to emerge from *in vivo* studies of resin elution or degradation of collagen matrices in aged resin-dentin bonds [1,139–141]. In 2003, Hashimoto and co-workers [142] demonstrated the degradation of acid-etched dentin collagen in water storage for 500 days *in vitro*. While the role of MMPs in the progression of dentin matrix of caries lesion had been suggested earlier [35,47,64,67,68], the first direct evidence of this occurring also in non-carious dentin collagen came when Pashley and co-workers demonstrated that dentin demineralized by acid etching slowly degrades in the absence of bacteria [143]. Collagen matrices incubated in artificial saliva were almost completely degraded within 250 days, while those stored either in artificial saliva containing enzyme inhibitors or mineral oil were practically intact [143]. The study also confirmed the intrinsic collagenolytic activity of dentin matrix indicated in the early paper by Dayan et al. [51]. After this groundbreaking report there have been several articles using different techniques that confirm that collagenolytic enzymes are present in human mineralized dentin, they can be active after demineralization with various methods, and that they have a role in the degradation of the hybrid layer collagenous matrix.

Biochemical techniques demonstrating the degradation of dentin collagen by extrinsic or intrinsic proteases

While Pashley et al. [143] clearly demonstrated the time-related collagen destruction with TEM, quantitative techniques to measure and quantitate the actual level of degradation have been used. Quantitative methods allow to test and compare different enzyme inhibitors within reasonable time frame (e.g. four weeks) and evaluate their potential before time- and labor-consuming *in vitro* or *in vivo* bond strength testing. These techniques include measuring the release of hydroxyproline (HYP) in hydrolyzates of media or C-terminal telopeptides (ICTP, CTX) into incubation medium.

The rationale for evaluating collagen degradation by means of HYP assays relies on the fact that type I collagen contains about 10 mass % HYP, but most other proteins contain little or none of this amino acid [144]. During type I collagen degradation, the release of HYP from the incubation solution can be measured with colorimetric methods or HPLC. Urine HYP has been used as a marker of bone resorption, but due to its lack of sensitivity and specificity to bone collagen and availability of more sensitive and specific methods, there is little clinical use of HYP any more [145]. With dentin, HYP release analysis was used for the first

time by Veis and Schlueter in 1964 [146] in their classical study characterizing dentin collagen. Carrilho and others [121] analyzed the effect of CHX on HYP release, along with modulus of elasticity, of demineralized dentin beams. Thirty min preincubation of demineralized dentin beams in 2% CHX resulted with significantly reduced HYP release compared to the controls, indicating decrease in collagen degradation. In addition, no changes in modulus of elasticity occurred in CHX-pretreated samples, while with controls a significant and time-dependent decrease was evident already after one week incubation in artificial saliva [121]. The study further confirmed the enzyme-inhibiting activity of CHX, but also demonstrated that HYP release can be used to quantitate collagen degradation in demineralized dentin. The method has since been used in several studies with different approaches to inhibiting demineralized dentin collagen degradation [122,147–150].

Matrix metalloproteases (MMPs)-1, -8 and -13 are true collagenases. They attack native collagen at a specific peptide bond and cleave collagen molecules into a ¼ and ⅓ segment [39]. A larger number of MMPs including -2, -9 and -13 can attack type I collagen telopeptides to release a long C-telopeptide segment called ICTP [38]. This segment includes at least two cross-linked telopeptides and the first phenylalamine of the phenylalamine-rich region. When MMPs-2, -9, -13 and -14 were added to partially demineralized human bone powder, ICTP telopeptide fragments were formed *in vitro* but they did not release the smaller eight amino acid C-terminal peptide, CTX [38]. However, when those authors incubated exogenous cathepsin K with demineralized bone powder, the major telopeptide that was released was CTX. No CTX was released from bone powder incubated with MMP-2, -9, -13 or -14 [38]. All MMPs (at least the above-mentioned MMPs-2, -9, -13 and 14 [38]) and cysteine cathepsins (at least cathepsins B and L [107,108,151]) that release ICTP or CTX telopeptide fragments from type I collagen would be called telopeptidases rather than collagenases. Cathepsin K is a unique protease in that it is both a telopeptidase and a collagenase. It can cleave telopeptides as well as the helical region of collagen [39].

Transmission electron microscopy

Transmission electron microscopy (TEM) is a commonly used and suitable method to examine the hybrid layers because of the small size (50–100 nm wide) of collagen fibrils and because the structural integrity can usually be evaluated by the characteristic banding of collagen. Also the interfibrillar spaces that are only approximately 20 nm wide can be seen. Numerous experiments both *in vitro* and *in vivo* have demonstrated the loss of the hybrid layer collagen structure in aged samples [2,4,5], in which collagen network and structure appear more or less disorganized or even lost, leaving empty spaces to be filled with sample embedding resin (Figure 9). Comparison of the samples prepared for TEM immediately or after aging, or comparing the control and experimental groups (as in Figure 9), offers a powerful tool to evaluate the effect of aging on the hybrid layer integrity. Similarly, TEM is frequently used to evaluate the effect of enzyme inhibition on the hybrid layer integrity. Figure 9 demonstrates the dramatic differences in the hybrid layers of the control and experimental (CHX-treated) primary teeth after six months in service *in vivo.* This was the first study that directly indicated the clinical efficacy of enzyme inhibition on hybrid layer preservation [152].

Few investigations of resin-dentin bonds use TEM because it is technically much more demanding than SEM. The ultrastructure of dentin hybrid layers was first described by Van Meerbeek et al. [153–155] and by Tay et al. [156–158]. They showed that the collagen fibrils exhibited 67 nm periodicity, took up heavy metal TEM stains and had 20 nm interfibrillar spaces between the collagen fibrils, presumably filled with infiltrated resin. Armstrong and co-workers [159] were the first to carefully examine such hybrid layers in resin-bonded dentin that had been stored in water for 5 years. Their TEMs revealed that

almost 70% of the collagen fibrils had disappeared! They had a "ghost-like" appearance. The other 30% of the hybrid layers appeared to be normal in TEM appearance with 67 nm cross-striations that stained well with heavy metals. This was the first solid evidence of why resin-dentin bond strengths fell over time. The collagen fibrils in the hybrid layer that anchor the overlying adhesive resin and composites to the underlying mineralized dentin had been destroyed. Such collagen fibrils are the only continuous structural elements connecting adhesive fillings to underlying dentin. Their loss coincided with decreases in measured bond strength [160,161].

This ultrastructural observation was an important breakthrough. The presence of MMPs in dentin had been reported by Martin-De Las Heras et al. [41] in a biochemical study. They extracted MMP-2 from normal dentin. This was well-received by developmental biologist who confirmed the observation and explained the relevance of MMPs in embryological development. This observation was completely ignored by restorative dentists who had little interest in developmental biology.

Effect of adhesives on purified and dentin matrix-bound enzymes

The use of 35% phosphoric acid (pH 0.7 to -1), to acid-etch dentin in the E&R approach, was initially thought to inactivate pro-MMPs trapped within the mineralized dentin due to its low pH which was believed to denature MMPs [143]. The subsequent application of resin monomers constituting the adhesive blends of either E&R [120] or SE adhesives [119] have been shown to reactivate the quenched collagenolytic/gelatinolytic activities of the endogenous enzymes. This phenomenon occurred both in coronal [119,120] and in radicular dentin [162]. The studies were performed to detect gelatinolytic/collagenolytic enzyme activities within the dentin powder before and after the sequential application of the phosphoric acid-etchant and an E&R adhesive [120] or treating mineralized dentin with a SE adhesive (i.e. the solid phase) [119,162]. These data were obtained using powdered dentin on internally quenched fluorescent collagen/gelatin substrate.

This experimental setup clearly revealed a specific gelatinolytic activities of the dentin bonding system/dentin powder mixtures, but they did not clarify which type of endogenous enzymes (MMPs, cathepsins or others) were involved in the degradation process. Thus such activity could have not been related to a specific enzyme. For this reason, more recently, different efforts have been devoted to identify the specific MMPs activated by the bonding agents, using immunohistochemical, gel electrophoresis, and confocal laser fluorescence techniques. With field emission in-lens-scanning electron microscopy (FEI-SEM) and TEM MMP-2 and -9 [55] and MMP-3 [57] were localized in partially EDTA-mineralized dentin (Figure 10). In particular correlative biochemical and immunohistochemical approaches at TEM and high resolution-SEM with MMP-2 immunolocalization in mineralized dentin, phosphoric acid-etched dentin and adhesive-treated acid-etched dentin support the hypothesis that MMP-2 may be one of the most relevant dentin-bound MMPs involved in hybrid layer degradation [163]. MMP-2 was found to be entrapped within the hybrid layer created by a two-step E&R adhesive system and aged in artificial saliva for 12 months. The amount of MMP-2 available for detection varied as a function of dentin treatment, being minimal in 0.2% CHX-treated samples [163]. The colorimetric antibody-specific assay allowing direct measurement of specific protein levels (QuantiSir) confirmed the electron microscopy findings. Interestingly, MMP-2 expression and activity varied depending on the step of the bonding procedure: while phosphoric acid etching reduced the detection level by 43% compared to mineralized dentin, treating the acid-etched dentin with SB 1XT increased the detection by 58% compared to mineralized dentin and by 179% compared to acid-etched dentin [163]. The results support the previous finding that adhesives can reactivate the enzymes present in dentin after acid etch etching [120]. Furthermore, pretreatment of acid-etched dentin with 0.2% CHX before the adhesive application decreased detection level by

27% [163], demonstrating the binding of CHX to the enzyme in demineralized dentin matrix and its inhibition.

These results were further confirmed using zymography assays performed on protein dentin extracts after different treatments of dentin powder. In gel zymography, gelatin is copolymerized in an SDS-PAGE gel as a substrate. When the protein extracts are run into the gel, proteins are separated by their molecular weight. The gel is then incubated in activation and incubation solutions, allowing gelatinolytic enzymes to degrade the gelatin in the gel, and the proteins can be detected and identified by the location of the bands in the gel [164]. While no enzymatic activity was found in proteins extracted from mineralized dentin, zymograms of phosphoric acid-demineralized dentin extracts showed bands at 66 kDa (MMP-2 active form), a fainter band at 86 kDa (active form of MMP-9) and other minor gelatinolytic bands with lower molecular weights [165]. Zymograms of adhesive-treated dentin showed an intense increases both in active (band at 66 kDa) and proform of MMP-2 (band at 72 kDa), together with a slight increase in MMP-9 (fainter band at 86 kDa) [165]. However, when SE adhesives were used, the zymographic assay was unable to reveal enzyme activation similar to what could be obtained with E&R adhesives [166,167].

Despite all these findings however, no assay has been able to evaluate the proteolytic activity in hybrid layers *in situ* due to the intrinsic difficulties to detect, precisely localize, and measure enzyme activities within the tissues, because zymography requires extraction of the proteolytic enzymes from the tissue prior to analysis. Recently Mazzoni et al. [58] using a correlative approach with an ELISA assay and *in situ* zymography, quantified and localized the MMPs activity within the hybrid layer created by an E&R adhesive. This was performed using quenched fluorescent-labeled gelatin showing intense and precise fluorescent localization indicating enzyme activity within the hybrid layer [58] (Figure 11). This may also be due to the presence of cysteine cathepsins [102] derived from the dental pulp via dentinal fluid and possibly activated by adhesive resin monomers.

The intense enzymatic activity reported along the dentinal tubules [58] also supports the hypothesis shown by Lehmann et al. [133] that a SE adhesive can increase MMP-2 synthesis in human odontoblasts, possibly increasing MMP-2 penetration into the hybrid layer via dentinal fluid. Similarly Orsini et al. [138] showed that unreacted monomers eluted from polymerized adhesives are capable of inducing human pulp fibroblasts to overexpress MMP-2.

Conclusions

Identification of the presence and activity of dentinal enzymes has provided basic knowledge that allows us better understand their roles in dental hard tissue pathologies. Matrix metalloproteinases and cysteine cathepsins in mineralized dentin and dentinal fluid have been shown to contribute to dental caries [35,67,68] and erosion [168,169], but the field with most interest has been the enzymatic degradation of the composite adhesive hybrid layer collagenous matrix. This, in turn, has already led to several experimental strategies that aim to improve the durability of the resin-dentin bond strength by inhibiting the enzymatic degradation of collagen in the hybrid layer. Even though work remains to be done e.g. to identify the key enzymes and to find the optional means of inhibition that would not affect immediate bond strength but would still retain the effect for years, the currently available data already holds excellent promise that stable resin-dentin bonds will be routinely available in a daily clinical setting already in a near future.

Acknowledgments

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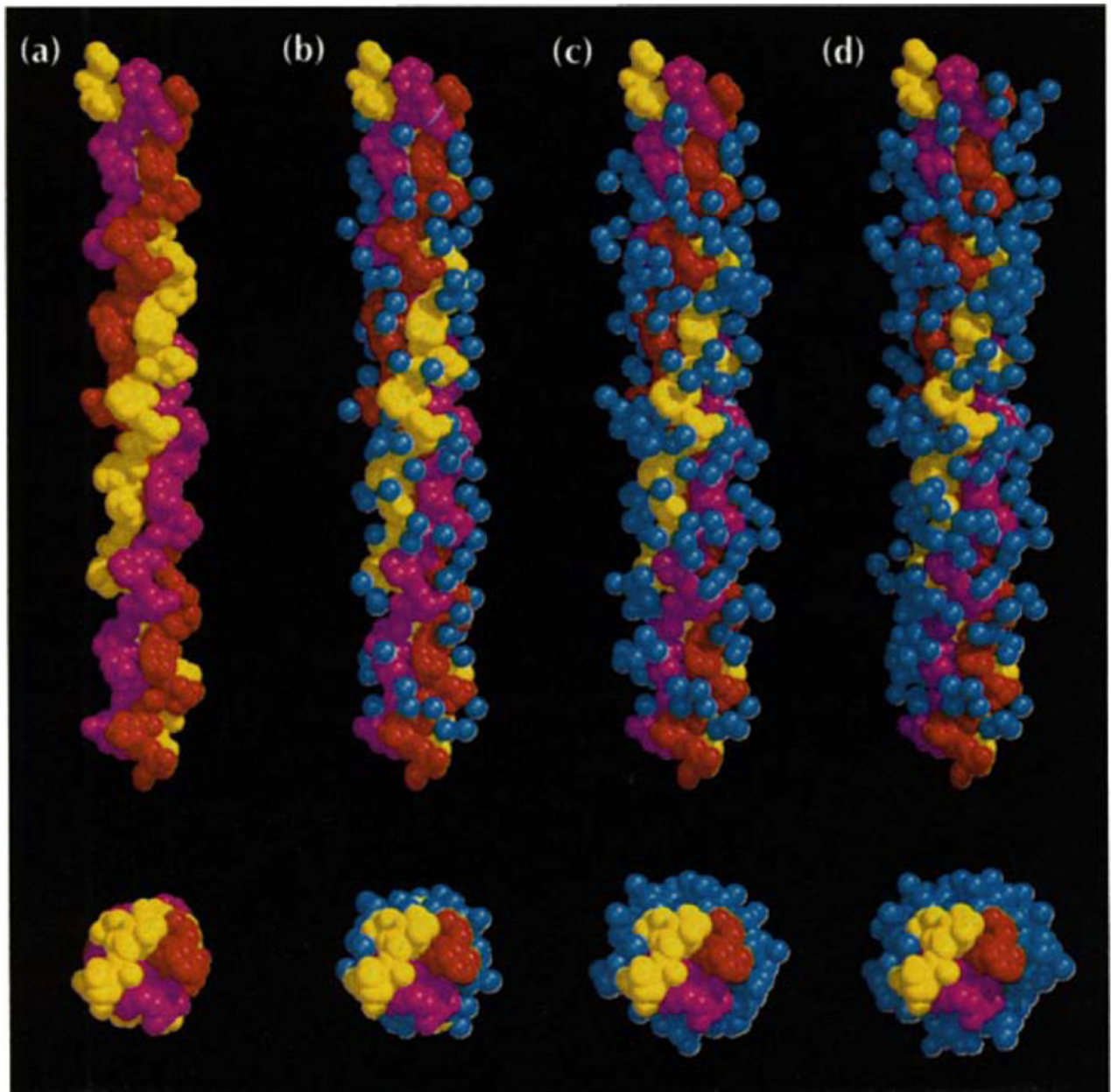170. Nakabayashi, N.; Pashley, DH. Hybridization of Dental Hard Tissues. Chicago: Quintessence Publishing; 1998.

Figure 1.
Representation of the progressive hydration of the collagen Gly-Ala peptide. Top row presents the perpendicular and the bottom row parallel view to the molecular axis at the same hydration level. (a) A view of the non-hydrated collagen, with the three peptide chains shown in different colors. (b) The first shell of water molecules (blue spheres), directly hydrogen-bonded to carbonyl, hydroxyl or amide groups on the peptide surface. (c) The second shell of water molecules, hydrogen bond to the water in the first shell, demonstrating the filling of the superhelical groove. (d) The third shell of water molecules. (Reproduced from [8] with permission.)
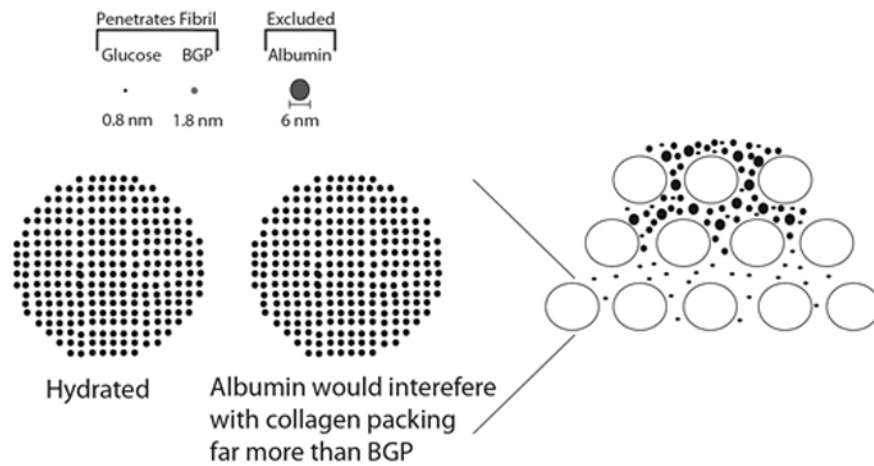
Figure 2.
Schematic of parallel collagen fibrils in demineralized dentin cut in cross-section to show the size of interfibrillar spaces. These spaces are not empty but contain proteoglycan hydrogels that may act as molecular sieves. Many dental adhesives are mixtures of comonomers. The large black dots on the right indicate dimethacrylates like BisGMA, while the smaller black dots indicate HEMA. BisGMA seems to only penetrate the top half of hybrid layers (reproduced from [170], with permission).

The schematic on the left shows that the interior of collagen fibrils is made up of hundreds of collagen molecules separated by water-filled spaces about 1.8 nm wide. Albumin, with a molecular diameter of 6 nm cannot enter "collagen water" but would be excluded as being too large (MW 68 kDa). Glucose (MW 180 Da), BGP (bone Gla protein or osteocalcin, MW 5700 Da) can equilibrate with collagen water even though the molecular diameter of osteocalcin is the same as the width of intermolecular spaces (1.8 nm in both cases) because collagen molecules are in constant rotational vibration (modified from [15]).
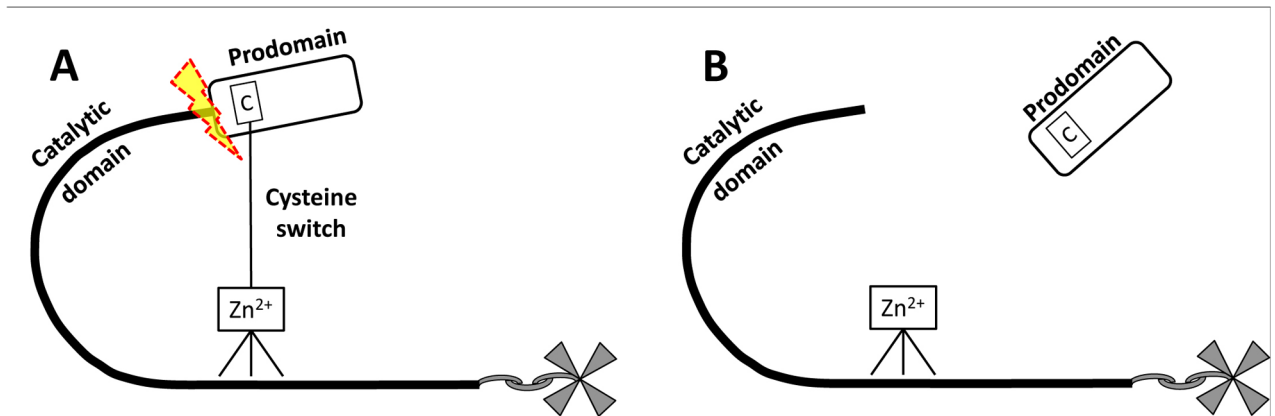
Figure 3.
A conserved cysteine residue (C) in the pro-domain coordinates with the Zn^{2+} ion at the functional site of the catalytic domain. The pro-domain is removed by cleavage in the pro-domain and between the pro-domain and the catalytic domain. The "propeller" is a hemopexin domain contained by most MMPs, and is attached to the catalytic domain by flexible hinge domain. The hemopexin domain e.g. mediates protein–protein interactions, contributes to substrate recognition, enzyme activation, and protease localization. Modified from [25]).
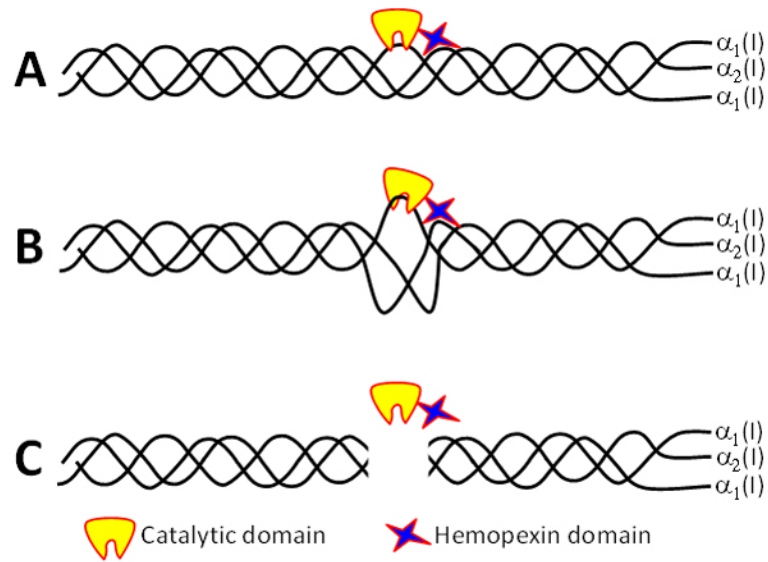
Figure 4. Steps involved in collagenolysis by collagenase
A) Collagenase bound to triple helical collagen via cooperative interaction of catalytic and hemopexin domain, but is unable to cleave in its native state due to the smallness of the cleft of the active site.
B) Unwinding the triple-helical conformation allows single α-chain to be presented to the active site of the catalytic domain for peptide hydrolysis.
C) All three α-chain are successively hydrolyzed, resulting with collagen molecule to be cut into ¼ and ⅓ fragments. (Adapted from [27]).
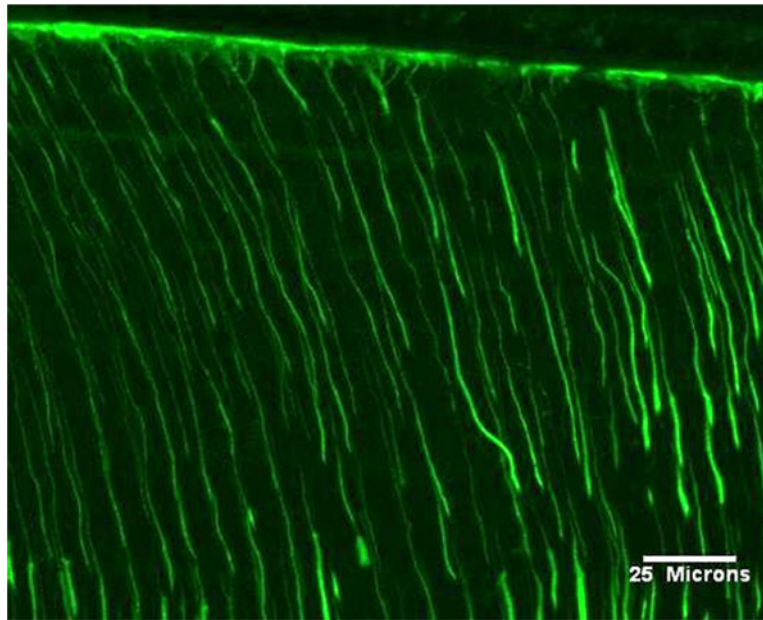
Figure 5.
Gelatinolytic activity in dentinal tubules (green fluorescence) and in the hybrid layer at the top of the tubules of intact human tooth as seen with confocal fluorescence microscope.
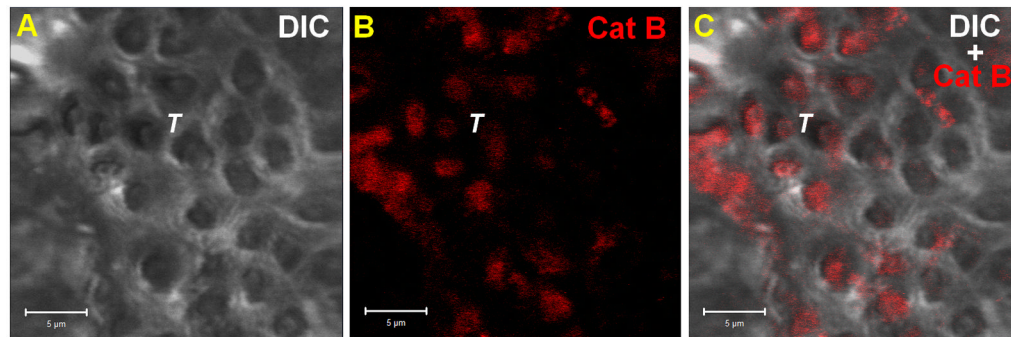
Figure 6.
Cathepsin B in dentinal tubules. Dentin tissue cross-section visualized by Differential Interference Contrast (DIC) (**A**). Intratubular Cathepsin B were immunolabeled using rabbit anti-human cathepsin B as primary antibody and mouse anti-rabbit IgG conjugated with Alexa Fluor 594® as a secondary antibody at the red channel (**B**). Merged Images (**C**). T = Tubules and scale bar = 5μm.
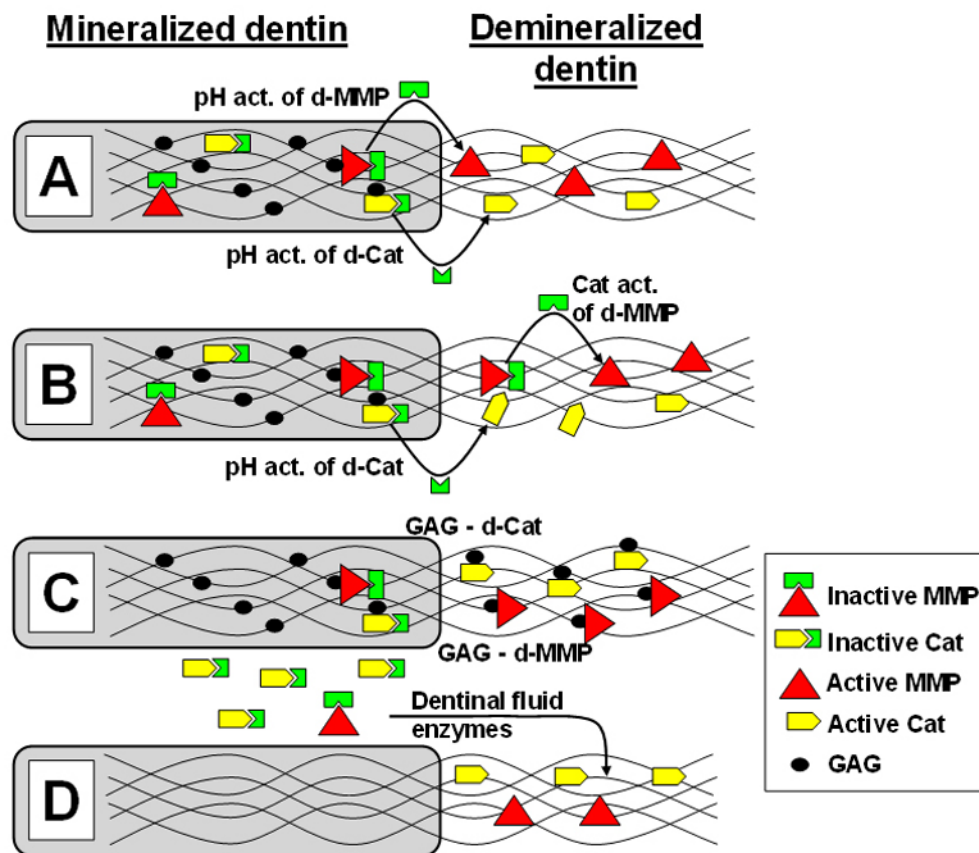
Figure 7.
Schematic presentation of possible mechnisms of activation and function of cysteine cathepsins and MMPs in dentin (modified from the Online-only Appendix 2 in [37]). Green blocks with MMPs (red triangles) or cathepsins (yellow arrowheads) indicate inactivity of enzyme either as a proform or in complex with specific (TIMPs, cystatins) or non-specific (e.g. α2 macroglobulin) inhibitors. Activation of protein (removal of green blocks) may represent either elimination of inhibitor, transform from latent to active form, or both. Glycosaminoglycans (GAGs) in dentin may also affect the enzyme activity either by activation or inhibition, depending on enzyme and GAGs in question [106,127].
A) pH changes caused by etching acid or acidic monomers convert dentin-bound proMMPs and/or MMP-TIMP complexes (d-MMP) into active MMP. Respectively, dentin-bound cathepsin (d-Cat) becomes active in acidic pH.
B) At least cathepsin B directly cleaves and inactivates MMP-specific tissue inhibitors TIMP-1 and TIMP-2 [36], changing the balance between MMPs and their inhibitors. Acidic pH activates cysteine cathepsins, which in turn either proteolytically activate proMMPs or degrade TIMP inhibiting MMP, or both, resulting with active MMPs and functional activity after neutralization of pH.
C) Glycosaminoglycan (GAG: black dot) activation and stabilization of cathepsin (and possibly MMPs), allowing functional activity of cathepsins even in neutral pH.
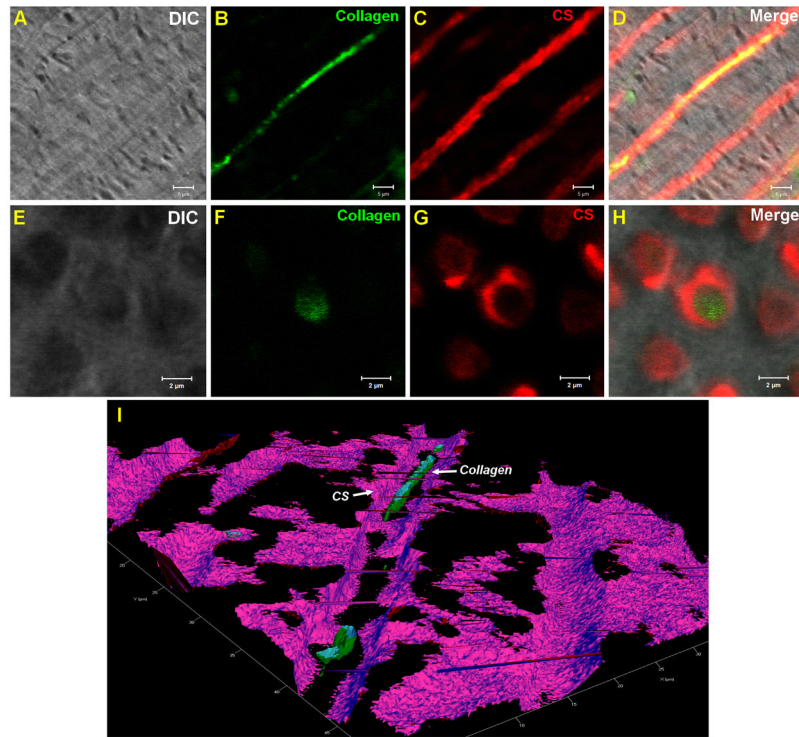**D)** Odontoblast- or pulp-tissue derived enzymes may enter the hybrid layer.

Figure 8.
Collagen and chondroitin 6-sulphate distribution in dentin. Dentin tissue was visualized by confocal microscopy in longitudinal- (**A, B** and **C**) and cross-section (**D, E** and **F**). Dentin morphology was shown by Differential Interference Contrast (DIC), (**A** and **E**). Molecularly well- structured collagen was visualized by its intrinsic fluorescence at the green channel (**B** and **F**). Chondroitin 6-Sulphate were immunolabeled using rabbit anti-human chondroitin 6-sulphate as primary antibody and mouse anti-rabbit IgG conjugated with Alexa Fluor 594® as a secondary antibody at the red channel (**C** and **G**). Colocalization between collagen and chondroitin 6-sulphate can be seen as the yellow color formed by the overlapping channels green and red in merged images (**D**) or interacting in the tubular area (**H**). The three-dimensional model was constructed from the upper panel's images and shows how collagen and chondroitin 6-sulphate molecules are anatomically distributed on dentinal tissues (**I**). CS = Chondroitin 6-Sulphate; scale bars = 5 μm (**A**, **B**, **C** and **D**) or 2 μm (**E**, **F**, **G** and **H**).
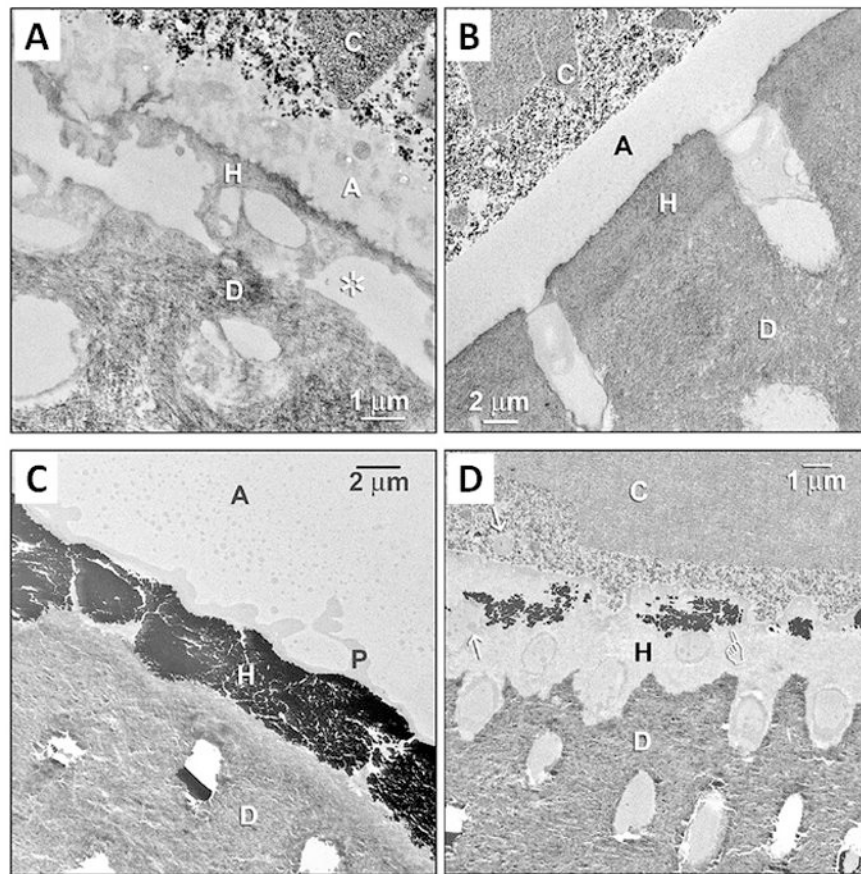
Figure 9.
TEM analysis of hybrid layer in primary molars restored six months earlier without (controls) or with 2% CHX pretreatment (experimental group), using 2-step E&R adhesive and composite resin. **A)** Demineralized section of the control tooth hybrid layer stained with phosphotungstic acid and uranyl acetate. The hybrid layer (H) is almost completely degenerated and partially even missing, with empty regions occupied by epoxy resin (asterisk). C: resin composite; A: adhesive; H: hybrid layer; D: dentin. **B)** The section respective to **A** from the experimental (i.e. CHX-treatment) group. The hybrid layer (H) appears normal. **C)** Undemineralized, unstained, silver-impregnated section from the control tooth demonstrates a degraded hybrid layer with extensive leakage seen as black silver deposits that almost completely fill the hybrid layer. P, polyalkenoic acid copolymer. The silver filled the water-filled spaces that replaced collagen fibrils. **D)** An experimental tooth pretreated with CHX, with sparsely distributed silver deposits (pointer) in the hybrid layer. Arrows: polyalkenoic acid copolymer that is characteristic of 3M-ESPE adhesives. (Reproduced from [152], with permission.)
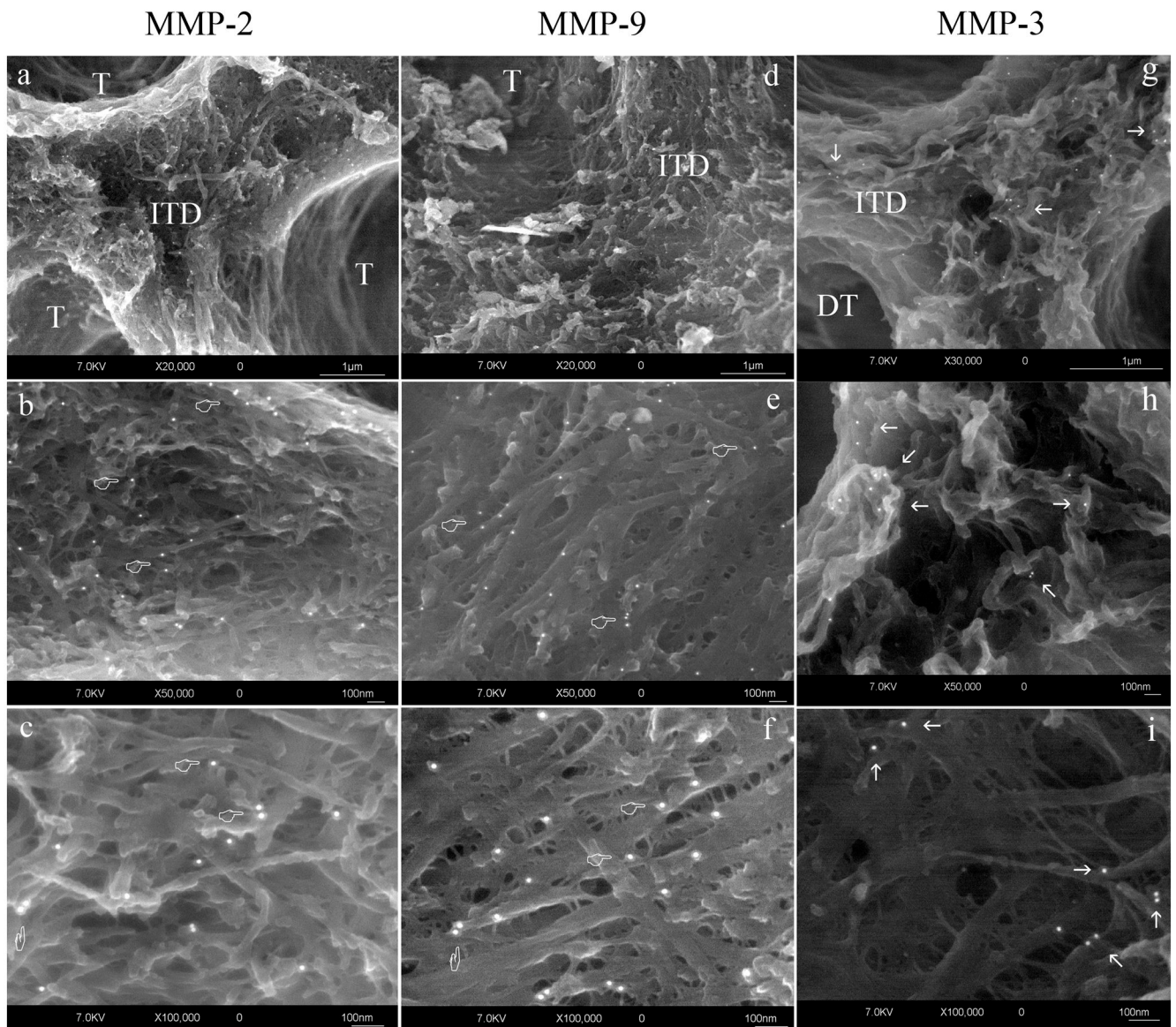
MMP-2   MMP-9   MMP-3

Figure 10.
Field emission in-lens SEM (FEI-SEM) micrographs of unfixed, partially decalcified dentin after a pre-embedding immunolabeling with antibodies for MMP-2, -9 and -3. Labeling is seen as electron-dense white spots (pointers). (**a,d**) Low magnification view (20 000x) of the partially decalcified dentin surface showing open tubular orifices (T) surrounded by a thick collar of organic matrix and intertubular porous dentin (ITD). MMP-2 and -9 labeling mainly localized in peritubular dentin. (**b,e**) A higher magnification (50 000x), showing MMP-2 (**b**) and -9 (**e**) along the collagen fibrils. (**c,f**) High magnification (100 000x) FEI-SEM micrographs, revealing the relationship between MMP-2 and -9 and the collagen meshwork. The specimen shows a moderate labeling for MMP-9 (**e**), which is uniformly weaker than MMP-2 staining (**c**). (**g–i**) FEI-SEM micrographs of partially decalcified dentin after a pre-embedding immunolabelling an antibody for MMP-3. (**g**) Low magnification (30 000x) showing a patent dentinal tubule (DT) and the porous intertubular dentine (ITD). MMP-3 is mainly localized within the intertubular dentine. (**h**) Higher magnification (50 000x) showing MMP-3 located along the collagen fibrils. (**i**) High magnification FEI-SEM

micrograph (100 000x), revealing MMP-3 distribution within the intertubular dentine. (Figures a–f reproduced from [55] and g–i from [57], with permission).
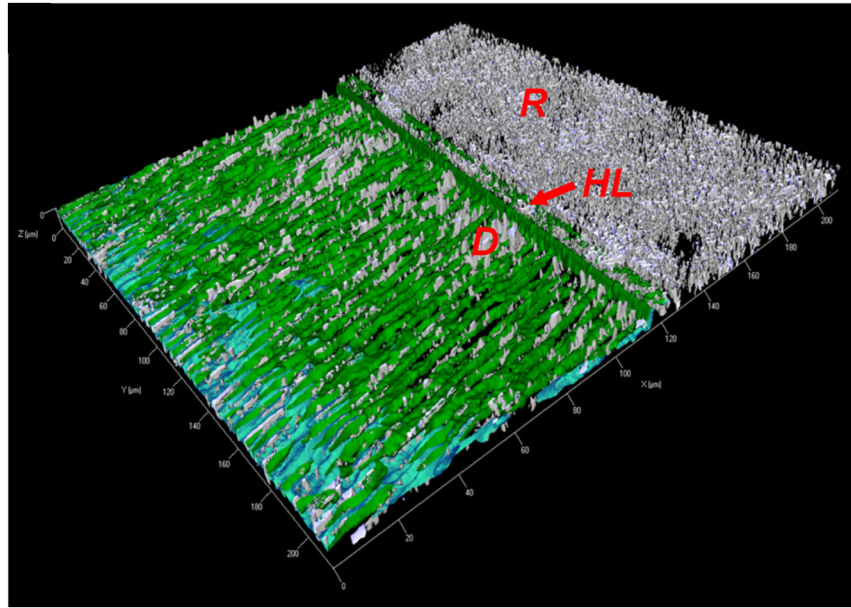
Figure 11.
Three-dimensional model of the acquired image using *in situ* zymography with the cross-section of composite resin (R), hybrid layer (HL: arrow) and dentin (D). In *in situ* gelatin zymography, samples are overlayed with quenched florescent-conjugated gelatin gel, and any gelatinolytic activity in the sample releases fluorescence (green color). Intense gelatinolytic activity is present in dentinal tubules and especially at the bottom of the hybrid layer as a 1- to 2-µm-thick, well-defined layer. (Reproduced from [58], with permission).