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Direktor: Prof. Dr. med. dent. Reinhard Hickel

Bioactive agents and their effect on dentin bond strength: Considerations for clinical applicability – an in vitro study

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Berichterstatter:	Prof. Dr. Dipl. Ing. Nicoleta Ilie
Mitberichterstatter:	Prof. Dr. Dipl. Ing. (FH) Bogna Stawarczyk, M.Sc.
	Priv. Doz. Dr. Dr. Gerson Mast
	apl. Prof. Dr. rer. nat. Mechthild Stöckelhuber
Dekan:	Prof. Dr. med. Thomas Gudermann
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Abbreviation list

10-MDP
10-methacryloyloxydecyl dihydrogen phosphate 23
4-META
4-methacryloyloxyethy trimellitate anhydride 20
Ala
alanin 27
bisEMA
ethoxylated bisphenol-A dimethacrylate 25
bisGMA
bisphenol A diglycidylether dimethacrylate 21
BisHPPP
2,2-bis[4(2,3-hydroxypropoxy)phenyl] propane 25
BPA
bisphenol A 25
CAD/CAM
computer-aided design/ computer-aided manufacturing 11
CC
cystein cathepsin 27
CHX
1,1′-hexamethylene-bis-5-(4-chlorophenyl)biguanidide 30
DC
degree of conversion 21
DEJ
dentino-enamel junction 12
DMSO
dimethyl sulfoxide 33
DPIHP
diphenyliodonium hexafluorophosphate 33
E&R
etch-and-rinse 19, 22
ECM
extracellular matrix 27
EGCG
(–)-epigallocatechin-3-O-gallate 31
GA
glutarylaldehyd 31
Gly
glycine 13
H_3PO_4
phosphoric acid 17
HCl
hydrochloric acid 20
HEMA
2-hydroxyethylmethacrylate 20
HPN
hesperidin 31
Hyp

Publication list

1. Franziska Beck, Nicoleta Ilie.

   Antioxidants and Collagen-Crosslinking: Benefit on Bond Strength and Clinical Applicability

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1. Contribution to both publications

1.1 Contribution to paper I

F.B.: Literature review, study design, pre-tests, sourcing of the used materials, collection of teeth, laboratory tests (preparation of specimens, preparation of test solutions, bonding of the specimens, organization of the immersion, testing of the specimens, changing of the immersion solutions), data curation, statistical analysis of the data, writing of the manuscript, visualization of the data and preparation methods.

N.I.: Study idea and conceptualization, methodology, study design, resources, supervision of pre-tests, organization/ sourcing of the used materials, data curation, supervision of statistical analysis, review and editing of the manuscript, project administration.

1.2 Contribution to paper II

F.B.: Literature review, study design, pre-tests, sourcing of the used materials, collection of teeth, laboratory tests (preparation of specimens, preparation of test solutions, bonding of the specimens, organization of the immersion, testing of the specimens, changing of the immersion solutions), data curation, statistical analysis of the data, writing of the manuscript, visualization of the data and preparation methods.

N.I.: Study idea and conceptualization, methodology, study design, resources, supervision of pre-tests, organization/ sourcing of the used materials, data curation, supervision of statistical analysis, review and editing of the manuscript, project administration.

1.3 Presentation of study results

1.3.1 15. VOCO Dental Challenge

First study results were presented at the 15th VOCO Dental Challenge in Cuxhaven on the 29th of September 2017. The presentation with the title "Antioxidants: Can they improve long-term bond strength?" *("Antioxidantien: Hoffnung gegen den enzymatischen Verbundsverlust?")* won the first prize among all submitted research projects and was rated by a jury of university professors, Prof. Dr. Stefan Rüttermann (Goethe-Universität Frankfurt), Priv.-Doz. Dr. Christian Meller (Universität Tübingen) and Prof. Dr. Dr. Andree Piwowarczyk (Universität Witten/Herdecke).

1.3.2 2019 IADR/AADR/CADR General Session - Vancouver, Canada

The research project and its results were further presented at the 2019 IADR/AADR/CADR General Session in Vancouver (Canada) in an oral session on the 19th of June 2019.

2. Introduction

The demand for invisible, tooth-colored aesthetic dental restorations has risen in recent years [1,2]. Integral for the success of tooth-colored restoration materials like direct resin composites, resin-based and hybrid CAD/CAM (computer-aided design/ computer-aided manufacturing) materials, as well as ceramics (CAD/CAM or conventionally manufactured), is the success and longevity of the adhesive bond because it is only indirect restoration materials, with a flexural strength exceeding 350 MPa, that may be cemented non-adhesively [3]. Especially for direct, posterior restorations, adhesive dentistry offers a shift from restorative material-oriented towards defect-oriented preparation designs [4]. Early studies in the 1980s confirmed the preventive effects of resin-composite fillings on tooth structure by reducing restoration extensions on the occlusal surface on average by 80 % as compared to amalgam [5,6]. Furthermore, the adhesive bond provides a stabilizing effect on cusp fracture resistance, as shown by Ausiello et al. for endodontically treated teeth [7]. Other than that, resin-based composites provide advantageous reparability [8] and are described by Hickel et al. as the repair material of choice for various defects and restorative materials [9]. Precisely since adhesive dentistry offers many possibilities to reduce tooth loss while simultaneously satisfying patients' aesthetic wishes [10], new research on how to improve the longevity of the adhesive bond is mandatory. Even though some studies report comparable mean annual failure rates for both amalgam (2.2 %) and direct composites restorations (3.6 %) in posterior, stress-bearing cavities [11] and similar data for their longevity [12], there is still evidence showing reduced longevity for resin-based composite restorations (9.4 ± 5.4 years) compared to amalgam (15.3 ± 6.6 years) [13]. An accelerated replacement rhythm, due to higher annual failure rates or reduced longevity of the restorations, naturally leads to an increase in cavity size and tooth loss [14,15]. The most common reasons for replacement of direct resin-based composite restorations are secondary caries, fractures in bulk and in margins [16]. Gaengler et al. even reported marginal deterioration effects as early as in the first three years after restoration placement [17]. The degradation effects seen in vivo and in vitro are mainly linked to the susceptibility of the resin-dentin hybrid layer to hydrolysis of both collagen and resin components [18-20]. Especially the proteolytic activity of host-derived enzymes poses a threat to the long-term durability and integrity of resin-dentin bonds [21]. To better understand the challenges adhesive dentistry faces today, a comprehensive understanding of the specifics of dental structure and basics of adhesion science is essential.

2.1 Dental hard tissue

The tooth principally consists of three different mineralized tissues, enamel, dentin and root cement [22]. As the first two are primarily subjected to adhesive conditioning, the following paragraph will focus on those with respect to their structure and composition.

2.1.1 Enamel

Mature enamel - forming the outmost layer of every tooth - is characterized by its brittleness and exceptional hardness [23,24], due to its high content in inorganic material measuring up to 96 wt %, further 3 wt % water and only a small fraction of 1 wt % representing organic compounds [25-29].

Physical properties of enamel are closely associated with the mineral content and its distribution throughout the enamel layer. It is generally accepted that the ratio of inorganic content continuously decreases towards the dentino-enamel-junction (DEJ), while the amount of organic material simultaneously increases [26,30]. Whereas the fracture toughness of enamel presents an inverse development compared to the degree of mineralization and, therefore, rises towards the DEJ, the indentation hardness develops proportionally to the inorganic content and, thus, displays its highest values at the outmost layer of enamel [23].

The mineral phase mostly constitutes of non-stoichiometric, carbonated hydroxyapatite crystals forming flattened hexagonal columns (also referred to as enamel rods or prisms) that span from the DEJ to the enamel surface, though showing an undulating growth pattern in inner enamel [22,31], which can be light-microscopically recognized as the so-called Hunter-Schreger-stripes.

However, the outmost enamel layer does not share this characteristic pattern. The so-called aprismatic zone presents more uniformly oriented crystallites, their c-axes standing practically perpendicular to the enamel surface [32]. The width of this laminated zone differs depending on tooth type and is postulated to range from 20 to 220 µm [32,33]. Though it can generally be concluded that the aprismatic layer is thicker in deciduous than in mature teeth. It increases from the anterior to the posterior dentition and is unevenly and asymmetrically distributed on the buccal, lingual, mesial and distal surfaces [34].

For the inner enamel, rod and interrod enamel can be differentiated based on the differing crystal orientation and alignment: The interrod crystals display a 60° angle to the rod's long axis [35], while being highly co-oriented in their c-axes. By contrast, the rod crystals are all morphologically aligned along the long axis of the rod, though showing misorientations in their c-axes from 30° to up to 90° [36]. This means that the elongation direction of a nanocrystal does not correlate with the crystallographic c-axes of nanocrystals.

Beniash et al. suspect a toughening mechanism against crack growth through the crystal misorientations, as their finite element analysis displayed successful crack deflection especially when the c-axis of the affected crystal was misoriented from 1 to 30° compared to the elongation direction [36].

2.1.2 Dentin

Dentin is the supporting biocomposite [37] underlying the enamel shell, building the bulk phase of the tooth. 70 wt % of dentin is comprised of carbonated apatite, which can be divided into intrafibrillar or extrafibrillar apatite, as it either fills the gaps between the fibrils or is attached to the fibril surface [38-40]. The apatite crystals found in dentin usually present plate-like or cylindrical shape with dimensions varying between 5 and 20 nm [38,41,42]. Dentin shows a rather complex structure being streaked with microscopic tubules (~ 1-2 µm in diameter) and can therefore be divided into intertubular – rich in organic matter – and hypermineralized peritubular dentin [43,44]. Furthermore, the tubular structure, with its increasing tubule diameter and density from the DEJ (diameter: 0.5-0.9 µm; density: 15 000- 20 000/ mm²) towards the pulp chamber (diameter: 2-3 µm; density: 45 000 – 65 000/mm²) [45], causes a differ-

ing distribution of humidity, permeability and calcium concentration at different levels in dentin [46-48]. This results in differing compositions of the smear layer depending on the preparation depth of the cavity and therefore the dentin level and proximity to the pulp chamber [49,50]. Thus, it challenges adhesive systems to be adaptive to multiple bonding conditions.

Collagen type I represents the majority of the dentin organic material with around 90 wt % [22]. While apatite crystallites are considered to provide strength [51], the collagen matrix provides toughness [52]. The collagen molecules exhibit a specific primary structure with repetition of the characteristic (X -Y-Gly)$_x$ amino acid sequence, X and Y being mostly substituted by proline and 4-hydroxyproline residues [53-55]. The tropocollagen molecule of collagen type I is formed through the intertwining of three left-handed helices, two α_1 and one α_2 polypeptide chains [53,56-58]. The right-handed triple helical structure emerges from the amino acid sequence and is inherently stabilized by the repetition of Gly residues at every third position and the high content of sterically restricted, inflexible imino acids [53,57-61]. Furthermore, interchain hydrogen bonding, foremost between the amide group of the glycine residue and the carbonyl group of the residue in the X position of the adjacent chain, ensures the aggregation into the helical conformation [53,58,59,62]. Also, the content of post-translationally modified 4-hydroxy-proline (Hyp) residues induces a positive impact on molecule stabilization and supports thermostability through establishing intra- and intermolecular water bridges between the peptide chains and the surrounding water network [63-65], thus, emphasizing the importance of the hydration structure of the collagen network to maintain conformation and macromolecular assemblies [65,66]. Every collagen molecule is about 300 nm long and exhibits non-helical telopeptides at both C- and N-terminal end, which consist of 16 to 26 amino acids each [67-71]. Five collagen molecules ($\sim$1.4 nm in diameter) assemble in a 1D ($=$ 67 nm) staggered and pleated shape to form the basic building block in the hierarchy of collagen's supramolecular structure: Microfibrils, with a diameter of $\sim$ 4-5 nm, are comprised of a tropocollagen pentamer aggregating in a quasi-hexagonal packing mode with a right-handed supertwist (first described in the 1970s and later specified by Orgel et al.) [69,72]. Recent research supports the theory that substructural units, categorized as "microfibrillar bundles" with a diameter of approximately $\sim$ 20 nm, function as an intermediate superstructure between microfibrils and the d-periodical collagen fibril [73]. Fibrillogenesis is an entropy-driven process that is realized through the lateral-packing of micro-fibrils in parallel to their long-axes. The fibril diameter is - inter alia - dependent on the tissue type itself and its hydration status: For hydrated dentin collagen, around 100 nm have frequently been reported [38,45,74,75].

Additional stabilization of the supramolecular fibrillar and microfibrillar structures is achieved through intra- and intermolecular lysine and hydroxylysine based crosslinks: Posttranslational enzymatic hydroxylation of lysine residues in both helical and telopeptidyl domains constitutes the first step of collagen crosslinking and is of crucial importance for the functional stability of connective tissues [76]. During fibrillogenesis, telopeptidyl lysine or hydroxylysine are oxidatively deaminated by the enzyme lysyl oxidase and are transformed into reactive aldehydes that further condense either with juxtaposed aldehydes or with unmodified lysine or hydroxylysine residues resulting in immature divalent intra- and intermolecular crosslinks [77-79]. Maturation into non-reducible, multivalent crosslinks may occur through additional reaction with amino acid residues including peptidyl histidine [80-82]. Depending on whether the crosslink formation is initiated with a lysine or a hydroxylysine residue, two mature crosslinking pathways can be differentiated: The former leading to the initial formation of aldimines, whereas hydroxylysine-based crosslinks are rearranged into keto-imines [83]. Crosslinks found in bone and dentin are mainly derived from the hydroxylysine-based pathway [84]. The supramolecular structure of collagen is not only stabilized by the generation of collagen crosslinks, but itself strongly influences it, as the corrugated structure poses strict structural limitations, which only permit crosslinkage between the telopeptides and specific helical (hydroxy)lysine residues at position 930 and 87 for the α_1- and 933 for the α_2-chain [71,81,85,86].

2.2 Adhesion science

Adhesion is defined as the tendency to form attractive forces on the interface of two differing substrates between dissimilar molecules or particles [87-89]. Cohesion, by contrast, describes force distributions and molecular interactions within a substrate between like molecules [90]. To establish intermolecular interactions, adhesion requires intimate contact between an adherend and an adhesive. Therefore, the wettability of a substrate is of crucial importance as to generate chemical (e.g. covalent, ionic, chelation bonds), physical (Van-der-Waals-/dipol forces) or mechanical bonding.

Also, McBain and Lee proposed in 1927 three requirements for successful adhesion, namely wetting, solidification and sufficient deformability to reduce the generation of elastic stresses during the formation of an adhesive bond [91,92]. While solidification and elastic deformability are inherently connected to the characteristics and chemistry of the adhesive, wetting is the result of interaction between adherend and adhesive and may therefore be influenced by both parties. Wettability is defined as the ability of a liquid to wet and optimally spread on a surface. It is dependent on the characteristics of the respective liquid, surface, and situational conditions:

In theory, the wetting behaviour of a liquid on a solid may be explained by a force equilibrium between the surface tension of a solid γ_{sv° towards vapor phases – which can be equated to its surface free energy for simplification – the surface tension γ_{lv° between a liquid and vapor and the interfacial tension of both γ_{sl}, and is measured by the contact angle θ that is formed by the liquid on the surface. A contact angle above 90° predicts non-wetting properties of a solid-liquid system, for $\theta < 90°$ the surface is wetted, and if θ assumes 0° the solid is optimally wet, as the liquid spreads on its surface [93-95].

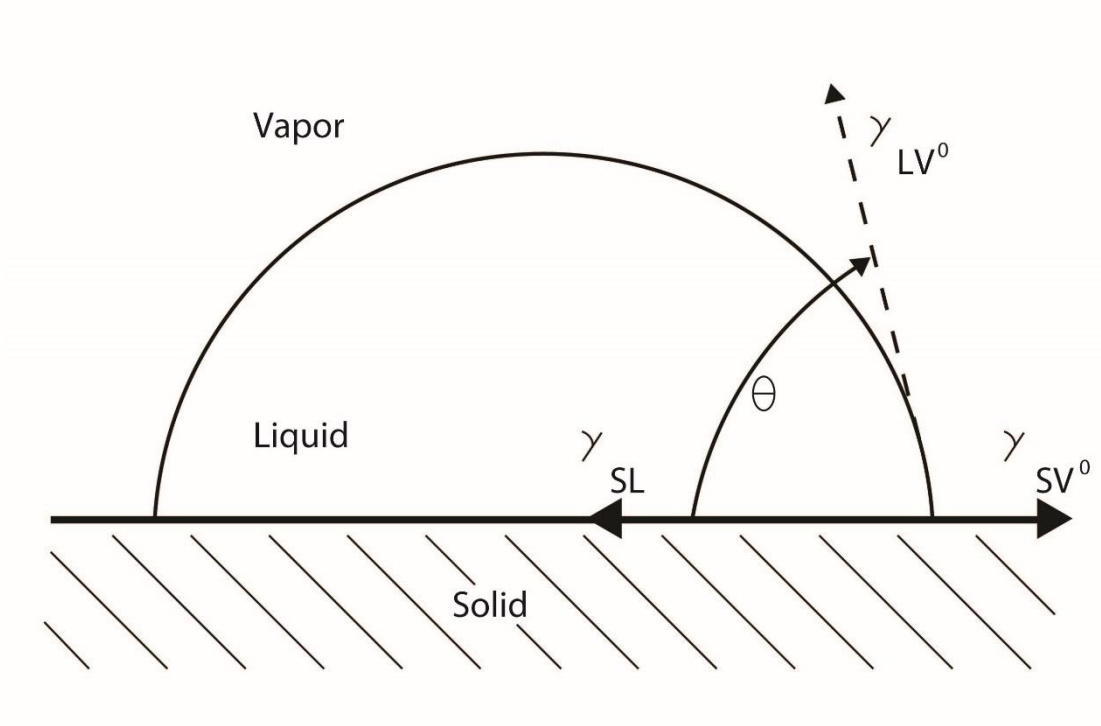

Figure 1 Theory of force equilibrium and the forming equilibrium contact angle after Baier 1968

Wetting in general can be considered as a thermodynamical process, as it results in a net increase or net decrease of free energy, dependent on whether the solid surface is wetted by a liquid with a higher or a lower specific surface free energy in comparison to the solid itself [96,97]. As every physical system aims

for a state of minimum free energy, wetting is therefore favoured when it results in a net decrease of free energy, thus when the surface free energy of the solid exceeds the liquid's. Moreover, the difference in energy level of the system also determines the wetting speed of the solid: The higher the net energy reduction of the system, the more readily a liquid spreads on a solid. Wenzel consequently deduced that surface enlargement leads to an amplification of the wetting properties of a solid due to a greater change in energy level: Therefore, solids with positive wetting properties, presenting contact angles < 90°, will improve towards smaller contact angles. However, non-wetting substrates, displaying contact angles exceeding 90°, will show a negative tendency towards even higher contact angle values, when roughened [98].

This information may be summed up by the following equation, where r represents the roughness factor, θ_1 stands for the measured and θ_2 for the true contact angle:

$$r = \frac{\cos\theta_1}{\cos\theta_2}$$

Generally, surface free energy γ is defined as the amount of energy that must be spent to create a surface of a bulk material and equals the totality of energy for the disruption of all intermolecular bonds. Thus, depending on the resulting specified surface, surface free energy can be equated to one half of the free energy of cohesion. Consequently, hard, high-melting solids, particularly with a high-energy crystalline structure and strong intermolecular bonds, also present a high surface free energy [99-101].

However, following Langmuir's concept of "independent surface action", wetting is primarily influenced by the bonds, forces and packaging of the outmost atoms and molecules of solid and liquid, due to their highly localized nature [102-104]. This principle was confirmed by extensive research by Shafrin and Zisman [99,105-107], even though they simultaneously elucidated exceptions to that common rule (as the residual fields of forces are less localized when either ions or uncompensated dipoles are present in the outermost layer of either liquid or solid). A change in surface free energy and concurrently in wettability is thus related to a change in atomic constitution as well as distribution and physical packing of substituents in the outmost surface layer [107-109].

2.2.1 Critical surface tension

In order to simplify comparisons among various substrates, Zisman et al. introduced γ_c, the critical surface tension, as a characteristic parameter of a solid, as a linear relation between $\cos\theta$ and γ_{lv} for homologous series of organic liquids was discovered [110]: The critical surface tension γ_c can be depicted as the intercept point of an extrapolated straight line graph that is computed by plotting $\cos\theta$ versus γ_{lv} and the horizontal line $\cos\theta = 1$, thus θ itself equals 0. Therefore, the determination of γ_c specifies the maximum surface tension of a liquid to still spread over the solid's surface spontaneously. Even for non-homologous liquids plotting $\cos\theta$ against γ_{lv} resumed in the graphics of a straight line or a narrow rectilinear band of intersection points. Generally, empirical data suggests that as long as low-energy surfaces do not interact with test liquids through hydrogen bonds or other interactive forces a graph of $\cos\theta$ versus γ_{lv} will always result in a straight line graph (or narrow rectilinear band) [111]. In those cases, a conclusive value for γ_c is obtained. Hence, if γ_c is assessed through a heterogenous group of pure liquids (of different reactivity and polarity), it may be used for comparing the wettability of different low-energy surfaces, as it serves as an approximation for the specific surface free energy γ_{s° of a solid [99,108,110,112].

From left to right, the following sequence displays elements that progressively influence critical surface tension γ_c either negatively – towards smaller values – (left) or positively by increasing wettability (right):

Fluorine – Hydrogen – Chlorine – Bromine – Iodine – Oxygen – Nitrogen

Consequently, an outermost chemical substituent based on these elements equally decreases (e.g. - CH_2, - CH_3, -CF_3, -CF_2H) or enhances wetting (e.g. phenyl, -OH, -SH, -COOH, -NH_2) [107-109]. Furthermore, this explains the adverse effects of fluoride application previous to adhesive procedures.

2.2.2 Mechanisms that improve or impede adhesion

The adsorption of so-called "abhesives" (= not adhesive) on high energy surfaces leads to poor wetting and consequently to the formation of interfacial voids, which further local stress concentrations and facilitate adhesion failure as shown by Griffith et al. [113]. Ultimately, the more γ_{lv° of a liquid exceeds γ_c of the abhesive, the greater the equilibrium contact angle θ, the poorer the wetting and the greater the void formation [114]. Common abhesives posess an amphiphatic structure, whose polar groups permit adsorption upon high-energy surfaces and whose outermost terminal groups (such as -CH_3 or -CF_3) simultaneously form a non-wetting monolayer presenting extremely low γ_c values. Thus, any adventitious contamination with organic films is to be avoided to maintain adequate adhesion.

Transferred to an oral environment, high energy surfaces like instrumented enamel easily attract low-energy (organic) contaminants, for example saliva, collagen debris, and blood, that reduce the adherence of an adhesive and, therefore, probably the integrity of a restoration on tooth structure [115-119]. Hence, any contamination on the tooth structure after tooth preparation, and especially etching, is to be prevented. The importance of perfect control of the operating field, preferably by absolute isolation through rubber dam usage [120], is stressed.

Many investigations confirm that even the adsorption of a monolayer of water on high-energy surfaces decreases γ_c considerably [114,121-123]. Furthermore, the more water is adsorbed, the more the critical surface tension γ_c resembles that of bulk water, which is at 22 dynes/cm [124].

Thus, spreading of hydrophobic liquids devoid of polar substituents is inhibited on moist surfaces [92,125]. Dependent on the constitution of the adherend and more so of the adhesive, simple organic components, oxides and even water may serve as abhesives [114,126], impede adhesion and result in poor wetting. As adventitious contamination may not be avoided, Zisman and his colleagues developed a strategy to displace contaminants and further endorse adhesion by introducing coupling agents specifically designed for certain adhesive-adherend combinations [92,114,127-129]:

The first step is the displacement of the abhesive coating irrespective of its chemical origin, as the prerequisites for displacement are universal for both organically as well as for water-based films: For maximum efficacy the displacing agent should present both a large initial spreading pressure $S_{b/a}$ [130,131] and a high equilibrium spreading pressure $F_{b/a}$

$$S_{b/a} = \gamma_a - (\gamma_b + \gamma_{b'a'})$$

$$F_{b/a} = \gamma_a - \gamma_{a'}$$

Above, γ_a and γ_b signify the surface tensions of a liquid a that is to be displaced by a liquid b and $\gamma_{b'a'}$ is introduced as the interfacial tension of b and a with the prime superscripts referring to a reciprocal satura-

tion of a and b. Consequentially, to ensure that the two expressions $S_{b/a}$ and $F_{b/a}$ are as large as possible, both the surface tension of the displacing agent γ_b and the interfacial tension $\gamma_{b'a'}$ must be as small as possible: Following Pound's rule, $\gamma_{b'a'}$ becomes negligible or small if liquid b presents to be soluble in a [132,133].This makes solubility another mandatory requirement for effective liquid displacement.

After decontamination of the solid surface, so-called coupling agents are utilized to further prevent adventitious contamination of the high-energy adherend either by adsorption/physisorption of atmospheric or liquid-based contaminants. Coupling agents behave as surface active agents as they adsorb on, alter and, to withstand displacement, interact strongly with both adherend and adhesive. The interaction is either chemically or at least physically based and forms a protective monolayered coating. To be as efficient as possible, coupling agents present a certain structure as schematically depicted below: The outermost part determines the wettability properties of the adsorbed monolayer. Thus, the outmost chemical substituent must be chosen accordingly. The opposite part should interact strongly with the adherend surface and is often composed of one or more polar groups that are selected adequately for the respective solid, in order to resist hydrolysis and displacement by solvents, surfactants or surrounding contaminants. A hydrophobic spacer is used to render the coupling agent water insoluble and prone against water permeation [92,114,127,128,134].

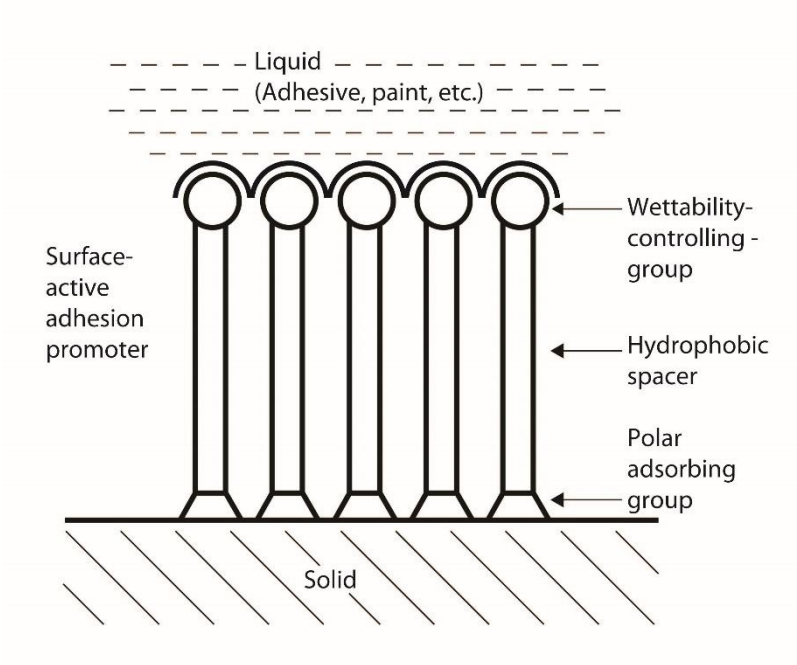

Figure 2 Coupling agents - Structure and function after Shafrin 1968

2.3 Adhesion to enamel

Michael G. Buonocore revolutionized dentistry when in 1955 he published his paper on a "simple method" to increase the adhesion of acrylics (acrylic filling materials) to enamel surfaces [135], where the use of 85 % orthophosphoric acid (H_3PO_4) led to prolonged adhesion of acrylic filling resin on otherwise untreated enamel. A microretentive surface formed due to the difference in acid solubility of the prism center, periphery and the interprismatic enamel, probably linked to the variance in crystal orientation [36,136,137]. Thus, etching patterns are less defined in untreated enamel and more so in posterior teeth

due to the increasing width of the aprismatic zone [34,138]. Buonocore's discovery was then refined in numerous studies regarding the used acid agent, concentration and etching duration [136,139-141]:

Hence, today the most recommended protocol for the exclusive etching of enamel is the application of 30 – 40 % H_3PO_4 for 30 s [142,143], even for uninstrumented, aprismatic enamel [144], although 60 s were previously endorsed in the 1970s/1980s [139,145,146]. More and more studies propagate a possible reduction in etching time to 15 s, provided that the enamel was priorly instrumented [145,147-149], thus, facilitating the simultaneous etching of enamel and dentin as propagated in the "total-etch"/"etch-and-rinse" technique [150,151]. Barkmeier, Erickson et al. recently demonstrated superior roughness values for extended etching duration (60 s) in comparison to the recommended 15 s, however, without a significant improvement of bond strength [152]. While early studies stressed the importance of gaining an optimal etching pattern [136] to guarantee micromechanical interlocking and the formation of resin tags [153], more recent studies propose that mere surface roughening might be sufficient for adequate bonding success [138,154], as only a weak correlation can be seen between penetration depth of etched enamel and resin-enamel bond strength [155,156]. Moreover, well-defined etching patterns occur quite rarely and suboptimal etching is most commonly encountered, particularly on the buccal surface [157]. As previously stated, surface roughening influences the wettability of a solid surface in a manner that amplifies the wetting properties of the respective substrate [98]. Therefore, as enamel possesses high surface energy due to its crystalline structure and strong intermolecular bonds [158], it presents a positive wetting tendency, which is even improved by surface enlargement. This is verified by the research of Retief et al. demonstrating higher wettability (i.e. reduced contact angles of the resin/enamel interface) for etched versus unetched enamel [159]. Furthermore, the study group showed significant advantages when enamel etching with various concentrations of H_3PO_4 was executed on ground enamel compared to the wettability that was obtained solely by etching with respective H_3PO_4 concentrations without previous grinding [160].

2.4 Adhesion to dentin

As elucidated above, dentin presents a more complex structure in comparison to enamel. Pashley [47] described intact dentin as a dynamic substrate as related to its differing distribution of minerals, organic network, water content and permeability (dependent on dentin depth), thus, creating a unique bonding substrate dependent on the given distance to the pulp [46]. Age-related or pathologic changes in dentin structure and morphology as induced by sclerosis or caries pose additional challenges for dentin bonding [161,162]. Both sclerotic and caries-affected dentin are associated with occlusion of dentinal tubules by mineral precipitates impeding resin infiltration and tag formation [163-165].

Comparable to enamel bonding, dentin adhesion is primarily accomplished through micromechanical interlocking of the resin with the microporous dentin surface [166-168].

2.4.1 Smear layer

The smear layer is the inevitable product of tooth instrumentation [169] and is formed as a result of a physicochemical process including the mechanical shearing of and thermal degradation of the collagen network [170,171]. Its morphology varies in thickness, roughness, composition, degree of attachment and density depending on the type of instruments used (carbide bur/diamond bur), method (wet/dry), type of irrigation solution, geometry of the cavity and proximity to the pulp chamber [169,170,172]. In clinics,

the common depth of the smear layer measures between 1 and 5 µm [49,173]. The particles themselves are sized between 0.05 and 10 µm and mostly present irregular shapes, which may assume plate-like configurations in bigger particles. The composition of the smear particles reflects the dentinal structure they were generated from in its distribution of inorganic and organic content. Though, in contrast to sound dentin, the organic matrix may exhibit signs of surface denaturation due to thermochemical alterations of its structure [47,169,174-177]. The smear debris leads to the reduction of dentin permeability by up to 86 % as it occludes tubule orifices with so-called “smear plugs” [178]. Thus, the smear layer forms a barrier, that hinders the direct interaction between the dentin substrate and the adhesive resin, and consequently, impedes the bonding process [179]. As early dentin adhesives were applied directly on the smeared surface, the resulting bond strengths mirrored the low cohesive forces between the smear particles [180-182]. The associated bond strength values commonly ranged between 3 and 7 MPa (no test settings mentioned, summary of various studies) [89] and were too low to withstand polymerization shrinkage of the restorative resin composite [183] leading to marginal gap formation [183] and consecutively to microleakage [184].

Therefore, the smear layer must either be removed, modified, or thoroughly impregnated to ensure adequate bonding by allowing resin penetration into the dentin tissue underneath [46,185,186].

Today’s adhesive systems may be subdivided into two categories depending on how they interact with the smear layer:

1. Etch-and-rinse (E&R) adhesives rely on simultaneous acid etching of both enamel and dentin, commonly with 30-40 % phosphoric acid, resulting in the removal of the smear layer and superficial mineral which is then thoroughly rinsed away [151].
    a) Three-step-etch-and-rinse adhesives (1. etching agent 2. primer 3. bonding agent)
    b) Two-step-etch-and-rinse (1. etching agent 2. combination: primer + bonding agent)

2. Self-etch (SE) adhesives are based on an acidic monomer that is applied directly on the smear layer without previous etching. The adhesive systems simultaneously etch and impregnate by making the smear layer permeable and incorporating it into the hybrid layer [179,187,188].
    a) Two-step-self-etch adhesives (1. acidic primer 2. bonding agent)
    b) One-step-etch-and-rinse (1. all-in-one-combination: acidic primer + bonding agent)

A frequently neglected point is the inability of acids to dissolve organic matter [47,189], which is naturally present in the smear layer. The proportion of collagenous and non-collagenous proteins in the smear layer is thus gelatinized/denatured, incapsulated by the resin and incorporated into hybrid layer [190,191]. Consequently, both approaches, even the etch-and-rinse, despite the use of phosphoric acid, lead to the incorporation of gelatinous collagen remnants into the resin-dentin bond.

The acid treatment of the smear layer strongly influences the fluid permeability of the dentin, due to the acid-based removal of the smear plugs in the tubule orifices that (before) lowered the fluid flow to nearly zero. Hence, the smear plug removal leads to a by more than 90 % enhanced outward fluid movement due to pulpal pressure [47,192,193], which according to Pashley equals up to 20-70 cm/H_2O [194]. The following increased contamination of the adhesive interface with dentinal fluid might not only impair the wetting of the dentin surface [195], but also polymerization by the dilution of primer and bonding agents [196,197]. Though, studies demonstrate that several commercially available adhesives can achieve solid bond strength values with long-term durability despite the augmented moisture level [198,199].

2.4.2 Hybridization

The hybrid layer – or resin interdiffusion zone – was first described in Nakabayashi et al. [166], which identified a HCl-resistant fusion zone beneath the adhesive interface consisting of monomer-penetrated intra- and intertubular dentin. The study suggested that the formation of this new entity was associated with the use of amphiphilic monomers and led to an improvement of resin-dentin bond strength up to 18 MPa, tested in a macro tensile test setting [166]. About 10 years after the first identification of a hybridized zone in dentin, Nakabayashi et al. concluded that "[w]hen true hybridization occurs, bond strength (particularly to deep, wet, highly tubularized dentin) increases dramatically" [167].

Generally, three basic processes are imperative to achieve hybridization [167,200]:

1. Decalcification of the dentin network without denaturing the organic matrix
2. Replacement of the mineral face with adhesive resin with total encapsulation of the collagen network
3. Perfect *in situ* polymerization of the adhesive resin

The objective is to achieve the "ideal hybrid layer", an entity Spencer et al. described as a "3-dimensional polymer/collagen network that provides both a continuous and stable link between the bulk adhesive and dentin substrate" [200].

As mentioned earlier, wetting is one of three prerequisites for successful adhesion, as proposed by McBain and Lee in 1927 [91], as well as for successful hybridization. It can therefore be deduced that γ_c of dentin must be at least equal or optimally somewhat higher than the surface tension of the applied adhesive to achieve proper wetting [89,108-110,112,114,201]. In contrast to enamel, where surface energy rises after acid conditioning [159], research confirmed that etching of smear-layer covered dentin (γ_c= 42 dyne/cm) leads to a decrease in critical surface tension down to about 27 dyne/cm depending on the used etchant [202]. An explanation for this phenomenon is the cumulation of organic matter (collagen, gelatine, non-collagenous proteins) on dentin surface following acid etching [89], since the collagen of the smear layer cannot be dissolved by acid treatment [190,191], but remains as a gelatinous layer [190]. The discrepancy between the surface energy of acid-conditioned dentin ($\sim$ 27 dyne/cm) and the approximated surface tension of an adhesive resin ($\sim$ 40 dyne/cm), impedes effective wetting and concurrently effective hybridization [195]. In conclusion, conditioning improves accessibility of the dentin structure by removing or transforming the smear layer, while it does not enhance wettability [203].

Priming agents are used to overcome the surface energy mismatch between conditioned dentin and adhesive resin. Sharing the basic concept of coupling agents [92,114,127,128,134], primers display a surface tension $\leq \gamma_c$ (less or equal to the critical surface tension) of conditioned dentin [89,203,204] and contain amphiphilic, bifunctional monomers that can mediate between the hydrophilic dentin surface and the hydrophobic resin to promote adhesion [89]. The promotion of tooth-resin adhesion by amphiphilic monomers was first observed in the late 1970s and early 1980s by the research group around Nakabayashi with the development of an adhesive system based on 4-META [166,205,206].

Moreover, Sugizaki et al. assessed the effects of different dentin conditioners as well as priming solutions on the hybrid layer thickness, surface level and collagen configuration, and found that while acid conditioning led to a denser collagen fiber arrangement, the following primer treatment seemed to alter the fibrillar configuration in a way that promotes penetration [207]. 2-hydroxyethylmethacrylate (HEMA) is an amphiphilic monomer commonly used in dentin primers to improve adhesion to the dentinal surface [89]. Its positive effect on hybridization [167,208] is attributed to both a rise in surface energy level of the dentinal interface as described by Attal et al. [203] as well as promoting the diffusivity of the dentin sub-

strate as confirmed by Nakabayashi et al. [209]. Both of these characteristics are linked to HEMA's low molecular weight, its small molecule size and high hydrophilicity due to a short carbon chain attached to a hydroxyl group [199].

As elucidated above, thorough impregnation is a prerequisite of successful hybridization. Optimal impregnation is achieved if all voids resulting from demineralization and all the dentin water content (bound as well as unbound) is displaced by monomers. The aim is the three-dimensional encapsulation of collagen fibers in adhesive resin [167,200]. Common co-monomers, e.g., triethyleneglycol dimethacrylate (TEGDMA) and bisphenol A diglycidylether dimethacrylate (bisGMA) are sparingly soluble in water [210]. Pashley's research group quantifies water solubility for TEGDMA and bisGMA at 9.5 x 10^{-3} [211] and 1 x 10^{-3} g/ml (water) [Pashley, unpublished research], respectively, greatly limiting the ability to displace intermolecular water [212]. HEMA as well as ethanol are therefore used as solvents for TEGDMA or bisGMA to increase their water-miscibility and ensure resin impregnation into intrafibrillar spaces [212]. However, HEMA raises the risk for water sorption due to its structure-based hydrophilicity which then again leads to plasticization effects on the polymer network [213].

The collagen structure, as mentioned earlier, is organized in a highly hierarchical manner, the smallest unit being the tropocollagen molecule, formed by three individual left-handed helices into a right-handed triple-helical structure [53,56-58], stabilized by interchain hydrogen-bonding [53,58,62] and the tightly-bound surrounding hydration structure [59,63-65]. The research group around Bertassoni et al. challenged the assumption that single monomer molecules like HEMA, TEGDMA, and UDMA (urethane dimethacrylate) could infiltrate intermolecular spaces at all [214], as they are indicated to level around 1.3 nm [69], while an extended, single TEGDMA molecule is approximated to 2.2 nm in length [215]. Takahashi et al. were able to prove the accessibility of water-saturated intermolecular spaces to both HEMA and TEGDMA molecules [212]. Further, on the basis of Torchia's research [216], they suggest that the collagen network is not static, but each molecule has sufficient mobility to rotate freely within a fully-hydrated collagen fibril and allows the intrafibrillar penetration of seemingly "too big" molecules [212]. Though, both research groups ultimately agree that "to date, there is no evidence that water in the intrafibrillar compartment of adhesive joints is completely replaced by resin" [212,214].

As mentioned above, solidification of the adhesive layer is one of the three basic requirements for successful adhesion as described by McBain and Lee in 1927 [91]. Therefore, to maintain the encapsulation of collagen fibres and the integrity of the hybrid layer, effective polymerization, and a high degree of conversion – from monomer to polymer – are imperative [200,217].

Adequate polymerization and solidification of the adhesive layer can be measured by the degree of conversion (DC), which is quantified by the proportion of remaining aliphatic -C=C- double bonds in the resin after polymerization in relation to the total number of -C=C- double bonds in the resin material before curing [218].

The DC of dental adhesives is one of the most validated parameters when it comes to evaluate their performance, as a low conversion rate shows detrimental effects on mechanical properties [219] and advances monomer elution [220,221]. A low DC renders the hybrid layer prone to water sorption [222-224] and permeability [225,226], which enables and furthers hydrolysis, the primary cause for resin degradation within the hybrid layer [227].

Multiple factors may adversely affect polymerization, such as residual water in the adhesive layer [197] and outward fluid movement due to pulpal pressure [196]. Though, especially in amphiphilic resin blends, more potential risks for insufficient conversion have been identified, which also facilitate nano phase separation [228]: Presence of water/water retention [229], adhesive hydrophilicity [230], monomer structure and functionality [231,232], solvent type and concentration [230,233,234] and choice of photo-

initiating systems [223,235]. Nano-phase-separation is a phenomenon frequently recognized in amphiphilic resin blends, particularly for simplified adhesives, describing the formation of heterogenous layers in the hybrid layer following the separation into hydrophilic and hydrophobic resin phases [223,236-238]. Especially the hydrophilic resin phase often shows insufficient DC, which was linked – by various studies – to the incompatibility of the hydrophobic camphorquinone-based photo initiator system with hydrophilic monomers, like HEMA [223]. Study results therefore propose a combination of hydrophilic photo initiators, e.g. Lucirin TPO (2,4,6-trimethylbenzoyldiphenylphosphine oxide), with the conventional camphorquinone/amine system, which demonstrably improves the conversion rate of both hydrophilic and hydrophobic monomers [217,223,235] and, therefore, diminishes nano phase separation [236].

In summary, the hybridization process is a very complex process itself, disregarding any operator-induced impacts. Even with perfect execution and control of the operating field, the micromolecular mechanisms on which optimal hybridization relies, precisely wetting, impregnation and polymerization, are affected by clinically incontrollable micro-and nanomolecular events associated with the dentin-inherent composite structure. This makes optimal hybridization a nearly impossible task and, thus, degradation management a compelling necessity for the preservation of the hybrid layer/adhesive interface.

2.4.3 Morphology of the hybrid layer

The micromorphological structure of the hybrid layer is greatly dependent on the adhesive it is created with. Today's most frequently used classification of dental adhesives originates from Van Meerbeek's Buonocore memorial lecture in 2003 and divides between E&R adhesives and SE adhesives regarding the required number of application steps [239].

Both approaches differ fundamentally in how the adhesive interacts with the smear layer or the dentin tissue itself. Therefore, scanning and transmission electron microscope (SEM/TEM) imaging presents a different micromorphology of the hybrid layer as linked to etching depth, infiltration, and the depletion of hydroxy apatite crystals [239].

In dentin, due to the simultaneous conditioning of enamel and dentin, E&R adhesives commonly exhibit an etching depth between 4 and 6 µm, which exposes a microporous collagen network with nearly complete depletion of hydroxy apatite crystals [199]. The propagated total removal of the smear layer by phosphoric acid etching must be considered as incorrect, because, due to the incapability of acids to dissolve organic tissue, the E&R approach leads to an incorporation of collagen remnants into the hybrid layer [190,191].

Self-etch (SE) adhesives are subdivided into "ultra-mild" (pH> 2.5), "mild" (pH≈ 2), "intermediary strong" (pH= 1-2) and "strong" (pH<1) depending on their acidity, which in turn affects their depth of etch. While "strong" self-etch adhesives performed well on enamel and created a depth of etch similar to E&R adhesives (up to 3 to 4 µm), dentin bonding failed due to the (1) absence of chemical bonding, (2) instability of the deeply etched collagen network lacking support by organised mineral, (3) low degree of conversion of highly hydrophilic resin monomers, and (4) destabilization of the hybrid layer due to the incorporation of hydrolytically instable calcium phosphates [199]. As a result, various clinical studies attested this type of adhesive significantly poorer survival rates in comparison to reference adhesives, which was linked to accelerated ageing resulting from the abovementioned drawbacks [199,240-242]. Today's recommendation is the use of mild and ultra-mild SE adhesives, which combine effectively chemical bonding with micro retentive interlocking and establish the most reliable and durable bond to

dentin among the group of SE adhesives. The shallow depth of etch of ≈ 1µm and the simultaneous etching/infiltration ensures thorough resin diffusion even with short clinical application times [199].

However, the differences in micromorphology cause inferior bonding effectiveness to enamel: High crystallinity, superior degree of organization of hydroxyapatite crystal in enamel both impedes the availability of Ca^{2+} for chemical bonding. Concomitantly, the lower etching effect of acidic monomers in (ultra)mild formulations and the reduced chemical reactivity result in inferior nanolayering of 10-MDP (10-methacryloyloxydecyl dihydrogen phosphate) with hydroxyapatite crystals/enamel tooth structure [243-245]. To achieve an adequate etching effect and thus a promising bond durability on enamel, selective enamel etching with phosphoric acid is the current recommendation for SE adhesives [199].

Generally, the micromorphology of the hybrid layer as well as the primary bonding mechanism is dependent on the adhesive approach that is applied. Moreover, the thickness of the hybrid layer does not reflect on the success of dentin bonding, rather it is the quality of the hybrid layer, specifically the susceptibility to micro-/nanoleakage due to voids and insufficient infiltration [199].

2.4.4 Microleakage

Microleakage describes the formation of diffusion channels originating from a gap formation between the restorative and the cavity walls. The marginal maladaptation and post restorative gap formation [246] is claimed to be mainly the result of a force mismatch between the bond strength of the adhesive interface and polymerization shrinkage of the resin restorative [247-249]. Up to the early 1990s, microleakage was usually detected macroscopically through inspection of dye penetration [250,251], which was rejected by various sources as it fails to identify the exact location of microleakage [251-253]. Then, the development of new adhesive systems, which supported true hybridization in vivo and in vitro through total-etch conditioning, was associated with the reduction of microleakage. Despite the application of these modern adhesive methods, postoperative sensitivity, the clinical manifestation of hydrodynamical fluid movement following microleakage, persisted [254,255]. Subsequently, Sano et al. identified a new pathway of microleakage via SEM/TEM imaging. In the absence of marginal gaps, a microporous zone beneath/within the hybrid layer showed indication of lateral, submicron leakage, termed "nanoleakage"[252,253,256,257].

2.4.5 Nanoleakage

As opposed to microleakage, nanoleakage is a degradation phenomenon localized beneath or within the hybrid layer in the absence of marginal gap formation [252,253,257]. Dependent on the adhesive systematic used (SE or E&R), the identified micro porosities (between 20 and 100 nm) in the hybrid layer differ in localization and distribution. In an immunohistochemical analysis of the resin envelopment of collagen fibrils in the hybrid layer, the research group surrounding Breschi et al. found distinctive differences in the labelling patterns of SE and E&R adhesives. While the E&R approach led to a gradient increase of gold particles from the top to the bottom of the hybrid layer, the reference SE adhesive presented a weak but uniform distribution pattern [258]. Ultimately, the labelling index (defined as number of particles per area unit) for the E&R bonding systems was increased when compared to the SE adhesive [258].

As confirmed by VanMeerbeek's newest status perspective on dental adhesion, E&R adhesives are especially prone to nanoleakage, owing to the disparity between demineralization and infiltration depth due to decoupling acid conditioning and infiltration [199]. The nanoleakage phenomenon is less prominent for

the SE approach, as the forming hybrid layer mostly appears to be significantly more homogenous as conditioning and infiltration occur simultaneously. However, nanoleakage is multifactorial and was previously associated with incomplete polymerization, nano phase separation, water sorption due to low-molecular-weight oligomers and hydrophilic monomers [259]. Simplified, so-called “one-bottle” adhesives that condense the three functions of etching, priming, and bonding into one step, often contain a high proportion of HEMA or solvents to keep amphiphilic resin blends in solution [260-262]. A high HEMA content, in turn, leads to the formation of poly(HEMA) hydrogels, which render the hybrid layer into a semi-permeable membrane allowing for higher water sorption and thus, a drop in desired physical properties [229,261]. On the other hand, for HEMA-poor/free “all-in-one” adhesives, an increased risk for phase separation is documented [262].

Nanoleakage, when resulting in water penetration and hydrolysis, is considered as the primary degradation mechanism in the dentin interface [263-265].

2.5 Degradation of the hybrid layer

In summary, insufficient infiltration, incomplete solvent evaporation and inadequate polymerization or rather monomer/polymer conversion are the primary causes for nanoleakage and therefore render the resin-dentin bond prone to degradation [200,252,266-268].

In clinics, the degradation processes are noted in the marginal wear and leakage, recurrent marginal decay of composite restorations and ultimately may result in their premature failure [269-271]. In general, two degradation pathways can be distinguished – the degradation of the hybrid-layer’s own methacrylic components and the degradation of the tooth structure itself – which both lead to the disruption of the adhesive bond.

2.5.1 Degradation of methacrylates

Methacrylates in the oral environment, regardless of originating from either adhesive, a resin-based cement or a resin-composite restorative, are subjected to various ageing processes [213]. Ambient conditions, physical and chemical, strongly influence the interactions with and effects on any exposed substrate. The oral cavity especially presents very specific challenges for any material: A humid to aqueous environment due to saliva, which is comprised of water, glycoproteins (mucin), lactoferrin, histatins, statherins, cystatins, electrolytes and includes various enzymes and antibodies, all of which fulfil different functions from remineralisation and buffering to digestion and pathogen defence [272]. Furthermore, as the oral cavity is the first part of the gastrointestinal tract, every material is subjected to a variety of exogenous chemicals, such as salts, alcohols, bases, acids, and oxygen all derived from food and beverages [213,273]. This is also the reason for a permanent change in ambient conditions, for instance in temperature and pH, which may lead to the softening of the polymer matrix measured as a decrease in surface hardness [274] .

Also, the material itself, in this case methacrylate-based polymers, and their chemical and mechanical characteristics, have a significant influence on their own behaviour in an aqueous environment: Important parameters are crosslinking density and hydrophilicity of the polymer network, the filler type/content and the discrepancy of the solubility parameter between the polymer and the solvent (in dentistry: saliva) [213].

For scientific clarity, the process of polymer degradation defines the cleavage process in which polymers are split through chain scission, first into oligomers, then into monomers, and can therefore be viewed as the opposite to polymerization [275]. Sorption of the surrounding solvent and elution into the solvent are antecedent phenomena that initiate polymer chain degradation/cleavage [213]. Both are greatly facilitated by structural hydrophilicity: If the polymer network is constituted of hydrolytically susceptible groups like ester, ether and urethane linkages or hydroxyl groups, those may attract water with potentially deleterious results. These groups offer the potential for hydrogen bonding and polar interactions, which affect the solubility parameter (δ) of a polymer, as it is defined as the cohesive energy of the solvent molecules per unit volume. As like dissolves like [276,277], the availability of polar groups and linkages in a polymer network is a key reason for elution and water sorption. In a good solvent, the attractive forces between the solvent and the chain components surpass the interchain forces and lead to their disruption and ultimately to solvent uptake, plasticization, or elution [213,278].

The water uptake in rate and extent is further enhanced by a porous network and can therefore be reduced if the crosslink density of a polymer network is improved. Though, in this respect again, the chemical nature of the crosslinking agent is important, as Arima et al. report: If the agent exhibits hydrophilic groups or linkages that permit hydrogen bonding, water sorption may still be enhanced despite a denser network [279,280]. Also, the excessive addition of crosslinking agents reduces mechanical properties and increases the quantity of unreacted components, since at higher agent concentrations the reaction efficiency reaches a point of stagnation as most of the binding sites are saturated [279,281]. As methacrylate polymers are deemed to be insoluble in water, the solubility potential is strongly dependent on the proportion of unreacted monomers and initiators [282]. Unreacted monomers not only lower mechanical properties through plasticization [283], but may also elute from the solid/resin material and thus pose the risk for various adverse health effects including any kind of tissue reactions in patients [284]. Several studies already documented the accumulated risk for contact allergies and respiratory hypersensitivity in dental personnel, which was linked to the regular exposition to methacrylates [285-287].

Studies estimate the amount of elutable, unreacted monomer to rank only between 2 and 10 % of the total of the remaining -C=C- double bonds [220,288,289], since the vast majority of unreacted double bonds are part of a dimethacrylate molecule that has at least reacted on one end and is thus covalently bonded to the polymer network [213]. Thus, the amount of elutable methacrylate material is reciprocally associated with the DC of a resin composite (RC) [290], so thorough polymerization is an effective measure to prevent unnecessary exposure to (co)monomer eluates. Sevkusic et al. have documented the possible release of 19 eluates from commonly used, commercial resin composites [291]. The study also confirmed the release of bisphenol A (BPA), which is a frequent concern, as BPA induces oestrogen-like effects on human cells [292].

The main chemical principle in polymer degradation is the scission of condensation-type linkages, such as ester, ether, or amide bonds, in reaction with water, therefore named hydrolysis. Hydrolysis can either proceed passively or actively through enzymatic catalysation.

Various studies document the activity of salivary cholinesterases on polymer chains of bisGMA and TEGDMA resulting in the cleavage products 2,2-bis[4(2,3-hydroxypropoxy)phenyl] propane (BisHPPP) and methacrylic acid (MA) [293-296]. Two main types of cholinesterases, cholesterol esterase and pseudocholinesterase, have been associated with the degradation of dental materials in the oral cavity [293,295,297]. Finer et al. could even prove sufficient esterase activity in human saliva for the effective hydrolysis of ester linkages in dental polymers [293]. Furthermore, the study confirmed the special affinity of cholesterol esterase for long-chain substrates, adding to its suitability for polymer degradation [293]. TEGDMA has proven to be more vulnerable to enzymatic degradation than bisGMA [298,299] and bisEMA (ethoxylated bisphenol-A dimethacrylate) [300]. This confirms the higher resistance of monomer

structures that contain either bulky hydrophobic structures like phenyl rings or show branching points close to the ester linkage [300].

Passive chemical hydrolysis is facilitated by changes in the oral environment as shown with food-simulating liquids or pH variations [273,301,302]. The reaction velocity for passive hydrolysis further increases with rising water uptake [275]. Additionally, oxidation processes on resin composite materials further the release of formaldehyde [303,304] and may result in discoloration of the fillings [305].

Both, passive and active degradation, result in the increased release of cleavage products furthering elution and material loss. As an overall effect, we observe a decrease in surface hardness as well as wear resistance in resin composite restorations for enzymatic [306-308] and passive hydrolysis [273,291,302]. All described processes – swelling, elution, active and passive hydrolysis – reciprocally influence each other and continuously lead to an increasingly instable polymer network. Naturally, these mechanisms affect the long-term stability of the hybrid layer, as the plasticization effects on the resin composite and the bonding material induce stress on the interface and challenge its integrity. Furthermore, the continuous displacement of interfacial adhesive resin by water leads to hydrolysis. Clinically, this frequently results in restoration loss [18,309].

2.5.2 Degradation of tooth tissue

The most common degradation of mineralized tooth tissue is caries. The cariogenic process is known to be initiated and mediated by cariogenic bacteria, e.g., mutans streptococci and lactobacilli. It describes a demineralization effect, which is the result of the acidogenesis of cariogenic bacteria following their metabolization of carbohydrates [310]. Though, acidic demineralization does not explain the breakdown of the teeth's organic matrix, which was traditionally linked to bacterial proteases. However, Tjäderhane et al. provided evidence that the proteolytic enzymes MMP-2, -8 and -9 are not only present and activated by the acidic environment in dentin caries lesions, but also prove their ability to degrade dentin organic matrix [311]. This finding supported previous research, which doubted the capacity of bacterial collagenases to effectively degrade dentin collagen on their own during dental caries processes [312,313].

As stated above, adhesive treatment, irrespective of the systematics used, does not entail perfect hybridization in dentin, but causes micro voids and leaves collagen fibrils unencapsulated within and at the bottom of the hybrid layer [44,212,252,253,257,259]. Nanoleakage not only furthers the degradation of resin components; a prolonged water immersion, as Hashimoto et al. depicted, leads to hydrolytic effects on the exposed collagen fibrils, which can be noted in a smaller fibril diameter, disorganisation of the collagen network and widened interfibrillar spaces [20].

2.6 Enzymatic degradation

In 2004, Pashley et al. proposed the possibility of the degeneration of denuded collagen matrices in the hybrid layer by host-derived enzymes utilizing nanoleakage channels. Their research has proven the degradation of collagen fibrils in the absence of bacterial colonization, thus implying the importance for effective collagenase inhibition to improve the longevity of adhesive-based restorations [21]. Therefore, in recent years, dental research – inter alia – focused on identifying the culpable proteolytic enzymes for collagen degeneration to better understand the underlying processes and correlations [314-327].

2.6.1 Proteinases

Matrix metallo proteinases (MMPs) and cystein cathepsins (CCs) form families of proteolytic enzymes known and specialized for the degradation of extracellular matrix [328-330]. In recent years, dental research linked MMPs as well as CCs to the collagen proteolysis in caries lesions [311,323,327] and beneath dental restorations which furthers caries progression and impairs bond stability [21,326]. To better understand the micromolecular processes that trigger enzymatic collagenolysis in dentin and subsequently how to prevent it, comprehension of structure and function of MMPs and CCs is crucial [326].

2.6.1.1 Matrix metalloproteinases

MMPs derive from the enzyme family of metzincins [331] and are involved in extracellular matrix (ECM) degradation as physiologically necessary in tissue turnover and morphogenesis, for example during growth, reproduction and in wound healing [332-336].

The ECM does not only function as a stabilizing scaffold, but also as a depot for biologically active agents, for instance growth factors [337]. Moreover, research suggests that the ECM itself and its cytoarchitecture regulates cell signalling and thereby influences cell growth and apoptosis [338-340]. Hence, MMPs indirectly control the same processes by remodelling and changing the organizational structure of the ECM. Then again, the released degradation products are biologically active [341]

A close regulation of these proteinases is organized at the transcriptional, post transcriptional and at the protein stage which additionally controls secretion, zymogen activation and proteinase inhibition [342]. Latter is mainly modulated by human TIMPs (tissue inhibitors of metalloproteinases), a protein family of four (TIMP-1, -2, -3, -4), which share a similar protein structure and fold [343]. They form tight, non-specific [344] 1:1 ratio complexes with MMPs through the inhibitory binding site that is localized in the N-terminal loops of the TIMP molecule [345]. Disruption at any of the regulation levels causes degenerative pathological processes as recognized in tumour progression [346-348], arthritis [334] and cardiovascular diseases [349,350], among others.

Human MMPs constitute a family of 23 zinc- and calcium-dependent peptidases, sharing sequence homologies in the propeptide and catalytic domain [337,351]. MMPs generally exhibit a N-terminal signal protein that is eliminated before leaving the cell and linked to a propeptide and a catalytic domain. At the C–terminal end a hemopexin-like domain is found, which is covalently bound to the catalytic domain by a prolin-rich hinge region [337,352]. The blockage of the catalytic center is preserved by a close bond between the Cys-73 residue of the prodomain peptide chain and the active site zinc atom/ion [353]. The disruption of this bond activates the zymogen by replacing Cys-73 as the fourth ligand of the zinc coordination sphere with H_2O, which emphasizes the dependency on water for the catalytic reaction [354], as previously suggested by Pashley et al [21]. The dissociation of the propeptide is a stepwise process mediated by various triggers, e.g., proteinases or non-enzymatic reactive agents like SH-reactive agents, mercurial compounds, reactive oxygen, denaturants, or acidic environments [355-358].

Collagenolysis by activated MMPs is a stepwise and complicated process [359] that is still not fully understood. Beside the collagenases MMP-1, MMP-8, MMP-13, MMP-18, also gelatinase A (MMP-2) [360,361] and membrane-type 1-MMP (MMP-14) [362] are capable of proteolyzing collagen specifically, which will be presented in the following [363]: The hydrolysis of the peptide bond of native collagen types I, II and III takes place after the Gly residue of a partial amino acid sequence Gly- [Ile or Leu]- [Ala or Leu] at a single locus approximately three-fourths from the N-terminus [364-367]. As several possible

cleavage sites consistent with those prerequisites exist, researchers hypothesize that the amino acid sequence surrounding the collagenase cleavage site is uniquely recognized by MMPs for binding (with their noncatalytic domains) and probably even facilitates hydrolysis [364]. For type I collagen a four-residue long sequence (RGER) was identified as binding locus for non-catalytic domains C-terminal to the site (the first R residue being on position 805 on α_1 and 798 on α_2). The theory is, as the hemopexin-like domain of MMP-1 seems to be in proximity with the RGER sequence, that both sequences probably interact with each other [368,369]. These interactions might further promote the dissociation of the peptide chains from the triple helix aiding the full insertion of the α_2 peptide backbone into the MMPs catalytic cleft [368]. Both the molecular and supramolecular collagen structure restricts the accessibility of the cleavage site situated in a narrow, only solvent-accessible cleft. Additionally, the α_2 peptide chain, which is the most dissociated and proteolytically vulnerable [370], is shielded by the C-telopeptide from proteolysis [368]. Recent research thus confirmed that the removal of the C-terminal end, enzymatically or non-enzymatically, enlarges the entry to the cleft and directly uncovers α_2 at the cleavage site. A possible non-enzymatic trigger could be a traumatic event or damage, which then results in structural changes, for instance fracture of the crosslinkages of the C-telopeptide, thus leading to the exposure of α_2 starting the cleavage process [368]. The cleavage of the triple-helix then proceeds successively, meaning that MMP1 hydrolyses each peptide chain one-by-one, after starting with the α_2 peptide [363]. The degradation is then furthered by gelatinases [342] supported by the tendency of collagen to disassociate at body temperature [371] after the cleavage is commenced by a collagenase [368,372].

2.6.1.2 Cysteine cathepsins

CCs are lysosomal proteases that belong to the subfamily of papain-like enzymes, due to their protein folding [373]. The human genome encodes for 11 CCs, in detail cystein cathepsin B, C, F, H, K, L, O, S, V, X, W [374]. Even though CCs are divided into cysteine cathepsin L- (CC-L) or cystein cathepsin B (CC-B)-like proteases [375], they share homologies in structure and composition as exemplified by CC-L [328,376-378]: CCs consist of two domains, referred to as the left (L-) or right (R-) domain. The proteolytic activity is provided by the catalytic site residues Cys25 and His163 (CC-L numbering) – each derived from a different domain – that form the catalytic thiolat-imidazolium ion pair in the center of the reactive site. The N-terminal end of the propeptide starts at the top of the enzyme at the "propeptide binding loop" (PBL), further running through the active-site cleft in reverse to the substrate, thus preventing the substrate from accessing the active site, to the N-terminus of the enzyme [377-380]. The propeptide fulfils multiple functions beside the inhibition of the catalytic activity: It supports proper folding and prevents the enzyme from inactivation or denaturation in neutral or slightly basic environment [328,379,381,382]. Except for cathepsin X (CC-X) [383], zymogen activation of CCs is an autocatalytic, step-by-step process triggered by low pH. In acidic environments the flexibility of the propeptide is enhanced, as the interactions with the mature enzyme are weakened [380,384,385], thus initiating a conformation change of the propeptide in the active-site cleft resulting in the activation of the latter/catalytic site. The definitive removal of the prodomain occurs in multiple steps, if activated and catalytically active zymogens come into close contact resulting in reciprocal cleavage [380].

Physiologically, CCs are associated with very different cell processes: Cathepsin S (CC-S), CC-L, CC-B mediate the MHC-II antigen presentation to immune cells [386-389] and cysteine cathepsin K (CC-K) is prominent in bone remodelling, being primarily involved in collagenolysis [390,391]. As in the case of MMPs, pathological processes are known to evolve from the dysregulation of CCs and of their activity. Thus, the prevention of potentially harmful and uncontrolled proteolytic processes is ensured through

different regulatory measures: Compartmentalization within organelles, zymogen activation, activity regulated by ambient pH and inhibition by protein and small-molecule inhibitors [328,392-395].

The mechanism of collagenolysis by CCs is best described based on the model of Schechter and Berger [396]: The nomenclature S4 to S3' labels the seven substrate binding subsites, the binding pocket of CCs is subdivided in. Again, each binding subsite interacts with their counterpart residues P4 to P3' on the substrate. The cleavage is initiated at the scissile bond between P1 and P1'. Interestingly, the substrate specifity of CCs is mainly determined by their S2 binding subsite [397,398]. So exemplarily, CC-K can be identified by its unique preference for a prolin residue on the P2 and a glycin residue at the P3 position of its substrate [398]. As the collagen type I molecule has multiple sequences fitting that preference [398], CC-K is the only mammalian collagenase able to cleave collagen I at multiple sites [391].

2.6.1.3 MMPs and CCs in dentin

Physiologically, the functions of interstitial proteases in dentin are not well understood. But in accordance with their role in tissue remodelling and turnover, they are associated with tertiary and peritubular dentin formation and the secretion of growth factors [399-401]. Even though dentin does not rely on extensive, gradual restructuring, as does bone, it still undergoes change resulting from functional demands, e.g., through modifications of predentin or non-mineralized tubular dentin [319].

Various MMPs are expressed by human odontoblasts and pulp tissue [402]. MMP-2,-8,-9,-20 are the most abundant metalloproteinases in human dentin [318,403-405]. Dentinal MMPs are secreted by odontoblasts during the formation of the dentin matrix and are probably involved in dentin formation.[319,400,402]. Due to mineralization of the dentin matrix, MMPs are then entrapped, until their reexposure due to cariogenic or restorative processes [399]. Tjäderhane et al. showed that the breakdown of demineralized dentin collagen matrix in carious lesions is closely related to the activity of endogenous MMPs [311]. For a long time, the destruction of the collagen network in carious lesions was thought to be caused by bacterial collagenases. However, Van Strijp et al. prove in their research, that bacterial collagenases, harvested from dentinal carious lesions, could neither degrade collagen in vitro nor showed sufficient activity, even when purified, in acidic environment [312].Therefore, it was concluded that mainly host-derived interstitial collagenases, namely MMPs and CCs, cause the destruction of the organic matrix in carious lesions [311,323,399].

Moreover, MMP's proteolytic activity was also proven in non-carious dentin in absence of bacteria. The associated study further highlighted the dependence of the proteolytic activity of these enzymes on water, as there was no evidence of collagen degradation after 250 days storage in mineral oil [21]. The activity of MMPs after adhesive treatment [21] is probably due to zymogen activation through low pH values during interface conditioning. This theory is supported for simplified and two-step etch-and-rinse [315,406] and all kinds of self-etch adhesives [315,320,407,408]. CCs are equally linked to the failure of adhesive restoration, as documented by various research groups [326,409]. Similar to MMPs, odontoblasts and pulp tissue exhibit a vast expression of CC genes in intact teeth [325], but definitive occurrence via antibody detection and verification of proteolytic activity in both carious and intact dentin was only demonstrated for CC-B and CC-K [323,327,410]. Both enzyme families can be triggered in acidic environment, though while CCs exhibit highest proteolytic activity at an acidic pH range, MMPs are known to be neutral proteinases [311,411,412]. Research indicates reciprocal activation between CCs and MMPs [413,414]. Further, Scaffa et al. prove close proximity between CC-B and MMP-2 on the collagen fibril [324]. Evidently, a close interaction and cooperation of both proteinase families in the proteolytic degradation of ECM must be assumed. Thus, collagenolysis in dentin in carious lesions and beneath dental restorations is probably the result of CCs' and MMPs' close interaction.

2.7 Prevention of enzymatic degradation

As activated proteases bear the potential of disintegrating the resin-dentin interface by degrading components of the ECM and support dental caries progression [21,311], dental research has placed a lot of emphasis on how to stop proteolytic hydrolysis [319,409,415-417]. Mainly, the two following strategies have been pursued: 1.) inhibition of proteolytic enzymes, via chelation or blockage of the catalytic center 2.) reinforcement of the collagen network by crosslinkage rendering it less assailable to proteolytic attacks.

2.7.1 Inhibiting agents

Biochemical science describes four different mechanisms for reversible enzyme inhibition, either competitive, non-competitive, uncompetitive or mixed competitive/non-competitive interaction [418]. The classification is dependent on how the inhibiting agent affects enzyme kinetics and interacts with the enzyme binding the enzyme in its free state, the enzyme-substrate complex, both or at its active site [418-420].

2.7.1.1 Chlorhexidine

Chlorhexidine (CHX), 1,1′-hexamethylene-bis-5-(4-chlorophenyl)biguanidide, is a surface active ammonium compound, which is well-known and commonly used as an antibacterial and plaque-reducing antiseptic in various dental applications [421,422]. Its bacteriostatic and bactericidal properties [423] have proven effective against a broad spectrum of gram-positive and gram-negative bacteria [424], furthermore it has shown effectivity against yeasts, dermatophytes and certain viruses [425]. Its antimicrobial characteristics derive from its ability to bind strongly to negatively charged bacterial cell walls, as its pK_a values (=2.2, =10.3) ensure dicationic charge over the entire range of physiological pH values [421]. The binding results in a dysfunction of the lipoprotein-based cell membrane and causes increased cell permeability. The severity of the cell dysfunction is depending on the CHX concentration used and leads to either bacteriostasis (low concentration) or to cell death (high concentration) [426-428]. CHX is commercially available as salt, e.g., CHX digluconate, diacetate or dihydrochloride, since the free-base is barely soluble and only exists above pH 12 [421]. The solubilization effectivity is dependent on the salt used. Since the use of CHX digluconate ensures high solution concentrations and presents to be advantageous compared to both CHX diacetate and especially CHX dihydrochloride, this chemical compound is usually the preferred choice [429].

CHX shows inhibiting effects on MMP-2, -8, -9 that are based on an unspecific chelating mechanism and interaction with sulfhydryl groups [430]. The chelating mechanism deprives MMPs of the Zn- and Ca-ions, that are integral to their function [431]. The inhibition of CC-B, -K, -L, by contrast, is linked to a direct interaction of CHX with subsites S2 to S2', proven by molecular docking analysis [432]. Early study results suggest a dose-dependency for the inhibitory effect on MMPs [430], the correlation between CHX concentration and bond strength is unfortunately not as clear [433]. A recent meta-analysis though showed that the application of 2 % CHX did not adversely affect immediate bond strength, both 0.2 and 2% CHX did improve the stability of bond strength during aging [434]. However, research data suggests that CHX' inhibiting effect might be reversible over time, as a significant decrease in bond strength can be noted - dependent on the bonding protocol and carious/ non-carious substrate – after 12 to 18 months in vivo [435,436]. Thus, probably due to the electrostatic nature of the CHX proteases interaction and

CHX binding, its efficacy is prone to leaching and displacement [437,438]. Nevertheless, CHX remains the best researched protease inhibitor in dentistry and allows for practicable applicability in clinics due to its easy availability and high efficiency. Limited application time as low as 30 s is sufficient to inhibit enzymatic activity on dentin [437].

2.7.1.2 Hesperidin

Hesperidin (HPN) belongs to the flavanones, a subfamily of flavonoids consisting of glycosides deriving from three main aglycones: hesperitin (4'-methoxy-3',5,7-trihydroxyflavanone), naringenin (5,7,4'trihydroxyflavanone) and eriodictyol (5,7,3',4'-tetrahydroxyflavanone) [439]. Chemically, HPN is the β-7- rutinoside, viz a disaccharide, of the aglycone hesperitin [440]. Flavanones are almost exclusively extracted from citrus fruits [441] and present various therapeutic effects on human diseases, including cardiovascular diseases, neurological and neoplastic disorders [442]. Hesperidin's antioxidant [443], anticarcinogenic [444] and antimetastatic [445] properties appear to be closely linked to its antiMMP activity. In dental applications, HPN exhibited beneficial effects in reducing proteolytic degradation, in preserving the hybrid layer and improving of micromechanical properties of dentin [446-448].

Due to its similarity in structure – based on the flavan moiety – HPN was initially falsely categorised as crosslinking agent same as the bioflavonoids epigallocatechin-3-*O*-gallate (EGCG) and proanthocyanidin (PA) [449]. Recent data however contradicts this categorisation, as neither the triple-helical structure nor thermal stability of collagen was altered by the administration of HPN [450]. This conversely means that the increased resistance against proteolytic degradation following the application of HPN, recognized in previous studies [446], is caused by an inhibitory mechanism rather than collagen crosslinkage [450]. The theory is that HPN might loosely bind to collagen, possibly blocking the proteases' binding and/or cleavage site and thus inhibiting their activity [450]. Like CHX, HPN might be subjected to leaching and displacement, especially as the interactions with collagen seem to be of non-covalent nature [450].

2.7.2 Crosslinking agents

An early study by Vater CM et al. in the 1970s proved that collagen crosslinkage decreases vulnerability of collagen to the degradation by mammalian collagenases [451]. In medicine, collagen crosslinking agents have been intensively studied in relation to bioengineering of tissue scaffolds and bio-protheses. Fixation of biological tissues improves the resistance against enzymatic degradation and reduces their antigenicity following implantation [452,453]. Traditional crosslinking reagents like formaldehyde, glutarylaldehyde (GA) and carbodiimide exhibit major disadvantages, such as high cytotoxicity, instability, or barely controllable crosslinking rates [452,454-456]. Naturally derived crosslinkers, e.g., PA, EGCG, and riboflavin (RB), offer new possibilities for tissue engineering and the stabilization of collagen scaffolds due to their low toxicity, high bioavailability, and favourable crosslinking capacities [456-458]. Early studies introducing these agents to dental applications confirmed their positive effects on dentin mechanical properties [446,459,460], proteolytic stability of collagen matrices [446,461], dentin bond strength [446,462,463] and inhibition of proteases [463,464].

2.7.2.1 Proanthocyanidin

PA is a naturally occurring nutrient, found as plant metabolite in fruits, vegetables, nuts, seeds, and bark [465,466]. PA is counted to the group of condensed tannins, which in turn is a subfamily of the bioflavonoids [465]. Its basic structural unit is phenolic flavan-3-ol ("catechin"), which can either exist in an oligomerised state (soluble), containing two to six catechin units, or in a polymerised state (insoluble; more than six units). The oligomerisation principally occurs through condensation of flavan-3-ol units on positions 4 and 8 [467,468]. Chemically, PA presents a heterogenic group of compounds relating to their various oligomerisation states, and thus is also referred to as oligomeric proanthocyanidin complexes (OPCs).

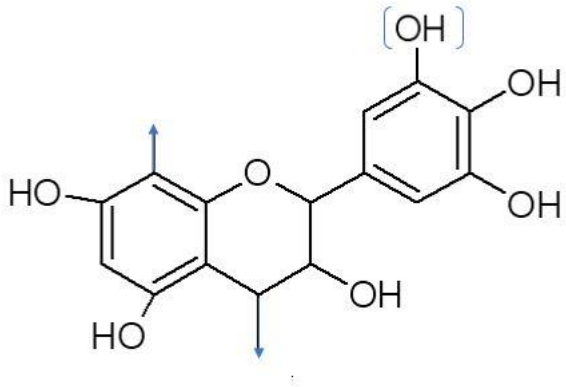

Figure 3 Flavan-3-ol oligomeric unit modified after Haslam et al. 1996

PA is preferentially extracted from grape seed [469] and is widely reviewed for its many positive biological properties and possible therapeutic applications: It shows antioxidant, antiviral, vascular protective, vascular dilatative, anti-inflammatory, and anticarcinogenic and potential [466,470-472]. Interactions with proteins include covalent binding [473], ionic [474], hydrophobic [456] and dipole-dipole attractions [475]. Detailed description about the nature of PA and proteins is summarized in the paragraph "Tannin-protein interactions" due to the similarity in that matter for both PA and EGCG.

PA-collagen crosslinkages mediate between different structural hierarchy levels depending on the degree of PA oligomerisation and thus on molecule length. Higher oligomeric PAs span crosslinkages not only intramolecularly, improving biostability, but also intermolecularly and inter-micro-fibrillarly, enhancing collagen's overall mechanical properties [476]. Furthermore, Epasinghe et al. proved PA to be a more effective protease inhibitor than CHX, showing dose-dependent inactivation of soluble recombinant MMP-2, -8, -9 at 90 %, of CC-B, -K at 70 to 80 % and reducing endogenous hydrolytic collagenolysis significantly [464].

Though, for the introduction to dental restorative processes, the actual efficacy at reduced application times is an important factor for clinical feasibility: Consequently, various studies confirmed PA's above-mentioned beneficial effects on dentin in clinically efficient time protocols, inter alia in enhancing the resistance of the hybrid layer to enzymatic degradation potentially preserving bond durability [477-480].

A complicating aspect for the usage of PA in restorative dentistry is its antioxidant capacity to scavenge free radicals [481], which in a restorative context can cause interference with the radical polymerization of classical dental adhesives [482,483]. At higher PA concentrations this leads not only to a reduction in

DC of the used adhesive, but may also significantly lower bond strength [483], which in turn challenges the possible application modes for PA: As a rinse-off application, possibly as additive to phosphoric acid [478], PA might indeed have little to no effect on the quality of cure, but also lacks sustained release of PA, which would possibly prolong its protective effect on dentin collagen [482]. Contrarily, the incorporation of PA into the adhesive saves an additional step, though a study by Epasinghe et al. raises concerns for lowered bond strength and more extensive nanoleakage at a PA concentration of > 3 % [483]. However, Liu et al. presented evidence that even with an incorporated concentration of 5 % PA the DC can be maintained above 65 %, if either a TPO based photoinitiation system or one based on camphorquinone/amine/DPIHP (diphenyliodonium hexafluorophosphate) with doubled iodonium salt concentration is used [482].

2.7.2.2 Epigallocatechingallate

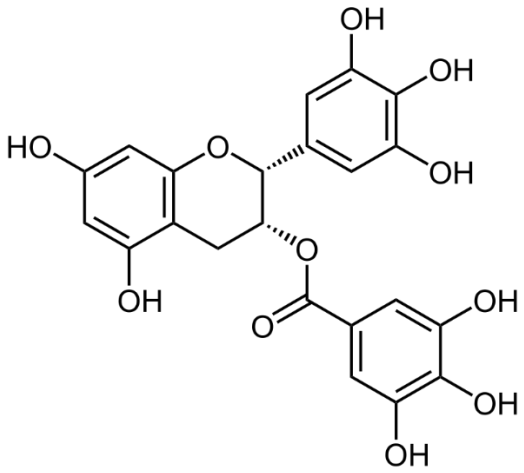

Figure 4 Structure of epigallocatchin-3-O-gallate

EGCG is the main catechin found in green tea [484,485]. The advantageous health benefits associated with the consumption of green tea, e.g. its anticarcinogenic, vascular protective, antioxidant, antiangiogenic and anti-inflammatory properties [486-488], are largely attributed to the EGCG moiety [489].

Studies in the early 2000s investigated the influence of EGCG on the expression, activation, and activity of various MMPs and CCs: Research of Demeule et al. indicates a certain inhibition specificity of EGCG on the activity of MMP-2, -9, -12 as well as proMMP-2 activation [490]. The results further stress the inhibition potency of EGCG in comparison with other green tea polyphenols [490]. Yun et al. even demonstrated significant inhibition of MMP-9 expression at a EGCG concentration as low as 20 µM [489]

Du et al. transferred the observations and applied them to restorative dentistry by incorporating EGCG in the formulation of a known E&R single component adhesive [491]. The results confirmed favourable antimicrobial effects while improving medium-term bond strength durability. Analysis of the DC of both experimental and unaltered adhesive further verified no adverse effects by EGCG on polymerization/ EGCG did not adversely affect polymerization [491]. Like PA, the radical-scavenging capacity of EGCG's phenol rings [487] might negatively interfere with the radical-based polymerization of a dental adhesive [482]. Though, a subsequent study on two self-etch adhesives did neither display significant differences in DC for varying EGCG concentrations (0 -100 µM) [492].

Early on, dental research attested EGCG effectivity against MMPS, CCs and bacterial biodegradation [493], improvement of dentin mechanical properties [493] and preservation of dentin bond strength at low-doses and reduced application times [491,492], making its use in clinics attractive. Also, EGCG presents adequate solubility in distilled water, ethanol and DMSO (dimethyl sulfoxide) enabling its use in various settings [494].

Due to the structural similarity and the close chemical relatedness, PA and EGCG share many properties. Therefore, the insights about their influence on enzyme activity and interactions with proteins are summarized below.

2.7.2.3 Tannin-protein interactions

Hydrogen bonds have been suggested to be the initiating driving force for protein-tannin complexes. As prolin-rich proteins (PRPs), like gelatin or collagen, are particularly strong hydrogen bond acceptors, this may explain – inter alia – the strong affinity between both moieties [495-497]. The formed hydrogen linkages are then additionally stabilized by adjacent hydrophobic pi-stacking between the proline's pyrrolidine and the phenolic rings [456,498]. Vidal et al. further provides information hinting towards covalent and covalent-like bond formation, i.e. Schiff-base (condensation product of aldehydes or ketones with primary amines), between PA and collagen [499]. This confirms the observations of Tang et al. in 2003, which stress the importance of hydrophobic interactions between polyphenols and collagen: They drew following conclusions from their research "…(1) the galloyl group of polyphenols is the functional group; (2) the strength of interactions are positively correlated with molecular size, the number of galloyl groups and the hydrophobicity of polyphenols; (3) the hydrophobic interactions are of great significance; and (4) the interactions are strongly dependent on the flexibility of galloyl groups…" [500].

Thus, it has been suggested that the interactions between tannins and collagen, as well as between tannins and MMPs are primarily mediated through the hydroxyl and galloyl moieties of their respective chemical structure [501,502]. This observation was much later confirmed for the inhibition of CCs by Vidal et al. [493]. The exact mechanism on how tannins, foremost PA, inhibit these enzymes is considered unspecific and it is still not fully understood, even though several hypotheses have been proposed on that matter: Similar to CHX, polyphenols possess a potent metal complexation capacity [467,503,504], which may deprive MMPs of Ca and Zn-ions, which are essential to their functionality [431].

Principally, 3D-conformational changes of the enzyme structure [505], direct blockage of either catalytic domain or an allosteric binding site [506] or the modulation of host-immune processes [507] were identified as possible target points for enzyme inhibition. Moreover, crosslinking stabilizes collagen fibril architecture and helical conformation, thus indirectly masking proteinase binding sites, e.g., the RGER recognition sequence, and restricting the accessibility for collagenolytic enzymes [368]. Furthermore, direct crosslinking of protease enzymes through covalent binding, e.g., interaction with prolin-rich hinge region of MMPs [337], might equally present a potential inhibiting mechanism that limits enzyme activity by restricting flexibility and mobility [463].

2.7.2.4 Riboflavin

By contrast, RB is a physical crosslinker and chromophore, characterized by its intensive yellow color and a non-toxic additive used in the food industry for vitamin supplementation or as a food dye [508]. Its crosslinking effect on collagen is attributed to its photosensitizing capacity that triggers both type I and type II photoactivation mechanisms when activated with light [509,510]. In the photochemical reaction of

light-activated RB with collagen the forming singlet oxygen, substrate radicals or ROS (reactive oxygen species: superoxide anion, hydroxylradical, hydrogenperoxide) [511] interact with amino acids along the collagen chain through oxidation resulting in the formation of covalent crosslinks. In ophthalmology, study reports documented increased mechanical rigidity of the corneal collagen network following the combined treatment of RB and UV- light (RB/UVA) [512]. The research group of Cova et al. successfully transferred the RB/UVA method to dental restorative applications to enhance and prolong the resin-dentin bond [463]. As RB shows various absorption maxima located in the UV and blue light spectrum at 446, 375, 265 and 220 nm [513] and previous studies confirmed beneficial effects of blue-light activated RB (RB/BL) on resin-dentin bond strength, formation and preservation of the hybrid layer and resistance to collagenolytic degradation [460,514]. In this context, blue light activation bears the advantage of easy clinical applicability and availability and shows a better tissue penetration compared to UV light and may therefore induce crosslinks at a deeper level in collagen [515].

2.7.3 Considerations for surface biomodification

As thoroughly discussed in the chapter “adhesion science”, every change in surface free energy, and concurrently in wettability is thus related to a change in atomic constitution as well as distribution and physical packing of substituents in the outmost surface layer [107-109]. By implication, every adsorbed film, applied additive or priming substance on a cavity surface, intentionally or unintentionally, potentially affects wettability and concomitantly successful adhesion either positively or negatively [91,92].

As crosslinking agents modify the collagen network and mechanical properties by inducing non-enzymatic crosslinks [516-518], it seems probable to assume that those changes also cause a shift in dentinal surface tension. Research by Leme et al. confirmed the hypotheses, at least for GA and PA, since the application of both crosslinkers reduced the hydrophilicity of demineralized dentin [519].

Though, this rule does not only enclose the bio-modifying agents, hence CHX, HPN, PA, EGCG, RB themselves, but also the chemical compounds that are used to solve them:

The adsorption of a monolayer of water on higher energy surfaces decreases γ_c considerably [114,121-123]. The more water is adsorbed, the more the critical surface tension γ_c resembles that of bulk water which is at 22 dynes/cm [124], which would also lead to a reduction in γ_c of smear-layer covered dentin, initially rated at 42 dynes/cm [202] and thus increase the mismatch in surface tension with adhesive resins ($\sim$ 40 dyne/cm) [195]. Contrarily, ethanol-saturation of dentin promotes resin-dentin bonding as first reported by Pashley et al. [520] followed by Tay et al. [521]. This approach displaces unbound [522] and in parts even bound water from collagen matrices [523], thus decreases residual water in resin-dentin bonds [524], enables infiltration of more hydrophobic resins/monomers [525,526] and allows for closer encapsulation of collagen by ethanol-solvated resins [228].

Also, DMSO, a polar, hygroscopic chemical compound with low surface energy [527,528], has in the past been used as solvent for crosslinking agents in various studies [446,529]. Recent dental research indicates improved immediate and preserved long-term bond strength, when DMSO is applied to dentin as a priming agent [530-534]. These favourable results are associated with an increased dentin wettability following DMSO treatment [535], which further betters adhesive penetration [531,536]. An additional advantage is the facilitation of radical polymerization by DMSO [228] leading to a significant increase of DC of commercially available dental adhesives [537], which might be another causative factor for the enhanced and more durable bonding performance.

In conclusion, inconsiderate surface treatment or usage of additives and solvents in bond strength studies may easily lead to distorted and biased results.

3. Summary (German):

Problemstellung. Der langfristige Verbund zwischen Dentin und Dentalkompositen birgt auch heute noch Herausforderungen, im Besonderen bei direkten Kompositrestaurationen. Einige Publikationen beschreiben bei Austausch weiterhin ein im Vergleich zu Amalgam vermindertes mittleres Restaurationsalter. Neben der generellen Anfälligkeit der Hybridschicht gegenüber Degradationsprozessen, beschäftigt besonders die enzymatische Schädigung des dentin-eigenen Kollagennetzwerkes durch aktivierte Matrix-Metallo-Proteinasen oder auch Cathepsine in den letzten Jahren die Fachwelt. Der Konsens hierbei ist, dass die Aktivität dieser Enzyme die Langlebigkeit von dentin-adhäsiven Restaurationen reduziert.

Zielsetzung. Die Überprüfung und Vergleich der Effektivität mehrerer bioaktiver Substanzen zur Verbesserung der mittelfristigen Verbundfestigkeit zwischen Dentin und Kompositharz mittels Scherfestigkeitstests. Dabei lag das Augenmerk auf der Entwicklung eines klinisch-orientierten und effizienten Klebeprotokolls (unter der Verwendung nicht-synthetischer Adjuvantien).

Methodik. Nach den Vorgaben eines Makro-Scherfestigkeitstests wurden 1100 dentin-basierte Probekörper hergestellt. Fünf ausgewählte, bioaktive Substanzen (Epigallocatechingallat=EGCG, Chlorhexidindigluconat=CHX, Proanthocyanidin=PA, Hesperidin=HPN, Riboflavin=RB) wurden entweder in destilliertem Wasser oder in der Primer-Phase eines kommerziell erhältlichen selbstätzenden Zwei-Schritt-Adhäsivsystems (Clearfil SE Bond 2, Kuraray Noritake, Japan) gelöst. Die Applikation der wässrigen Testlösungen auf das Dentin erfolgte vor, die der primer-basierenden Testlösungen als Teil des Adhäsivprotokolls. Ein modernes, schrumpfungarmes Bulk-Fill-Komposit diente als Restaurationsmaterial (Admira Fusion xtra, VOCO, Deutschland). Die Anwendung der Produkte Adhäsiv und Komposit ohne Veränderung des Protokolls und im Sinne der Gebrauchsanweisung diente als Kontrollgruppe. Die Prüfkörper der elf Testgruppen (n= 20) wurden in destilliertem Wasser bei 37 °C für eine Woche, einen Monat, drei Monate, sechs Monate oder ein Jahr gelagert und zuletzt in einer Universal-Prüfmaschine auf ihre Scherfestigkeit getestet. Im Anschluss erfolgte die Kategorisierung in die entsprechenden Frakturmuster (adhäsiv, kohäsiv und mixed) unter zehnfacher Vergrößerung. Die Daten wurden mittels Statistikprogramm (SPSS 24.0, Armonk, NY, USA) ausgewertet und anhand von 95%-Konfidenzintervallen, univariater ANOVA, Tukey HSD post-hoc Test ($p< 0.05$) und Weibull-Analyse verglichen.

Ergebnisse. Mittels univariater ANOVA wurden die Effektstärken der Parameter „Substanz“, „Applikation“ und „Lagerungszeit“ auf die Scherfestigkeit bestimmt: Dabei zeigte der Parameter „Substanz“ den größten Einfluss auf die Scherfestigkeit ($p< 0.001$; $\eta^2p= 0.074$). Interessanterweise war der kombinierte Effekt von „Substanz x Applikation“ ($p< 0.001$; $\eta^2p= 0.032$) größer als jener von „Applikation“ ($p< 0.009$; $\eta^2p= 0.007$) allein, was wiederum auf eine Abhängigkeit zwischen den beiden Faktoren hindeutet.

Für die unmittelbare Verbundfestigkeit, nach nur einer Woche Lagerung, wies die ANOVA keinerlei signifikante Unterschiede zwischen den Testgruppen auf ($p= 0.069$). Die post-hoc Analyse hingegen offenbarte signifikant höhere Scherfestigkeitswerte für HPNp im Vergleich zu RBp ($p= 0.016$). Jedoch konnte weder HPNp noch eine der restlichen Testgruppen nach einer Woche einen signifikanten Vorteil gegenüber der Kontrollgruppe präsentieren ($p> 0.05$).

Nach sechs Monaten Lagerungszeit, zeigte die post-hoc Analyse der Scherfestigkeitswerte von Substanz PA eine statistisch signifikante Überlegenheit gegenüber allen anderen Testsubstanzen an ($p\leq 0.05$) inklusive RB. Im Vergleich zur Kontrollgruppe ($m= 2.79 \pm 0.28$) konnte zu diesem Zeitpunkt zumindest eine

höhere Verlässlichkeit der Ergebnisse beider PA-Testgruppen konstatiert werden (PAp: m= 6.12 ± 0.86; PAs: m= 3.95 ± 0.21).

Während die Kontrollgruppe nach zwölfmonatiger Lagerung nur signifikant höhere Verbundfestigkeitswerte im Vergleich zu EGCGp (p= 0.014) zeigen konnte, präsentierte PAs signifikant höhere Werte gegenüber EGCGp (p< 0.001), CHXp (p= 0.019) and RBp (p= 0.020). Außerdem zeigten beide PA-Testgruppen die verlässlichsten Scherfestigkeitswerte nach diesem Zeitraum (PAp: m= 5.72 ± 0.60; PAs: 6.12 ± 0.86).

Sowohl die ANOVA-Werte als auch der Vergleich der Weibullgraphen, weisen auf eine höhere Stabilität und Konsistenz der Scherfestigkeitswerte der „Lösungs"-Testgruppen (EGCGs, CHXs, PAs, HPNs, RBs) im Vergleich zu den meisten der „Primer"-Testgruppen (EGCGp, CHXp, PAp, HPNp; außer RB: p= 0.792), über alle Lagerungszeiten hinweg, hin. In der post-hoc Analyse konnten jedoch alle Testgruppen abgesehen von EGCGp (p= 0.011) ihre Verbundfestigkeitswerte, zwischen einer Woche und einem Jahr Lagerung, verlustfrei halten.

Die übliche Kritik an der Validität von Scherfestigkeitstests aufgrund disproportional hoher Raten an Kohäsivbrüchen kann bei einem Anteil von 1,5 % aller Proben nicht bestätigt werden.

Schlussfolgerung. Mit Rücksicht auf die üblichen Limitationen einer in-vitro Verbundfestigkeitsstudie, kann konstatiert werden, dass die Einführung von PA in die adhäsive Zahnmedizin vorteilhaft sein kann. Die Daten weisen auf eine mögliche Verlängerung der Lebensdauer von direkten Kompositrestaurationen bei der Verwendung von PA hin. Während die unmittelbare Verbundfestigkeit nicht positiv beeinflusst wird, zeigen PAp und PA nach einem Jahr Lagerung verlässlichere Verbundfestigkeitswerte als die Kontrollgruppe. Die separate Applikation der Substanzen scheint zudem zu stabileren Ergebnissen innerhalb des Beobachtungszeitraumes zu führen. Allgemein betont die Studie die Notwendigkeit der Berücksichtigung chemisch-struktureller Besonderheiten, um negative Wechselwirkungen mit der Zusammensetzung des Adhäsivs zu vermeiden.

4. Abstract (English):

Statement of the problem. Achieving a long-term adhesive bond between dentin and resin-based dental composites remains a challenge, especially with direct resin-composite restorations. Some publications continue to report a reduced average restoration age in comparison to amalgam. Aside from the general susceptibility of the hybrid layer to degradation processes, the enzymatic degradation of the dentinal collagen network by activated MMPs or CCs has been an important concern for dental researchers in recent years. The consensus is that the activity of those enzymes compromises the longevity of dentin-adhesive restorations.

Objective. The aim is an experimental review and the comparison of various bioactive agents on their effectivity in improving the medium-term resin-dentin bond strength by means of a shear bond test (SBS). The focus lay on the development of a clinically effective, time-efficient protocol using mainly naturally sourced agents.

Methods. A total of 1100 human dentin-based specimens were prepared consistent with the requirements for a macro SBS test. Five agents (Epigallocatechingallate=EGCG, Chlorhexidindigluconate=CHX, Proanthocyanidin=PA, Riboflavin=RB, Hesperidin=HPN) were applied on dentin, either incorporated in the primer of a two-step self-etch adhesive (Clearfil SE Bond 2, Kuraray Noritake, Japan) or as an aqueous solution before applying the adhesive. A low-shrinkage bulk-fill composite (Admira Fusion xtra, VOCO, Germany) was used as restorative. Bonding protocol executed according to the manufacturer's information served as control. Eleven groups (n=20) were tested on a Universal testing Machine (MCE2000ST, Quicktest Prüfpartner GmbH, Langenfeld, Germany) with immersion times of one week, one month, three months, six months or one year (37°C, distilled water). The fracture pattern was then categorized (adhesive, cohesive, mixed) using ten-times magnification. Statistical analysis was carried out by means of statistic software (SPSS 24.0, Armonk, NY, USA) and the data was compared using 95%-confidence intervals, univariate ANOVA, Tukey HSD post-hoc tests ($p< 0.05$) and Weibull-analysis.

Results. Using a multifactorial, univariate analysis of variance (ANOVA, $p\leq 0.05$) with partial eta-squared statistics, the influence of the parameters "agent", "application mode" and "immersion time" on bond strength was assessed. The parameter "agent" showed the strongest impact on SBS ($p< 0.001$; $\eta^2_p= 0.074$). The combination "agent and application mode" ($p< 0.001$; $\eta^2_p= 0.032$) displayed a stronger effect on bond strength than "application mode" ($p=0.009$; $\eta^2_p= 0.007$) itself, suggesting a dependence between those parameters.

For immediate bond strength, after one week of immersion, the ANOVA did not display statistically significant differences ($p= 0.069$) among the test groups. However, post-hoc analysis revealed significantly higher SBS values ($p=0.016$) for the HPNp test group when compared to RBp. There was no significant difference between the control group and any test group ($p> 0.05$).

After 6 months immersion, post-hoc analysis showed superior SBS ($p\leq 0.05$) for PA compared to all other agents RB included and a higher reliability in both primer ($m=6.12\pm0.86$) and solution application ($m=3.95\pm0.21$) when compared to control ($m=2.79\pm0.28$).

While the control group, after twelve months of immersion, could only provide significant higher SBS values in comparison to EGCGp ($p= 0.014$), PAs exhibited significantly higher values when compared to

EGCGp ($p< 0.001$), CHXp ($p= 0.019$) and RBp ($p= 0.020$). Furthermore, after one year, both PA incorporated test groups demonstrated the most reliable outcome (PAp: $m= 5.72 \pm 0.60$; PAs: 6.12 ± 0.86).

Both the ANOVA-values as well as the interpretation of the Weibullgraphs indicated a higher stability of SBS values for the solution test groups (EGCGs, CHXs, PAs, HPNs, RBs) in comparison to the vast majority (exception RBp: $p= 0.792$) of primer test groups (EGCGp, CHXp, Pap, HPNp). In post-hoc analysis, apart from EGCGp, which showed a significant loss of bond strength in between 1 week and 1 year SBS ($p= 0.011$), all test groups, including control ($p= 0.996$), maintained their bond strength when 1week and 1 year SBS values were compared.

The common criticism that shear bond tests exhibit a high percentage of cohesive failures cannot be confirmed by this study because only 1.5% of all breaks were classified as cohesive.

Conclusion. Within the general limitations of a laboratory study, the results indicate possible advantages for the introduction of PA in adhesive dentistry as it may prolong the lifespan of direct resin-based restorations, thus reducing the replacement frequency, simultaneously leading to cost reduction and preservation of dental hard tissue. While the immediate bond strength is not positively impacted by PAp and PAs, both test groups, after one year of immersion, exhibited more reliable bond strength values than the control group. Furthermore, the separate application of agents appears to be associated with more consistent SBS results throughout the observation period. This research also stresses the importance of considering the chemical structure of an additive before incorporating it into the bonding process to avoid negative interactions with the formulation of the adhesive.

5. Publications

5.1 Paper I: Antioxidants and Collagen-Crosslinking: Benefit on Bond Strength and Clinical Applicability

Franziska Beck, Nicoleta Ilie.

Antioxidants and Collagen-Crosslinking: Benefit on Bond Strength and Clinical Applicability

Materials (ISSN: 1996-1944)

Journal Impact Factor: 3.623 (2020)

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5.2 Paper II: Riboflavin and Its Effect on Dentin Bond Strength: Considerations for Clinical Applicability—An In Vitro Study

Franziska Beck, Nicoleta Ilie.

Riboflavin and Its Effect on Dentin Bond Strength: Considerations for Clinical Applicability—An In Vitro Study

Bioengineering (ISSN: 2306-5354)

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Image Index

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