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New Index for the Stability of a Type I Collagen Affected by Hydrophobic Environment

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Effects of hydrophobic environment adjusted by various alcohols on the structural stability of calfskin collagen (CSC) were studied to elucidate the nature of collagen-monomer interaction in adhesion. The stability of CSC in aqueous alcohol solutions was represented by its denaturation temperature, T_d, measured by DSC. The hydrophobicity of the alcohol solutions was quantified with their specific dielectric constants, ε_r, calculated from their concentrations. The effect of each alcohol to stabilize or destabilize CSC was evaluated by the initial slope of each T_d *vs.* ε_r plot, denoted as $-(dT_d/d\varepsilon_r)_{ini}$ and termed as stabilization power. Results showed that a hydrophobic environment with a smaller ε_r lowered the stabilization power. Stabilization power ranged from −3 (strong destabilization) for phenol (ε_r=12) to +0.3 (weak stabilization) for glycerol (ε_r=47). In view of the encouraging results obtained in this study, the new index was therefore helpful in predicting the effects of new dental materials of known ε_r values on the stability of dentinal collagen.

Keywords: Collagen, Stability, Hydrophobicity

INTRODUCTION

With resin adhesive systems, primers are typically used to enhance dentin adhesion by the formation of a hybrid layer between dentin and bonding resin[1]. The hybrid layer, a new biological composite, is a result of dentinal collagen in contact with some organic agents — 2-hydroxyethyl methacrylate (HEMA) as a primer or ethanol and acetone as solvents for monomers. In other words, the initially hydrophilic environment of dentinal collagen becomes hydrophobic to some degree as a result of the primer treatment.

In general, the stability of proteins is very sensitive to the hydrophobicity of their environment because they are folded into the most stable conformation by a hydrophobic interaction among nonpolar segments in the molecular chain in an aqueous medium[2]. In this connection, it was reported that the stiffness of dentinal collagen was greatly affected by some organic liquids like acetone, ethanol, or HEMA[3]. In light of this finding[3], it is then reasonable to infer that the organic components in resin adhesives may affect the stability — or even the structure — of the fully hydrated dentinal collagen that has been exposed by etching prior to priming. This inference mandates further investigation and confirmation, since the integrity of the three-dimensional network structure of the exposed dentinal collagen is essential to enhancing the permeation of resin monomers and to forming a well-defined hybrid layer[4].

To date, the adsorption of a typical priming agent HEMA to a type I tendon collagen (like dentinal collagen) was proven by IR spectroscopy, and the amount of bound HEMA was directly related to the stability of collagen as determined by differential scanning calorimetry (DSC)[5]. In the same vein, it has been reported that the concentrations of HEMA and some simple alcoholic compounds affected the denaturation temperature of a tendon collagen, and likewise their alkyl chain lengths or number of hydroxyl groups[6]. In the report[6], the denaturation temperature of collagen was related to the hydrophilicity/hydrophobicity of alcoholic compounds as indicated by the hydrophile-lypophile balance (HLB) values[7] — although their physical meaning remained unclear.

However, thus far, not much information is available — qualitatively and quantitatively — in published literature on the relation between the structure of dentinal collagen and medium hydrophobicity/hydrophilicity; the latter being a result of treatment by organic dental materials in adhesive resins. Furthermore, information is lacking too on how the structural stability of collagen may impact adhesion efficacy. Against this backdrop of dire information scarcity, this study was undertaken with two specific aims for the new index: (1) it must be able to evaluate the effects of additives in altering the stability of a type I collagen; and (2) it must bear a clearer physical meaning unlike the empirical HLB values, and hence able to express the hydrophobicity of the medium surrounding the

collagen.

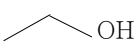
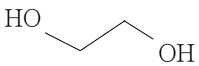
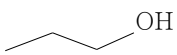
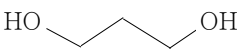
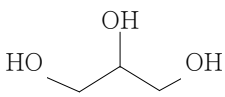
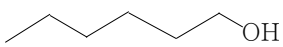
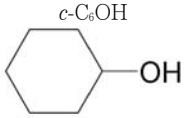
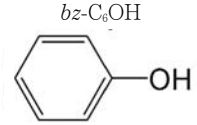
To adjust the hydrophobic environment systematically in the current study, various alcoholic compounds with different hydrocarbon structures and with different numbers of hydroxy groups were used. Some of which were closely related to dental adhesive components in clinical applications.

MATERIALS AND METHODS

Materials

Calfskin collagen type I (CSC; Sigma-Aldrich, St. Louis, MO, USA) was used in this study. Although it has less cross-links compared to insoluble dentinal collagen, the validity of its use would be mentioned in the Discussion section. To compare the effects of hydrophobic and hydrophilic structures in organic additives on collagen stability, the following alcohols were used: (1) monohydric alcohols with a straight alkyl group of different lengths (aliphatic alcohols) — methanol (*n*-C_1OH), ethanol (*n*-C_2OH), 1-propanol (*n*-C_3OH), 1-hexanol (*n*-C_6OH); (2) polyols with a straight alkyl chain — ethylene glycol (*n*-C_2OH_2), trimethylene glycol (*n*-C_3OH_2), glycerol (*n*-C_3OH_3); (3) monohydric alcohols with a hydrocarbon moiety of different types but with the same number of carbon atoms — 1-hexanol (*n*-C_6OH), cyclohexanol (*c*-C_6OH), phenol (*bz*-C_6OH). In the abbreviations given above in parentheses, the prefixes "*n*", "*c*", and "*bz*" stand for normal (straight), cyclic (ring), and aromatic (benzene

Table 1 Alcoholic compounds used in this study with the type of *x*-C_iOH_j

i \ *j*	1	2	3
1	methanol *n*-C_1OH 		
2	ethanol *n*-C_2OH 	ethylene glycol *n*-C_2OH_2 	
3	1-propanol *n*-C_3OH 	trimethylene glycol *n*-C_3OH_2 	glycerol *n*-C_3OH_3 
6	1-hexanol *n*-C_6OH 		
6	cyclohexanol *c*-C_6OH 		
6	phenol *bz*-C_6OH 		

ring) structures of a hydrocarbon group, respectively. The subscript indicates either the number of carbon atoms (i) or hydroxy groups (j) in the structure. These materials are summarized in Table 1 with their abbreviations. Molecular structures of these alcoholic compounds are also shown.

All the alcohols used in this study were of guaranteed reagent grade and purchased from Nacalai Tesque (Kyoto, Japan). They were used without further purification. Each alcoholic compound was diluted by distilled water to a desired concentration. The pH of the aqueous immersion solutions for CSC was adjusted using a dilute aqueous hydrochloric acid or sodium hydroxide of analytical grade. No buffer was used to preclude the influence of salts.

Differential scanning calorimetry (DSC) measurement

An approximate amount of 1 mg of CSC was immersed in 100 μL of aqueous alcohol solutions of various concentrations and left unstirred for an hour at 25℃. It was then blot-dried and brought into a sealable shallow aluminum cell so that an excellent thermal contact was achieved. An aluminum lid was placed on the cell and tightly sealed. It was used for differential scanning calorimetry (DSC) measurement conducted by a Pyris 1 DSC (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). Sample was scanned from 20 to 100℃ at 5 deg/min. In this study, the temperature giving an endothermic peak was chosen as the denaturation temperature of CSC (T_d). T_d values were averaged over three measurements for each condition.

RESULTS

Features of DSC profile obtained for CSC

A typical DSC thermogram obtained for CSC at neutral pH in the absence of any alcoholic compounds gave a denaturation-related endothermic peak at 47℃ with an enthalpy change (ΔH) of about 4 kJ/mol (Fig. 1). Although a pre-transition-like small shoulder appeared at a lower temperature side of the peak, it was not taken into account in the current study since it seemed to contribute little to the overall stability of CSC. At pH 3, the peak appeared at 43℃ with other features similar to those observed for neutral pH (Fig. 1).

Relation between denaturation temperature and alcohol concentration

Typical T_d *vs.* C (concentration) plots for ethanol (n-C_2OH) and ethylene glycol (n-C_2OH_2) systems in a full concentration range are shown in Fig. 2. While T_d decreased with increasing concentration of ethanol in the lower concentration region, it was otherwise for ethylene glycol. In other words, an opposing tendency in the stability of collagen — depending on the alcohol chosen — was illustrated here. Since such a distinct feature among the alcohols emerged in the lower concentration range, T_d *vs.* C plots hereafter would be limited to the lower concentration range for all the alcohols examined.

Figures 3A and 3B show the concentration dependence of T_d for a series of aliphatic monohydric alcohols (n-C_iOH) at acidic pH and neutral pH, respectively. T_d decreased with increasing alcohol concentration, and the degree of this tendency was pronounced for alcohols with a longer alkyl chain.

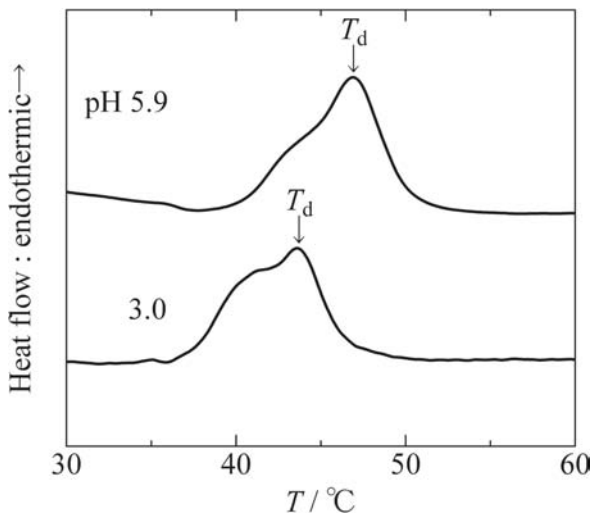


Fig. 1 Typical DSC thermograms obtained for CSC in the absence of any alcoholic compounds at acidic and neutral pH. Temperature giving an endothermic peak was chosen as the denaturation temperature, T_d, of CSC. While shapes of peaks were similar to each other, T_d was lower at the acidic pH.

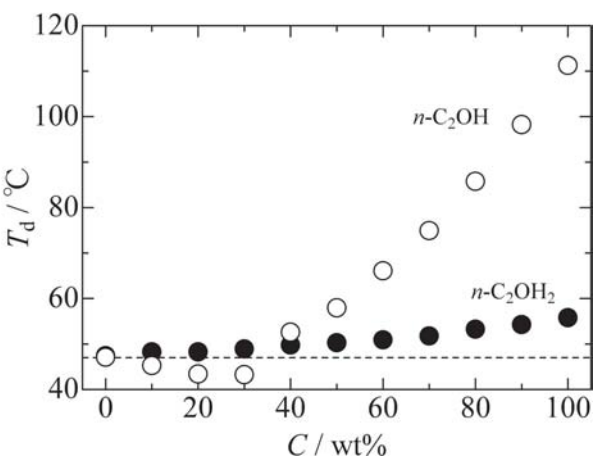


Fig. 2 Dependence of T_d on the concentration of either ethanol (open circle) or ethylene glycol (filled circle) in a wide concentration range. Ethanol showed a minimum in the plot while a monotonic increase in T_d was seen for ethylene glycol. Qualitatively important difference was observed in the lower concentration region.

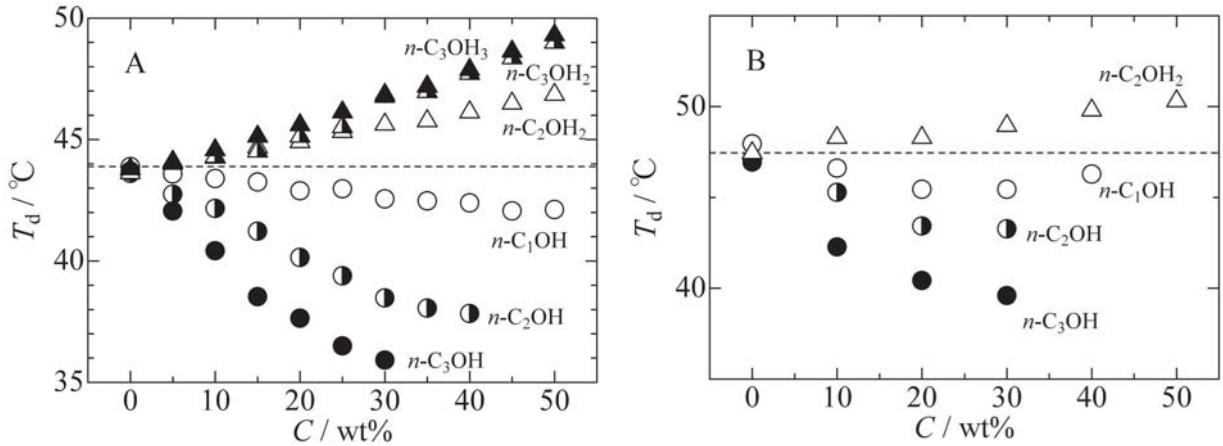


Fig. 3 Dependence of T_d on the concentration of alcohols with a straight hydrocarbon chain (circles) and various numbers of OH groups (triangles), measured at either pH 3 (A) or neutral pH (B).

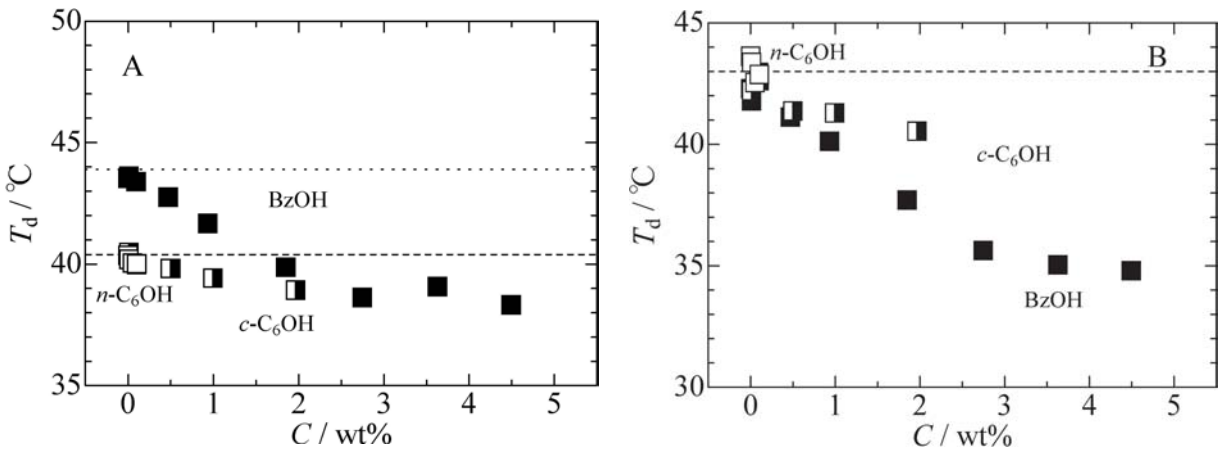


Fig. 4 Dependence of T_d on the concentration of alcohols with a hydrocarbon structure of various types composed of six carbon atoms, measured at either pH 3 (A) or neutral pH (B).

The dependence of T_d on alcohol concentration for a series of aliphatic polyols is also shown in Figs. 3A and 3B. In contrast to the monohydric alcohols, T_d increased with the concentration of polyols. In addition, the degree of increase in T_d was enhanced for polyols with more OH groups.

For the monohydric alcohols with different types of hydrocarbon moiety of the same carbon number (6), T_d drastically decreased with increasing alcohol concentration (Figs. 4A and 4B at acidic and neutral pH, respectively) compared to the aliphatic alcohols. The initial slope of T_d *vs.* C plot was steeper for aromatic alcohol (phenol).

pH dependence of T_d vs. concentration relations

Upon comparing the data at pH 3 against those at neutral pH, the most important difference was the overall shift in T_d. All T_d values were higher for the neutral pH group by *circa* 4℃ regardless of the concentration or type of alcohol. The tendency of concentration dependence of T_d was common between the two pH conditions.

DISCUSSION

Materials used in this study

While the CSC chosen in this study may differ from dentinal collagen in some ways, they too share many similarities. Both are of type I with a common amino acid composition and both have the same triple helix geometry composed of three α polypeptides which are alike: two α1(I) chains and one α2(I) chain. The key difference, then, between CSC and dentinal collagen seems to be the degree of crosslinking. In addition, our forthcoming spectroscopic study on the secondary structure of CSC affected by hydrophobic environment requires the collagen to be soluble. On these given grounds, a water-soluble CSC was used in this study instead of insoluble dentinal collagen.

Though most of the alcohols chosen here were not directly related to resin adhesive cements, they were used because of our objective to systematically adjust the hydrophilicity/hydrophobicity of different immersion media to investigate their effects on the

stability of CSC. Among the alcohols used in this study, ethanol is used in clinical applications as a solvent of monomers and phenol is structurally similar to hydroquinone — which is used in clinical applications as a monomer stabilizer.

Hydrophobicity index of immersion media

Dielectric constant, ε, is an electric property which is a measure of molecular polarity. While water has a specific dielectric constant (ε_r) that exceeds 80, many organic compounds have smaller ε_r values. The larger the discrepancy of ε_r between two materials, the lower is the affinity between them, and *vice versa*. Hence, hydrophobicity can be related to the difference in ε_r values between water and a material. Alternatively, it can be said that a material with a low ε_r is hydrophobic. For the alcoholic compounds used in this study, their specific dielectric constant values are listed in Table 2[8].

A merit of using ε_r is that hydrophobicity can be quantitatively expressed with a clearer physical meaning, as compared to other empirical parameters such as hydrophile-lypophile balance (HLB) values[7]. For this reason, we used ε_r instead of HLB values[6] to express the hydrophobicity of alcoholic compounds. The ε_r value has another advantage over HLB value in that while the use of HLB values is limited to pure substances, ε_r is applicable to a mixture of two or more materials of known ε_r values if the additivity of individual dielectric constants holds. For simple organic liquids such as ethanol or ethylene glycol, their ε_r values for mixture with water have been tabulated[9]. As shown

Table 2 Specific dielectric constants for the alcoholic compounds used in the current study[8]

Compound	Abbreviation	ε_r
methanol	*n*-C_1OH	33.0
ethanol	*n*-C_2OH	25.3
1-propanol	*n*-C_3OH	20.8
1-hexanol	*n*-C_6OH	13.0
cyclohexanol	*c*-C_6OH	16.4
phenol	*bz*-C_6OH	12.4
ethylene glycol	*n*-C_2OH_2	41.4
trimethylene glycol	*n*-C_3OH_2	35.1
glycerol	*n*-C_3OH_3	46.5

ε_r values were measured at 20℃, except for phenol (30℃).

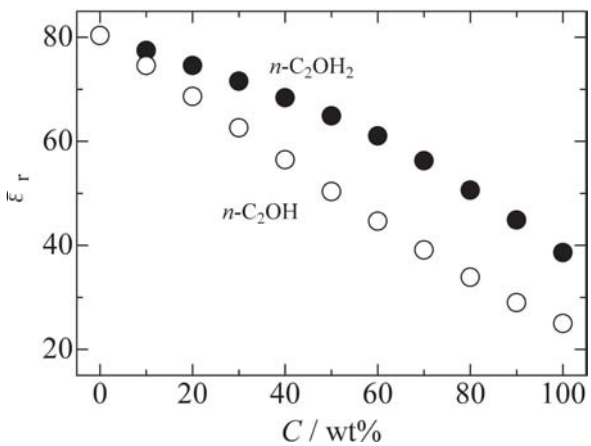


Fig. 5 Relation between concentration, C, and the corresponding specific dielectric constant, ε_r, for ethanol (open circle) and ethylene glycol (filled circle) in water. A linear relation was seen between C and ε_r at least within a limited concentration range. Data for the plots were taken from the Ref [9].

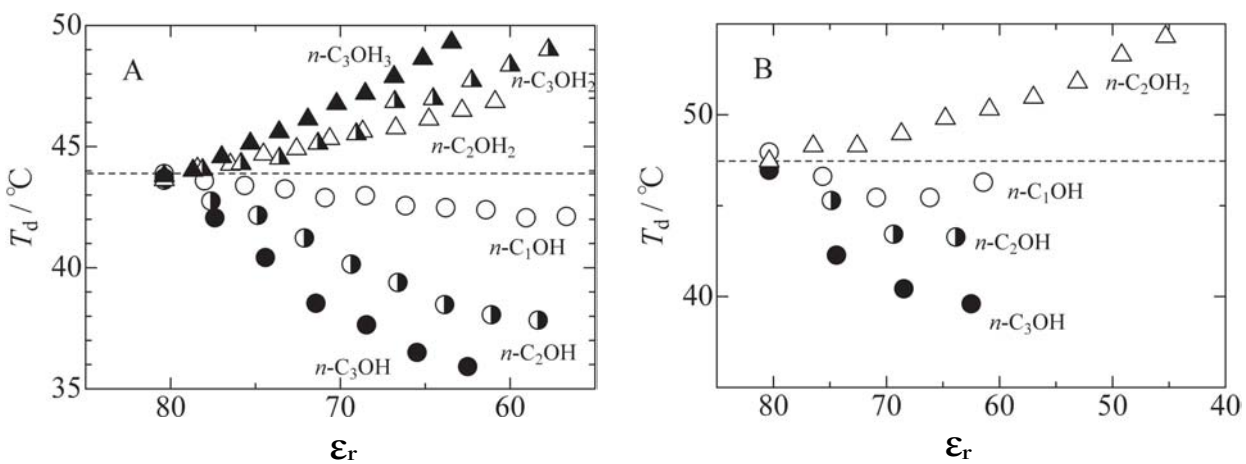


Fig. 6 Dependence of T_d on the specific dielectric constant of aqueous alcohols with a straight hydrocarbon chain (circles) and various numbers of OH groups (triangles), measured at either pH 3 (A) or neutral pH (B).

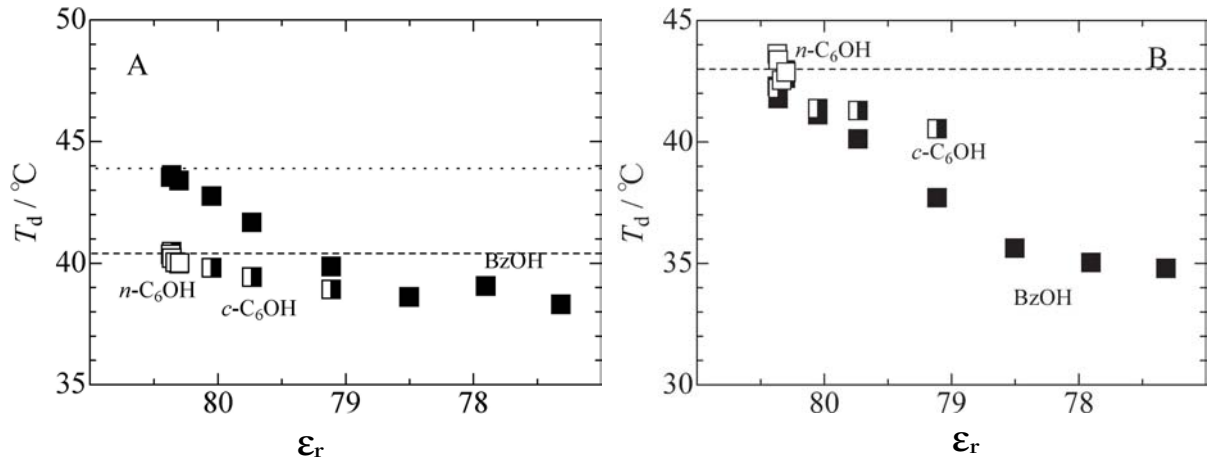


Fig. 7 Dependence of T_d on the specific dielectric constant of aqueous alcohols with a hydrocarbon structure of various types composed of six carbon atoms, measured at either pH 3 (A) or neutral pH (B).

in Fig. 5, ε_r is almost linear to C (concentration in wt%) at least within a limited concentration range. Assuming this linear relation between ε_r and C for a series of alcoholic compounds studied here, the concentration abscissas in Figs. 3A, B and 4A, B were replaced by ε_r, and T_d could now be directly plotted against the hydrophobicity of the medium (Figs. 6A and 7A at acidic pH, Figs. 6B and 7B at neutral pH). As expected from the quasi-linear relation between ε_r and C, the features in Figs. 6A, B and 7A, B resembled those in Figs. 3A, B and 4A, B, respectively.

Stability index of collagen

Figure 8 shows schematically a difference between the T_d of collagen in water and that at a minimum appearing in the T_d *vs.* C plot, ΔT_d, for ethanol (thick line)[6]. ΔT_d seemed to be a reasonable index to evaluate how strongly an alcoholic additive affected the stability of collagen, because the minimum became deeper as the additive bore a more hydrophobic nature[6]. However, ΔT_d — as a destabilization index — had the following shortcomings. Since a minimum appeared at the balancing point between the two opposing effects — that is, the decreasing (destabilizing) process as opposed to the increasing (stabilizing) process (see Fig. 8), it inevitably reflected mixed properties from these two opposing effects and which were inseparable. Furthermore, polyols could not be evaluated with this index because they showed no minimum in the T_d *vs.* C plot (Fig. 8, thin line).

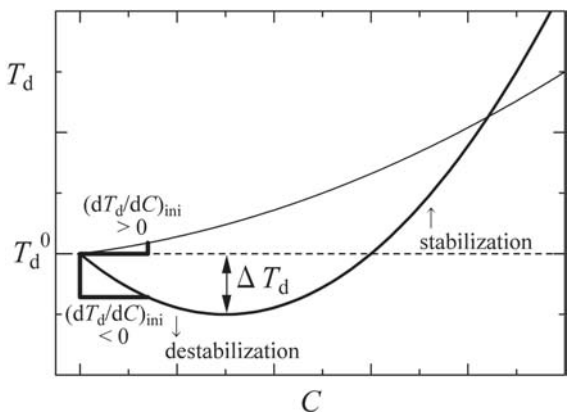


Fig. 8 Illustration of stability index, a measure of how strongly an additive affects the structural stability of collagen. ΔT_d: destabilization index defined before[6]; $(dT_d/dC)_{ini}$: stabilization power proposed in the current study. ΔT_d was defined as the difference between the T_d in water (T_d^0) and the T_d at the minimum of T_d *vs.* C profile as seen for ethanol (thick curve). Note that ΔT_d could not be defined in the case where no minimum appeared, as for ethylene glycol (thin curve). This shortcoming was overcome if an initial slope of the T_d *vs.* C plot, $(dT_d/dC)_{ini}$, was chosen, where its positive sign stood for a stabilizing effect and negative sign *vice versa*, and its magnitude represented the degree of stabilizing/destabilizing effect.

At this juncture, we thus proposed a new index to evaluate the effects of additives on the stability of collagen. The new index was defined as the initial slope of T_d *vs.* C (or ε_r) plot and denoted as $(dT_d/dC)_{ini}$ (or $-(dT_d/d\varepsilon_r)_{ini}$: a negative sign given for consistency sake with the tendency of dT_d/dC), as shown in Fig. 8. This new index had two features: (1) a positive value was directly related to the stabilizing effect, and a negative value *vice versa*; (2) the larger the value, the more pronounced was the effect because it was caused by even a small amount (or a small change in ε_r) of the additive. Hereafter, this initial slope would be termed as "stabilization power".

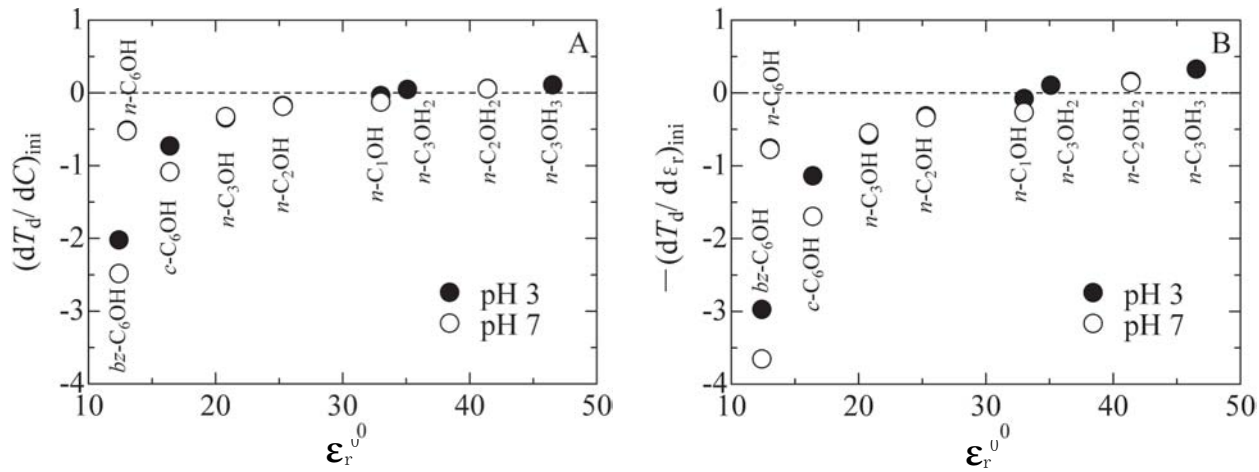


Fig. 9 Stabilization power (A: $(dT_d/dC)_{ini}$; B: $-(dT_d/d\varepsilon_r)_{ini}$) for various kinds of alcoholic compounds inspected in this study. The more hydrophilic (larger ε_r^0) an additive was, the more effectively it stabilized the collagen, and *vice versa*. Alcohols of similar type aligned in a straight line, while those with different hydrocarbon structures veered off the line.

Comparison of the stabilization power of alcoholic organic compounds bearing different hydrophobicity

For each alcoholic compound studied here, its stabilization power defined as $-(dT_d/d\varepsilon_r)_{ini}$ (or its original form, $(dT_d/dC)_{ini}$) was plotted against the ε_r of its corresponding pure compound (ε_r^0), as shown in Fig. 9 (Fig. 9A for $(dT_d/dC)_{ini}$ and 9B for $-(dT_d/d\varepsilon_r)_{ini}$). For the plots in Fig. 9, a smaller ε_r^0 value corresponded to a stronger hydrophobicity. Many features could be drawn from this plot pertaining to the relation between the hydrophobicity of additive and its power to either stabilize or destabilize the structure of CSC.

In general, the more hydrophobic an additive was, the more effectively it destabilized the helix structure of CSC, and *vice versa*. For example, the stabilization power $-(dT_d/d\varepsilon_r)_{ini}$ of phenol (ε_r^0 = 12.4) was 2.97 and that of glycerol (ε_r^0 = 46.5) was +0.33. Ethanol (ε_r^0 = 25.3) gave an intermediate value of −0.31. The specific dielectric constants, ε_r^0, of polyols are greater than those of monohydric alcohols with the same alkyl chain length (Table 2: ethanol < ethylene glycol; 1-propanol < trimethylene glycol < glycerol), and ε_r^0 is smaller for alcohols with a longer hydrocarbon chain (Table 2: methanol > ethanol > 1-propanol > 1-hexanol; ethylene glycol > trimethylene glycol). Therefore, it could be seen that the order of $-(dT_d/d\varepsilon_r)_{ini}$ corresponded to those of ε_r^0.

The hydrophobic interaction among amino acid residues folds a polypeptide strand into an ordered conformation. The energy gain of a hydrophobic interaction is larger in a more hydrophilic environment, and a hydrophobic interaction stabilizes the ordered structure of proteins. On the contrary, this stabilization effect may be reduced in a more hydrophobic environment, which could have resulted in the lower T_d for organic materials with smaller ε_r^0 values in this study. The stability of collagen helix is mostly explained by the hydrogen bonding among the three constituent polypeptide strands. Nonetheless, it should be mentioned that hydrophobic glycine residues are folded in the central (axial) position of the triple helix structure — and hence isolated from water. In other words, hydrophobicity of the environment soundly — and most pertinently — contributes to the structural stability of CSC. An almost linear relation between stabilization power and ε_r^0 was observed, except for C_6OH series with different hydrocarbon structures. Moreover, the slope seemed to reflect a structural or geometrical feature, although the detail is to be discussed in future. Nonetheless, these findings indicated that there were at least two factors affecting the stability of CSC: hydrophobicity of the medium as a global factor, and presumably a specific geometry-related interaction as a local factor.

In the current study, the effect of hydrophobicity on T_d, or the structural stability of collagen, was examined by DSC. However, the effect of hydrophobicity on the specific conformation of collagen is yet to be investigated. The latter could be done by some spectroscopic methods like nuclear magnetic resonance (NMR) or circular dichroism (CD) measurement. Of these two methods, CD would be more relevant to the current system since it can easily determine the conformation as well as the denaturation temperature of CSC. Therefore, besides the stability of CSC, we would soon disclose — by means of CD — how common alcohols affected the specific structure of CSC.

Relevance in the dental aspect

Some organic solvents, such as ethanol and acetone,

are used as diluents of adhesive monomers, and they are known to affect the stability of collagen[6,10,11]. Hydroquinone, which is typically added to a monomer component as a stabilizer, has a structure similar to phenol. While the content of hydroquinone in commercial products is quite small, it might be a potential substance that affects the stability of dentinal collagen even at a low concentration, deduced from the results of phenol used in this study. In the same vein, HEMA — a popular dentin primer — has an alcohol structure at the end of the molecule and is known to alter the stability of some collagens[5,6].

In the current study, the effect of inorganic salts was not inspected. Nonetheless, it should be another important factor since the dielectric constant of aqueous medium is affected by the addition of salts. On this note, it was reported that many types of salts lowered the T_d of CSC at a low concentration, while they raised the T_d at higher concentration by salting out[12]. In the present study, neither buffers nor salts were used to adjust and stabilize the pH of each solution. Instead, only a minimal amount of hydrochloric acid or highly diluted sodium hydroxide solution was added to accomplish the targeted pH. These solutions used to adjust pH contributed little to the ionic strength: at most 0.001 M for hydrochloric acid. At a molecular level, some adhesive-related substances were reported to interact with collagen[13-15]. It was highly expected that those substances would follow the general rule drawn from the current study in terms of hydrophobic interaction. Therefore, owing to the potentially significant bearing on practical dental applications, the validity of hydrophobic interaction for these compounds must be verified as well as for some general adhesive monomers.

As for the degree of crosslinking of collagen — including dentinal collagen, it is closely related to the aging of collagen in our body. It is generally known that as a creature gets older, its collagen exhibits a higher degree of crosslinking and greater stiffness[16]. In a previous study[17], the T_d *vs.* C relation was investigated for some collagens and their relatives with different degrees of crosslinking. It was revealed that as the degree of collagen crosslinking became more heightened, the following were observed: (1) the dependence of T_d on C became more prominent; and (2) the profile of T_d *vs.* C plot for a collagen was shifted to a higher temperature as a whole[17]. To investigate the aging effect of dentinal collagen on dentin adhesion using resin cements, a comparison between the collagen from a normal dentin and that from an aged dentin, in terms of sensitivity to a hydrophobic environment, would be necessary. A study for this comparison will be developed in near future. In addition, the relevance of current results to the permeability of fibrous dentinal collagen is also expected to be clarified.

It is well known that the adhesive strength to carious dentin is inferior than that to sound dentin. In the case of carious dentin, some acidic metabolites from cariogenic bacteria decalcify the dentin. As a result, dentinal collagen gets exposed to an acidic environment. In the same way, collagen treated at an acidic pH condition in the current study could be related to a case of decayed dentinal collagen. Our results showed that T_d values were lowered at all concentrations for each alcoholic compound by about 4℃ at pH 3, as compared to neutral pH. In other words, a lowered stability of collagen at an acidic pH was indicative of poorer adhesion on carious dentin. The stabilization power, $-(dT_d/d\,\varepsilon_r)_{ini}$, for each alcoholic compound was, however, scarcely dependent on pH (Fig. 9A or B). More specific interpretation of the relation between the stability of collagen and practical adhesion efficacy is currently under discussion.

CONCLUSIONS

Based on the findings of the current study, it was speculatively concluded that the structural stability and permeability of dentinal collagen might be affected by the hydrophilicity/hydrophobicity of the environment. A hydrophobic environment was caused by organic compounds added to adhesive resins to enhance dentin adhesion.

In the present study, hydrophobicity of the environment was represented in terms of specific dielectric constant of the medium, instead of empirical HLB values as proposed before. A new index was thus set up to measure the ability of adhesive-related organic substances in stabilizing or destabilizing the structure of collagen. Potentially, this new index could be used to predict the effects on the structural stability of dentinal collagen when a new dental material of known ε_r is applied.

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