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# The Interaction(s) between Calf-Skin Hyaluronic Acid (Hyaluronan) and Dermal Type I Calf-Skin Collagen under 254 nm UV Radiation: Ability of Hyaluronan to Alter Qualitative and Quantitative Dimerization of Collagen Tyrosine Residues

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## Abstract

The extracellular matrix (ECM) is the non-cellular component present within all tissues and organs, providing not only essential physical scaffolding for the cellular constituents and initiating crucial biochemical and biomechanical cues, required for tissue morphogenesis, differentiation and homeostasis. Roughly divided into two groups, these are 1) the main fibrous ECM proteins: collagens, elastins, fibronectins and laminins. 2) Classification of proteoglycans (PGs) is based on their location and binding. Although many different molecular interactions are possible, they depend on the cells' condition (*i.e.* "Normal", Aged, Wounded/Fibrotic, and cancerous). There is little or no data that addresses the influence of the surrounding ECM on dityrosine formation. As a simpler model, we have replaced total PG with hyaluronan (HA) and have used purified calf-skin collagen tyrosine, which forms dityrosine ($A_2$) under 254 nm UV in buffered solution and (near) physiological temperatures. Our results reveal a complicated temperature dependence involving factors relating to collagen HA structure, and collagen's photochemical activation parameters.

## 1. Introduction

The extracellular matrix in mammalian dermis (ECM) is the non-cellular component, providing not only essential physical scaffolding for the cellular constituents and initiating crucial biochemical and biomechanical cues. In mammalian dermis, the predominant molecules are 1) Type I and Type III (85:15) collagens and 2) modular proteoglycans (PGs) (*for review*, see [1] [2]). Although many different types of molecular interactions are possible, they depend on the cells condition (*i.e.* "Normal", Aged, Wounded/Fibrotic, and Cancerous). Furthermore, PGs themselves appear to bind to many cell-surface receptors with high specificity, thereby activating signaling pathways that control cell proliferation, differentiation, adhesion, and migration.

Previous studies document that collagen and HA interact with each other in the *ground state* under physiologically relevant conditions in rabbit synovium, [3] [4] [5] [6], *in vitro* [5] and in *aqueous solution* [7]. Results of rotary shadowing electron microscopy and computer simulation indicate that HA self-aggregates into highly-branched networks that can form two-fold aggregation structures at the ends of the helix. HA mixed with collagen *in situ* causes a shift in distribution of fibrils to smaller diameters [3]. Hyaluronic acid (hyaluronan, HA) does not bind covalently to collagen, but collagen and HA do interact by mutual steric exclusion. This noncovalent interaction enables HA to form non-ionic complexes [7].

As the presence of dityrosine is diagnostic for protein damage, its presence in proteins has been proposed as a molecular probe of UV-induced photodimerization (reviewed in [8] [9]). There is little or no data that addresses the effect of the surrounding ECM on the *photochemical* production of dityrosine via *excited state* tyrosine (A*).

We have used a model *in vitro* buffered collagen/HA system to study the influence of HA molecules on UV-induced photodimerization. Shimazu [10] found that the rate of photo-dimerization of tyrosine to dityrosine in aqueous solution at small irradiation times is quasi-linear and proportional to the initial tyrosine concentration [$A_o$]. Our preliminary results with collagen and HA reveal a complicated temperature dependence involving several factors relating to structure and conformation of the collagen, the HA, as well as photochemical activation parameters.

## 2. Experimental

### 2.1. Sample Preparation

**Collagens:** Type I acid-soluble calf-skin collagen samples (Elastin Products, Inc., Owensville, Missouri 65066) were prepared in phosphate buffer, pH = 7.4. Stock solutions of 25 ml of collagen + HA and matching collagen control (Coll (o) solutions were freshly prepared by prior to the photolysis for each experiment by separate 25.1 ml of collagen in 25 ml of buffer (1.0 mg/ml solution)) and 52.3 ml of Coll-HA in 25 ml buffer (2.0 mg/ml). Stock solutions were diluted 1:1 with

either buffer alone or buffer + HA solution to form either collagen alone (0.5 mg/ml) or 1:2 collagen-HA solution (1.0 mg/ml). If the collagen stock solutions are allowed to stand, both Coll(o) and Coll (HA) solutions show signs of autoxidation at the expense of tyrosine even when stored in the dark at 4°C.

## 2.2. Sample Characterization

**Fluorescence Spectroscopy:** Emission spectra of collagen samples were recorded on a Perkin-Elmer 650 - 40 fluorescence spectrometer in quartz cells (Hellma Cells, Inc., Plainview, NY, USA). The fluorometer was equipped with a thermostatted sample compartment, in conjunction with a circulating bath (Lauda, K-2R; Brinkmann Instruments, Westbury, NY, USA). Temperatures of the photolyzing sample were monitored inside the reaction cuvette with a calibrated model BAT-8 Thermometer (Bailey Instruments, Saddle Brook, NJ) equipped with a copper-constantan probe (Physitemp Instruments, Inc. Clifton NJ 07013).

**Irradiation Experiments:** Sample irradiation was carried out with a filtered 4 W UVG-11 hand lamp emitting primarily (≥95%) 254 nm radiation; total output 6.6 W/m$^2$ (UVP$^@$) (See **Appendix**). The amount of dityrosine was small at the short irradiation times used. The geometry was such that the irradiation impinged on the entire sample. We allowed the sample to equilibrate in the dark fluorometer for 5 - 10 minutes before recording fluorescence intensities. At a given temperature the slope of 400 nm fluorescence with irradiation time, $\Delta I_f(t)/\Delta t$, is proportional to the rate of [A$_2$] formation [10] [11]. Data are expressed individually and as the ratio $R$:

$$R = \frac{\text{rate of dityrosine formation in test sample}}{\text{rate of dityrosine formation in control sample}}$$

$$= \text{slope}(\text{test/control})_{400\text{nm}} \quad (1)$$

$R < 1$ signifies that the control sample degrades faster than the test sample, which denotes relative stabilization of collagen by HA; $R > 1$ indicates destabilization. Rates of A$_2$ formation were calculated for given temperatures between T = 8°C and T = 62.9°C for collagen Coll(o) and Coll(HA) samples.

## 3. Results

Photolysis of Collagen and Collagen + HA residues at 254 nm produces A$_2$ dimers in qualitatively similar manner to Shimazu's results at room temperature [10]. **Figure 1** shows that at short irradiation times, the initial formation of dityrosine is also linear with irradiation time, but the relative effect of HA depends on the temperature. At 8°C, HA *stabilizes* the collagen polymer ($R < 1$; **Figure 1(a)**), whereas at 51°C, HA *destabilizes* it ($R > 1$, **Figure 1(b)**).

**Figure 2** is a more detailed plot of the relative rates of dityrosine formation in Collagen (black circles) and Collagen + HA (white circles) from 8° ≤ T ≤ 70°. In the absence of HA, the rates of A$_2$ formation are more stable between ~20°C and
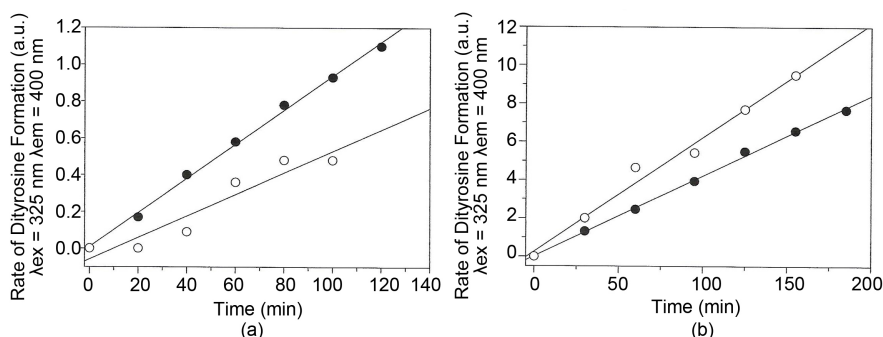
**Figure 1.** Formation of Dityrosine under 254 nm Irradiation of *Calf-kin Collagen* (1.0 mg/ml) and *Collagen + HA* (1.0 mg:2.0 mg) solutions in 0.1 M Phosphate Buffer, pH = 7.4, Black Dots-*Collagen Alone* solution; White Dots-*Collagen + HA* solution; Relative rate of dityrosine formation for each solution is given by the slope of the respective line in arbitrary units. (a) T = 8˚C Dityrosine formation is faster in the in the solution *without* added HA ($R$ = 1.8); T = 51.0˚C Dityrosine formation is faster in the in the solution *with* added HA ($R$ = 0.6).
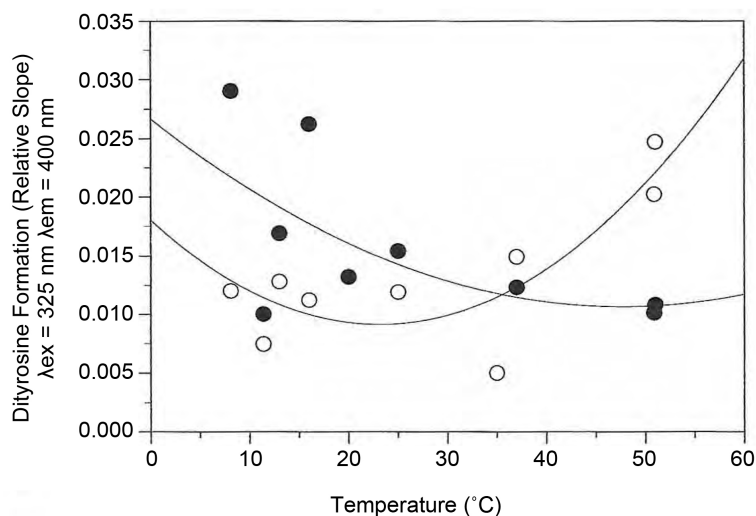


**Figure 2.** Effect of HA on UVC-Induced Dityrosine Formation at Temperatures Ranging from 8˚C - 50˚C under 254 nm. Irradiation of Calf-Skin Collagen (1.0 mg/ml) and Collagen-HA (1.0 mg/ml: 2.0 mg/ml) Solutions in 0.1 M Phosphate Buffer, pH = 7.4. Black Dots-*Collagen Alone*; White Dots-*Collagen + HA*. Fluorescence emission expressed in arbitrary units. The salient features of this figure are: 1) *Collagen Alone* (*Black Dots*): The stabilization of dityrosine formation in *Collagen Alone* at T < 10˚C is consistent with assertion of Leikina *et al.* that the energetically preferred state is the random coil [12]. At T < 10˚C, the collagen triple helix exists almost exclusively [12]. At 35˚C < T 50˚C stabilization by reversible predenaturational micro-unfolding of collagen "cooperative blocks" is significant and may result in the shape of the curve for collagen alone in **Figure 2**. 2) *Collagen + HA* (*White Dots*): The addition of HA *decreases* the rate of dityrosine formation below *Tm* (36˚C); above *Tm*, HA *increases* the rate of dityrosine formation. At T < 50˚C the collagen configuration in the *Collagen + HA* system may be restricted by the interacting HA molecules. Therefore, at T > 35˚C, the overall temperature dependence of dityrosine formation may depend primarily on photochemical activation parameters. With *Collagen-HA*, the overall rise in formation rate with at T > 35˚C may be attributed to a higher degree of micro-unfolding between 20˚C and 60˚C in Collagen-HA [12] [14], which favors photodimerization [12] [13] [14].

50˚C, but increase below 10˚C. On the other hand, in Collagen + HA, the rate of dityrosine formation is a minimum from 8˚C - 10˚C, and increases monotonically between 30˚C ≤ T ≤ 50˚C. At temperatures above 50˚C, both collagen samples start to denature and dissociate. The data for T < 20˚C for the collagen sample in the absence of HA is much less precise than at higher temperatures, resulting in a poor correlation coefficient ($r^2$ = 0.40) compared with $r^2$ = 0.77 for the companion collagen + HA sample.

Figure 3 is a plot of individual values of $R$ ratios as functions of temperature. The resulting curve is more "well behaved" than those in Figure 2 and can be reasonably be described by a 2nd order linear regression curve ($r^2$ = 0.69). At T < 15˚C, $R$ decreases markedly with rising temperature, having a value of 2.4 at 8˚C and decreasing to 1.4 at T = 20˚C. $R$ ~ 1.2 between 25˚C and 40˚C. At temperatures between 40˚C ≤ T ≤ 60 ˚C, $R$ < 1. Even here, however, the data tend to be scattered at T < 20˚C, although much less than in Figure 2).

## 4. Discussion

Previous literature has indicated that the collagen environment in the ground state is radically changed in the presence of HA [3] [4] [5] [6] [7]. Öbrink concluded from light scattering and turbidimetric studies under physiologic conditions that HA and collagen affect each other by a mutual steric exclusion [5]. In addition, there are several salient publications showing collagen-HA interactions in gels [4] [6]. In aqueous solution [7], there is visible evidence from electron microscopy that collagen increases the spacing between collagen in collagen HA co gels. Our results indicate a priori molecular interactions of collagen tyrosyl radicals in the excited state with ground state HA. Such a situation is,
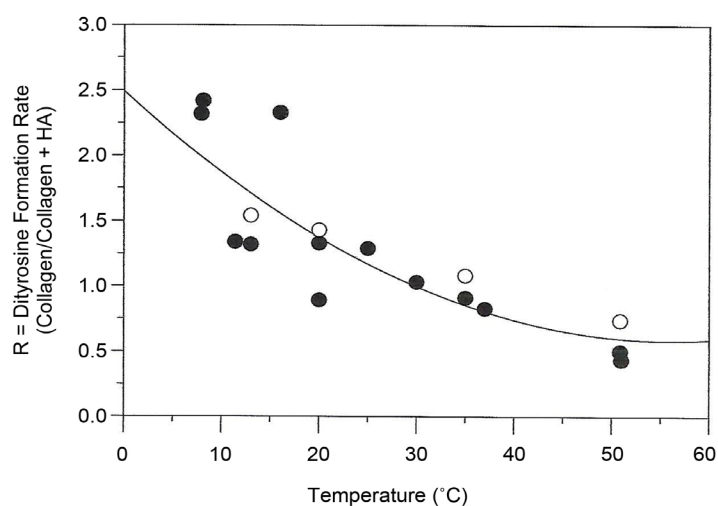


**Figure 3.** Effect of Temperature $R$, the (dimensionless) ratio of dityrosine ($A_2$) formation in the absence and presence of HA. $R$ = (Dityrosine formation in Collagen Alone)/(Dityrosine formation in Collagen + HA) (1). $R$ < 1.00 indicates that HA retards dityrosine formation (*i.e.* increases stability); $R$ > 1.00 indicates that HA increases the rate of dityrosine formation (*i.e.* decreases stability).

indeed, compatible with an intimate relationship between collagen and HA.

Our work demonstrates that collagen-bound tyrosine qualitatively behaves similarly to Shimazu's system in unbound tyrosine [10] [11] at physiological pH. Interactions between collagen and surrounding ECM could either facilitate or retard dimerization which results in the temperature-dependent shifts in *R* ratios, seen in Figure 2 and Figure 3. In the present case, the net result of collagen-HA interactions stabilizes tyrosyl residues by increasing its stability at temperature below ~36°C. Our previous work [13] shows that Skh-1 hairless mouse skin has different fading properties in solution than the present calf skin sample. This suggests that these two collagen samples may have a different overall geometry and/or chemical state and that the tyrosyl residues may therefore interact differently with their surrounding environment. It should be pointed out that effects of surrounding environment on polymer *stability* and *activity* need not be the same [15].

Type I collagen and its fluorophore tyrosine are inherently unstable. At physiological temperatures collagen in solution can form aggregates, gels or slowly autoxidize. At higher temperature, it can also irreversibly decompose to gelatin, dissociate, and/or change its conformation from helix to random coil. Because of this instability, it is necessary to prepare matched *Coll* and *Coll-HA* solutions within one day of an experiment using the same collagen stock solution for both control and experimental samples to minimize possible artifacts.

Figure 2 indicates that in the *collagen-HA system*, particularly at T < 50°C the collagen configuration may be restricted by the interacting HA molecules. Therefore, the overall temperature dependence of dityrosine formation in the *collagen alone* system may depend primarily on photochemical activation parameters at T ≥ 35°C. On the other hand, at T ≤ 10°C, the triple helix exists almost exclusively, and is less stable than the (micro)unfolded state [12] [13] [14]. This loss of stability could increase the rate of dityrosine formation, and it may also contribute to the relative lack of precision in dimerization rate at temperatures below 20°C (see Figure 2 and Figure 3). With *Collagen-HA*, the monotonic rise in formation rate with temperature at T ≥ 35°C may be attributed to a higher population of excited state molecules, which favors photodimerization. At T > 60°C, complete denaturation and dissociation to single coils ensue in both samples.

Collectively, the evidence seems to indicate that although there is no electrostatic interaction between collagen and HA, the two polymers are intimately involved with each other. This has the effect of stabilizing the collagen at a relatively wide range of temperatures. At higher temperatures, activation parameters play a more prominent role. At T > 60°C, there is complete conversion of collagen to a random coil and subsequent degradation, and this markedly increases the rate of photodimerization for both samples.

## Acknowledgements

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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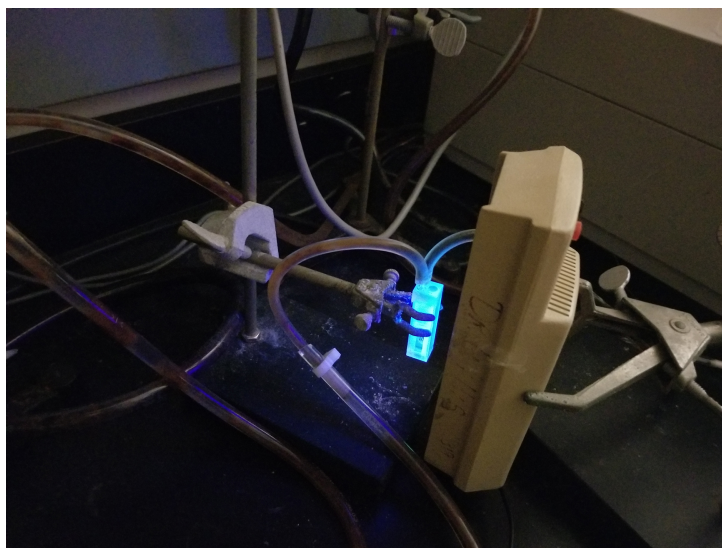
## Appendix



**Figure A1.** Irradiation of Collagen-HA System (described in manuscript). Collagen ± Hyaluronan solution were placed inside a thermo-statted fluorescence cuvette and were irradiated with a 4 W UVG-11 hand lamp emitting primarily 254 nm UV radiation (Ultraviolet Products Ltd.). As shown in this demonstration, a blue fluorescence attributable to dityrosine could be seen after prolonged irradiation. Our experimental irradiation period was much shorter, so the amounts of dityrosine generated were small with respect to the amount of tyrosine in the collagen sample *i.e.* [tyrosine] ≫ [dityrosine]. The blue fluorescence could not be seen after our relatively short irradiation period. The rates of dityrosine fluorescence increase under these conditions are linear (10), as shown by **Figure 1**.

## Abbreviations and Acronyms

Fluorescence excitation and emission wavelengths (nm): e.g. excitation at 325 nm emission at 400 nm = I(325)/400 nm; Normalized fluorescence excitation and emission wavelengths (nm): In(325)/400 nm

$[A_o]$: initial tyrosine concentration in collagen N-telopeptide;

$[A]$: tyrosine concentration;

$[A_2]$: dityrosine concentration;

$R(t) \equiv$ Rate of dityrosine formation in *control* sample/Rate of dityrosine formation in *test* sample;

*Tm*: Melting temperature of collagen.