Journal of Photochemistry and Photobiology A: Chemistry 310 (2015) 60–65

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

Aggregation and photoreduction in anaerobic solutions of flavin mononucleotide

Pavel Kamaev *, Mikhail Smirnov, Marc Friedman, Evan Sherr, David Muller

Avedro, Inc. 230 Third Avenue, Waltham, MA 02451, USA

ARTICLE INFO

Article history:
Received 11 February 2015
Received in revised form 21 April 2015
Accepted 28 April 2015
Available online 27 May 2015

Keywords:
Flavin mononucleotide
Riboflavin
Dimerization
Photobleaching
Anaerobic

ABSTRACT

Corneal cross-linking (CXL), a clinical photodynamic treatment for keratoconus and other ectatic diseases of the cornea, utilizes concentrations of flavin mononucleotide (FMN) which are known to cause dimerization (aggregation) and typically occurs in a low oxygen environment. The dynamics of these combined conditions have not been previously evaluated.

Using UV–vis absorption spectroscopy, we investigated dynamics of photobleaching of FMN in anaerobic conditions and demonstrated that the aggregation process of FMN, at clinical concentrations, plays a significant role in photochemistry.

Based on these results, we developed a reliable theoretical model of the aggregation process of FMN at concentrations typical for the present CXL clinical protocols and determined how the aggregation process affects photobleaching of FMN under anaerobic conditions. The derivation of this model is a key contribution toward the development of a more comprehensive model of corneal cross-linking.

1. Introduction

Flavin mononucleotide (FMN) and, to a lesser extent, riboflavin, are able to self-associate and to form stacking-type charge transfer complexes when at sufficient concentration in aqueous solutions [1–3]. Intermolecular interactions in such complexes lead to a decrease of the molar absorptivity of riboflavin (at 365, 450 nm wavelengths), the appearance of an isosbestic point at 488 nm, and a bathochromic shift in the area of 490–520 nm [4]. As a result, the yellow color of FMN solution becomes reddish at high concentrations. FMN or riboflavin appears to be converted to the semiquinoid form in complex [1,5], which is non-fluorescent in solutions at ordinary temperatures and able to quench light-excited riboflavin upon collision [6,7,3].

There is a vast amount of literature on FMN photochemistry at low concentration in which aggregation behavior appears absent [14–16,20,22–24,26]. FMN has also been investigated at high concentration for dimerization (aggregation), without photochemistry [1–7,17,19,27,29,30]. Analysis of the photochemical processes of riboflavin at high concentration and the subtleties of aggregation were inconsequential until now.

Over the past dozen years, a clinical photodynamic treatment for keratoconus and other ectatic diseases of the cornea called corneal collagen cross-linking (CXL), utilizes the combination of FMN and UVA light [8–11]. The first standard clinical procedure, called the Dresden protocol, includes removal of the corneal epithelium and instillation of a 0.1% w.v. FMN solution by drops onto the cornea every 3 min for half an hour. The cornea is then exposed to UVA light with a wavelength of 370 ± 5 nm and an irradiance of 3 mW/cm^2 for a total time of 30 min delivering a 5.4 J/cm^2 total dose [12]. More recently developed CXL protocols have utilized FMN formulations with increasing concentrations up to 0.25% w.v. of riboflavin [8,10].

The concentrations of FMN used in these procedures are at least 10–20 times higher than those used to evaluate FMN photoreduction and bleaching. At the concentrations used clinically, aggregation has a significant influence on the photochemical processes, affecting overall reaction efficiencies [22]. Our purpose was to develop a theoretical model of the aggregation process of FMN at concentrations typical for the present CXL clinical protocols and to investigate how it affects photobleaching of FMN under anaerobic conditions, which is the prevailing condition at depth within the cornea during a standard CXL procedure [13].

2. Material and methods

Sodium riboflavin-5′-phosphate was purchased from DSM Nutritional Products, Inc. (Parsippany, NJ). Analytical assaying by the manufacturer gave a riboflavin content of 77%.

* Corresponding author. Tel.: +1 781 768 3422.
E-mail address: pkamaev@avedro.com (P. Kamaev).

http://dx.doi.org/10.1016/j.jphotochem.2015.04.025
1010-6030/

Measurements of the association of riboflavin phosphate were carried out in distilled water at room temperature using quartz cells with 10, 0.5, and 0.2 mm light paths (NSG Precision Cells, Inc. Farmingdale, NY) and an Evolution 300 UV–vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Riboflavin solutions with concentrations of 0.01%, 0.05%, 0.10% and 0.50% (w.v. in distilled water, pH range of 6.1–6.3) were prepared and oxygen was removed from each 0.5–1 ml of solution placed in a small test tube by positioning it at the bottom of a 250 mL laboratory glass beaker filled with CO_2. Argon was then bubbled through the solutions for 10 min where the oxygen concentration was measured to be less than 0.05 mg/L, as determined by an oxygen microsensor (NTH-PSt1-L2,5-TS-NS and fiberoptic oxygen meter OXY-4 micro (PreSens Precision Sensing GmbH, Regensburg, Germany)).

In the same beaker filled with CO_2, de-oxygenized riboflavin solution was then placed in a 0.2 mm demountable UV quartz spectrophotometer cuvette (cat.# 20ES0.2, NSG Precision Cells, Inc. Farmingdale, NY). The cuvette was tightly closed and positioned in a cuvette mount (A20, NSG Precision Cells) and the riboflavin's absorption spectrum was recorded by Evolution 300 UV–vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

The riboflavin solution in the cuvette was then irradiated for various amounts of time with a 365 nm light source (UV LED NCSU033B[T]; Nichia Co., Tokushima, Japan) with a top hat beam (63% root mean square) at irradiances of 3 or 30 mW/cm^2 measured with a power sensor (model PD-300-UV; Ophir, Inc. Jerusalem, Israel). Immediately after the UV exposure, absorption spectrum of the riboflavin was measured again.

During anaerobic irradiation of riboflavin in solution some amount of the reduced form of riboflavin (dihydroriboflavin) is generated. In order to obtain its molar extinction coefficient, dihydroriboflavin was produced in a secondary experiment by the addition of sodium hydrosulfite powder (Sigma–Aldrich, St. Louis, MO) to freshly prepared riboflavin solution [6]. Sodium hydrosulfite was then added in equal molar amounts to the known concentrations of riboflavin. Absorptions of the prepared solutions were then immediately recorded and the absorptivity of dihydroriboflavin was calculated from a calibration plot.

3. Theory and calculation

Our theoretical and computational model includes the following processes: (a) UV light propagation in riboflavin solution with varying concentrations of regular riboflavin, *Rf*, and reduced riboflavin, RfH_2, (b) diffusion of riboflavin and its derivatives, in a water solution under anaerobic conditions, (c) optical excitation of riboflavin molecules to singlet state, Rf_1^*, followed by quantum transitions to triplet, Rf_3^*, and ground states, (d) chemical reactions, (e) aggregation of *Rf* and RfH_2 monomers into dimers, *A*, and (f) quenching of the singlet and triplet riboflavin by dimers. The list of transformations is:

$$Rf \rightarrow Rf_1^*, \ I \tag{1}$$

$$Rf_1^* \rightarrow Rf, \ \kappa_1 \tag{2}$$

$$Rf_1^* \rightarrow Rf_3^*, \ \kappa_2 \tag{3}$$

$$Rf_3^* \rightarrow Rf, \ \kappa_3 \tag{4}$$

$$Rf_1^* + A \rightarrow Rf + A, \ \kappa_{1a} \tag{5}$$

$$Rf_3^* + A \rightarrow Rf + A, \ \kappa_{3a} \tag{6}$$

$$Rf_3^* + Rf \rightarrow RfH^\bullet + RfH^\bullet, \ \kappa_4 \tag{7}$$

$$RfH^\bullet + RfH^\bullet \rightarrow Rf + RfH_2, \ \kappa_5 \tag{8}$$

$$Rf + Rf \underset{\kappa_a^-}{\overset{\kappa_a^+}{\rightleftarrows}} A_1, \tag{9}$$

$$RfH_2 + RfH_2 \underset{\kappa_a^-}{\overset{\kappa_a^+}{\rightleftarrows}} A_2 \tag{10}$$

$$Rf + RfH_2 \underset{\kappa_b^-}{\overset{\kappa_b^+}{\rightleftarrows}} A_3 \tag{11}$$

The notations *Rf* and RfH_2 represent monomers. It is assumed that riboflavin confined in dimers does not undergo light-induced transformations (as demonstrated by trapping and dissipating the exciting light energy [3], [22], [30], and by the absence of fluorescence of dimers [7], [6]). Relation (1) expresses the optical excitation of *Rf* molecules to the singlet state. The excitation rate is proportional to the light intensity, *I*, and the extinction coefficient, ε_{1m}. Accounting for the absorption of riboflavin, dihydroriboflavin, and associated riboflavin, the light propagation equation takes the form:

$$\frac{\partial I}{\partial x} = -\{\varepsilon_{1m} \times [Rf] + \varepsilon_{2m} \times [RfH_2] + \varepsilon_d \times [Rf_a]\} I \tag{12}$$

where ε_{1m}, ε_{2m}, and ε_d are the molar extinction coefficients at 365 nm for regular, reduced, and associated riboflavin, respectively. The extinction of RfH_2 is low [31] and association must make it even lower, therefore the extinction of RfH_2 dimers is neglected at this wavelength. $[Rf]$ and $[RfH_2]$ are the concentrations of monomers where square brackets represent molar concentrations and $[Rf_a]$ is the concentration of associated riboflavin given by:

$$[Rf_a] = 2[A_1] + [A_3] \tag{13}$$

Relations (2) and (3) show decay of the singlet state down to the ground and triplet states at rates k_1 and k_2, respectively. The triplet riboflavin decays to the ground state at rate k_3 (4). Next, both singlet and triplet riboflavin can be quenched by the interaction with dimers (5) and (6) via nonradiative energy transfer [3,7]. The triplet riboflavin also reacts with molecules in the ground state yielding semiquinone radical, $RfH^\bullet$ (7) [14,15]. These radicals quench each other yielding the stable forms of riboflavin—both regular and reduced (8) [14]. Reversibly, some amount of semiquinone radicals can be generated because of the interaction between *Rf* and RfH_2 but we disregarded this reaction in our scheme because the equilibrium constant for semiquinone formation is in the range of 0.0004–0.073 [28]. The light-induced set of reactions results in gradual decrease of the light absorption coefficient of the solution at 365 nm because of the depletion of the regular form of *Rf* and the accumulation of low absorbing hydroquinone: RfH_2 [14].

The dimers may be of the three different forms: the *Rf* dimers, A_1, the RfH_2 dimers, A_2, and the mixed dimers, A_3 [6,16,17]. It